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Spatial heterogeneity of bacterial communities in the mucus of *Montastraea annularis*

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ABSTRACT: Corals are known to contain a diverse microbiota; however, few studies have explicitly addressed the spatial variability of bacterial communities across individual, healthy coral colonies. This study applied culture-based and culture-independent methods to examine the spatial heterogeneity in bacterial communities in the mucus of 3 healthy *Montastraea annularis* colonies from Looe Key Reef, Florida Keys. Automated ribosomal intergenic spacer analysis (ARISA) results showed significant variability (up to 61% dissimilarity) in the composition of the total bacterial community at different locations only centimeters apart on individual coral colonies. Abundances of culturable *Vibrio* spp. determined by TCBS plating were highly variable across individual coral colonies, differing by up to 100-fold between different locations on the same colony. ARISA profiles indicated that intracolony variation rivaled intercolony differences in the composition of the culturable *Vibrio* community (i.e. types of culturable *Vibrio* spp. and their relative abundances). The high degree of spatial heterogeneity in coral-associated bacteria observed across individual colonies has implications for coral microbiology studies and coral restoration projects.

KEY WORDS: Community profiling · ARISA · Bacteria · Coral · Spatial heterogeneity · *Vibrio*

INTRODUCTION

Corals harbor abundant and diverse microbial communities, with approximately 100 million bacteria cm^{-2} of coral surface (Ritchie et al. 1994, Ritchie & Smith 1997, 2004, Rohwer et al. 2001, 2002, Wegley et al. 2004, 2007, Bourne & Munn 2005, Koren & Rosenberg 2006, Ritchie 2006). Bacterial concentrations in coral mucus are 100- to 1000-fold greater than those in ambient seawater (Ducklow & Mitchell 1979a, Wild et al. 2004), and the coral bacterial communities are often species-specific associations that are maintained over time and space (Ritchie & Smith 1997, Rohwer et al. 2002). Bacteria play a myriad of critical roles for coral ecology, including nutrient acquisition (Shashar et al. 1994, Lesser et al. 2004, Beman et al. 2007, Wegley et al. 2007) and antibiotic production (Ritchie 2006, Nissimov et al. 2009, Shnit-Orland & Kushmaro 2009). In addition, it has also been suggested that changes in the microbial community can allow corals to rapidly adapt to changing environmental conditions (Brown & Bythell 2005, Reshef et al. 2006, Kooperman et al. 2007, Rosenberg et al. 2007). Recent studies indicate that corals may regulate the activity of their associated bacterial communities (Breitbart et al. 2005, Kline et al. 2006, Rohwer et al. 2010). Certain factors, most notably the addition of dissolved organic carbon to concentrations above field-observed averages, cause this regulation of coral-related bacteria to break down, leading to coral decline (Kline et al. 2006, Smith et al. 2006). Shifts

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in bacterial community composition have been observed in bleached and diseased corals, with opportunists and pathogens present in these health-compromised states (Ritchie & Smith 1995, Kushmaro et al. 1997, Frias-Lopez et al. 2002, Pantos et al. 2003, Pantos & Bythell 2006, Ritchie 2006, Bourne et al. 2008).

The coral surface layers are extremely complex and dynamic (Ainsworth et al. 2006, Johnston & Rohwer 2007); however, few studies have examined spatial heterogeneity in coral–microbe associations across individual coral colonies. In diseased coral colonies, significant differences have been observed between the bacteria found in the healthy versus diseased portions of the colony (Frias-Lopez et al. 2002, Pantos et al. 2003, Breitbart et al. 2005, Gil-Agudelo et al. 2006, 2007, Pantos & Bythell 2006, Sekar et al. 2006). A limited number of studies have also demonstrated spatial variation in the bacterial community associated with healthy branching corals. Rohwer et al. (2002) demonstrated that bacterial communities were spatially structured on the branching coral *Porites furcata*, with specific bacterial taxa only found at the branch tips. In addition, bacterial community analysis in 6 replicate tissue samples from healthy *Pocillopora damicornis* colonies revealed spatial heterogeneity across some coral colonies (Bourne & Munn 2005), although some bacteria were uniformly found throughout an individual coral colony. Hansson et al. (2009) also demonstrated that the cold water coral *Madrepora oculata* exhibited spatial variation of bacterial communities within and among colonies, yet a *Spongiobacter* sp. was consistently found in all healthy samples.

The degree of spatial complexity in coral-associated bacteria has implications for our understanding of the health and ecology of the coral holobiont. The present study utilized both culture-based and culture-independent methods to determine the extent of spatial heterogeneity of the bacterial community in mucus sampled from various locations across the surface of individual *Montastraea annularis* colonies from the Florida Keys. In addition to the total bacterial community, the abundance and diversity of cultured *Vibrio* spp. was addressed, since this bacterial group has been extensively examined in coral microbiology research. The results showed a significant amount of spatial heterogeneity across individual colonies, although some members of the microbial community were present at all locations sampled on a colony. Abundances of culturable *Vibrio* spp. were highly variable across individual coral colonies, suggesting that this parameter should not be used to indicate coral health. The observed spatial heterogeneity of bacteria in mucus from individual coral colonies has important implications for coral microbiology studies and coral restoration projects.

MATERIALS AND METHODS

Sample collection. The surface mucus layers of 3 apparently healthy *Montastraea annularis* colonies (MA1, MA2, MA3) were sampled *in situ* at Looe Key Reef, in the lower Florida Keys (24° 32.75′ N, 81° 24.35′ W) in July 2009. Several random locations (approx. 12 cm^2 each, constrained through the use of 4 cm diameter PVC pipe) were sampled from the sun-facing tops of each coral colony (Fig. 1). Using sterile blunt-end syringes, the surface of the coral was briefly disturbed and 12 ml mucus samples were aspirated, with efforts to minimize the amount of surrounding

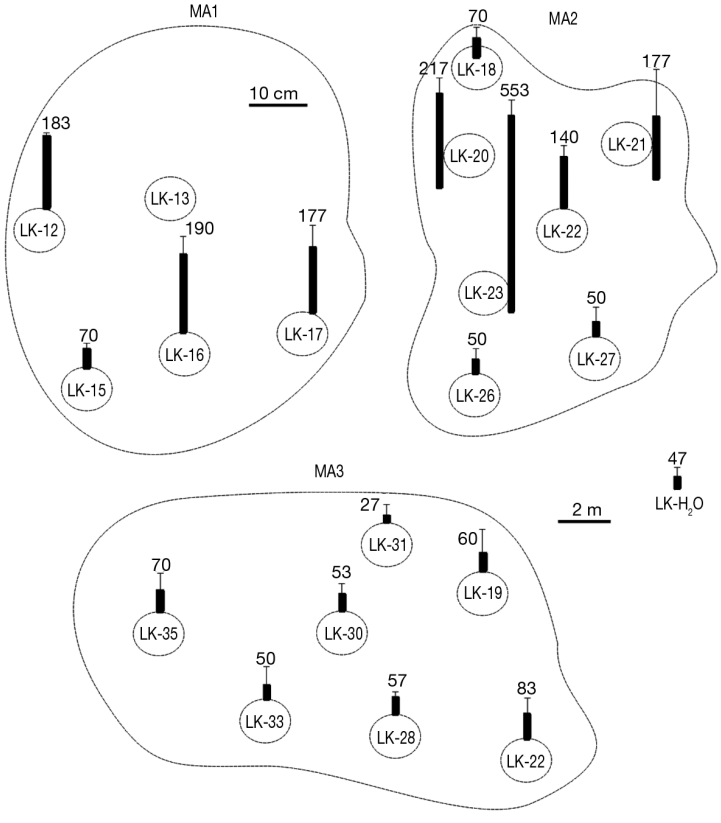

Fig. 1. Top-down view demonstrating approximate locations of each sample on individual *Montastraea annularis* colonies (MA1, MA2, MA3) as well as the spatial arrangement of the colonies at the Looe Key (LK) reef site. Distances between spots within a colony are measured at the 10 cm scale and distances between colonies are measured at the 2 m scale. Sample number is designated by LK-no. The bar size and number above the bar represent culturable *Vibrio* spp. abundance for each sample (colony forming units [CFU] ml^{-1})

seawater collected. MA1 and MA2 were located approximately 2 m apart, while MA3 was 5 m away; all colonies were at a depth of 5.5 m. An ambient water sample (1 l) was also collected approximately 1 m above the reef. Mucus and water samples were stored on ice until return to the laboratory (within 2 h), where they were processed immediately for culture-based analyses. The remaining fractions of all coral mucus samples were frozen at −80°C, and the water sample was filtered onto a 0.2 µm Sterivex filter (Millipore, Billerica) and then stored at −80°C.

Molecular profiling of total bacterial community by ARISA. Bacteria were concentrated from 10 ml of the coral mucus samples through centrifugation at 15 000 × *g* for 15 min. Mucus pellets from individual locations across *Montastraea annularis* colonies and the Sterviex filter from the Looe Key seawater sample were extracted with a PowerSoil DNA Kit (MO-BIO) according to the manufacturer's instructions, with an extended (30 min) bead beating step and elution of DNA into sterile water instead of kit buffer. DNA was stored at −20°C.

Automated ribosomal intergenic spacer analysis (ARISA) (Borneman & Triplett 1997, Fisher & Triplett 1999), which relies on the variable length of the intergenic spacer (ITS) region between the 16S and 23S ribosomal RNA, was used to profile the total bacterial community. ARISA profiling was chosen based on its low cost, fast sample processing, high level of reproducibility, and ability to detect differences in bacterial community composition (Hewson & Fuhrman 2004, Danovaro et al. 2006). Following a modified protocol of Danovaro et al. (2006), 50 µl ARISA-PCR mixtures contained 10 µM universal bacterial primers 16S-1392F (5′-GYA CAC ACC GCC CGT-3′) and fluorescently labelled 23S-125R-HEX (5′-HEX-GGG TTB CCC CAT TCR G-3′), 0.2 mM dNTPs, 100 µg ml^{-1} BSA, 1× Red Taq buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$), 1 U RedTaq DNA Polymerase (Sigma-Aldrich), and 5 µl of DNA template. Thermocycling conditions consisted of a 3 min initial denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a final extension of 10 min at 72°C. Triplicate PCR products were purified with a DNA Clean & Concentrator Kit (Zymo Research) and quantified using a Quant-IT™ Pico Green Assay Kit (Invitrogen).

Purified PCR products were normalized by diluting to 5 ng µl^{-1} for each sample and sent to the University of Illinois Urbana-Champaign for capillary electrophoresis runs. ARISA profiles were generated by combining 1 µl of cleaned ARISA-PCR product with ROX 1000 (Applied Biosystems) internal size standard, and deionized formamide. This mixture was denatured at 95°C for 2 min, and placed on ice. Each mixture was loaded into a separate capillary on an ABI Prism 3730xl Genetic Analyzer (Applied BioSystems), which separates DNA fragments by size (where each peak represents an operational taxonomic unit [OTU]), and detects fluorescence produced by those fragments (relative abundance). Control bacterial communities, ranging from a single bacterial isolate to a complex seawater sample, were run in triplicate to determine the accuracy of the ABI 3730xl (±1 bp, data not shown). Replicates performed on these controls demonstrated that this method is robust and reproducible.

ARISA profile analysis. Fragments were sized from the raw electropherograms using the Local Southern calling method in Peak Scanner v.1.0 software (Applied Biosystems). Since accurate sizing of peaks is typically limited to ≤1 kb and small fragments are typically considered artifacts (Fisher & Triplett 1999), only profile peaks between 300 to 1000 bp were retained for analysis. Raw profiles were further filtered using the following standardization criteria: (1) exclusion of peaks less than 5 times the baseline signal, (2) removal of shoulder peaks (Fisher & Triplett 1999, Danovaro et al. 2006, Luna et al. 2006), and (3) exclusion of peaks not present in 2 of 3 triplicate profiles (Hewson & Fuhrman 2004). Binning of ARISA data has been shown to correct for methodological variation that would otherwise be misconstrued as ecological variability (Hewson & Fuhrman 2006). The interactive binner developed by Ramette (2009) was used to apply a 2 bp bin across ARISA profiles to account for detector error and technical variation inherent to peak sizing. All profiles were subsequently examined visually to ensure proper binning of peaks. Binned peak data (OTUs contributing ≥0.09% of total fluorescence) was imported into Excel (Microsoft), where the average area under each peak was calculated between triplicates to produce a single composite profile for each sample, and peak areas were normalized to reflect the percent contribution of each peak to the total profile fluorescence (Hewson & Fuhrman 2004).

Statistical analyses were run in the PRIMER software package (Clarke 1993) and MATLAB v.7.8 (MathWorks) to determine the extent of similarity between bacterial community profiles from distinct locations across each coral colony. Square-root transformed ARISA fluorescence data was used to calculate Bray-Curtis distances (Bray & Curtis 1957), which represent similarity based on OTU composition and abundance. Resemblance matrices were generated from pairwise comparisons of these values and hierarchical cluster diagrams were produced to display dissimilarities between ARISA profiles. Bray-Curtis values from sample comparisons within the same colony were averaged to calculate the mean intracolony dissimilarity. The intercolony values were also calculated using

these same resemblance matrices. Analysis of similarity (ANOSIM), which compares ranks of distances, was performed to determine whether intercolony profiles were significantly different from intracolony profiles. An approach less inclined to produce Type I errors, but analogous to ANOSIM, is permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001). This test compares the means of distances among versus within groups to produce an *F*-statistic, and a p-value is generated through random permutation. Because PERMANOVA assumes equal variances for intracolony samples, nonparametric dispersion analysis (NP-DISP), which calculates distances to a colony centroid from each sample profile within each colony, was used to confirm that the ARISA data did not violate the assumption of homogenous dispersion (Anderson 2006). Five thousand permutations were conducted for all tests and a p-value ≤ 0.01 was considered significant. Similarity percentage (SIMPER) analyses were also conducted to determine which OTUs contributed to dissimilarity among samples.

Identification of ARISA OTUs. Taxonomic characterization of ARISA OTUs was performed using an identical PCR to that described above for ARISA, except the reverse primer did not contain the HEX fluorophore. Total community PCR products from samples LK-17, 21, and 33 (one from each coral colony) were cloned into a pCR4-TOPO vector, which were transformed into chemically competent *E. coli* cells following the TOPO TA kit protocol (Invitrogen). Clones were screened to identify insert sizes consistent with the OTUs of interest identified from the ARISA profiles using the Agilent Bioanalyzer DNA7500 assay, as per manufacturer's instructions. Multiple OTUs of identical sizes across different samples were also sequenced to validate that these peaks were produced by the same bacterial taxa. Cloned ARISA PCR products were sequenced bi-directionally with 1392F and 125R primers, and sequence data was trimmed for vector removal and quality in Sequencher (Genecodes). The complete sequence (accession numbers: HM209393-HM209399) from each clone was compared using BLASTN (www.ncbi.nlm.nih.gov/blast) against the National Center for Biotechnology Information (NCBI)'s GenBank non-redundant database to assess the degree of nucleotide identity to known sequences (Altschul et al. 1990). BLAST assignments of ARISA clones were further confirmed by the Ribosomal Database Project CLASSIFIER, a highly curated and annotated database that applies a Bayesian algorithm to classify 16S sequence data into taxonomic categories (Wang et al. 2007).

Abundance and diversity of culturable *Vibrio* spp. *Vibrio* is a genus of marine bacteria found in the mucus and tissues of healthy corals (Koren & Rosenberg 2006), where their presence and abundance varies seasonally (Ritchie 2006, Bourne et al. 2008). In addition, some members of this group are pathogens associated with a number of coral diseases (Kushmaro et al. 2001, Ben-Haim et al. 2003a, Cervino et al. 2008, Sussman et al. 2008). Kline et al. (2006) demonstrated that *Vibrio* spp. from healthy corals induced mortality when re-introduced at higher concentrations, suggesting that the concentration of *Vibrios* has important implications for coral health. The abundance and diversity of culturable *Vibrio* spp. at distinct locations across the coral colonies was examined by culturing mucus samples on thiosulfate citrate bile salts (TCBS, Neogen) agar, a growth media formulated to select for *Vibrio* spp. (Lotz et al. 1983). Immediately upon return to the laboratory, samples were vortexed and 100 µl of mucus from each sample was plated onto TCBS agar in triplicate. The number of colonies was determined after 48 h of incubation at room temperature. All colonies from 2 replicate plates for each sample were scraped into a single microcentrifuge tube with sterile toothpicks to create a culturable *Vibrio* community fraction for comparison to total bacteria ARISA profiles, and stored at −80°C. Ten colonies from the remaining replicate plate were also archived as individual isolates at −80°C. DNA was extracted from the culturable *Vibrio* community fraction using the DNeasy Tissue Kit (Qiagen) according to manufacturer's instructions, with final DNA eluted into sterile water instead of kit buffer. ARISA profiling was performed on the *Vibrio* community fraction and isolates as described above.

RESULTS

Total community profiles

To examine inter- and intra-colony heterogeneity in coral-associated bacterial communities, ARISA profiles were generated from mucus samples collected at multiple distinct locations on 3 different *Montastraea annularis* colonies (MA1, MA2, MA3) from Looe Key Reef in the Florida Keys. On average, 47 OTUs (size range 301–968 bp) were detected in the coral mucus samples, while the overlying water sample contained 70 OTUs (size range 301–962 bp) (Fig. 2). Some overlap in OTUs was observed between the ARISA profiles from the water column versus the coral mucus samples; however, Bray-Curtis dissimilarity values demonstrate that the coral mucus bacterial profiles were distinct from the bacterial community found in the overlying water column (Figs. 2 & 3). The 2 most abundant OTUs in the water sample (P491 and P595), which together accounted for 22% of total profile fluores-

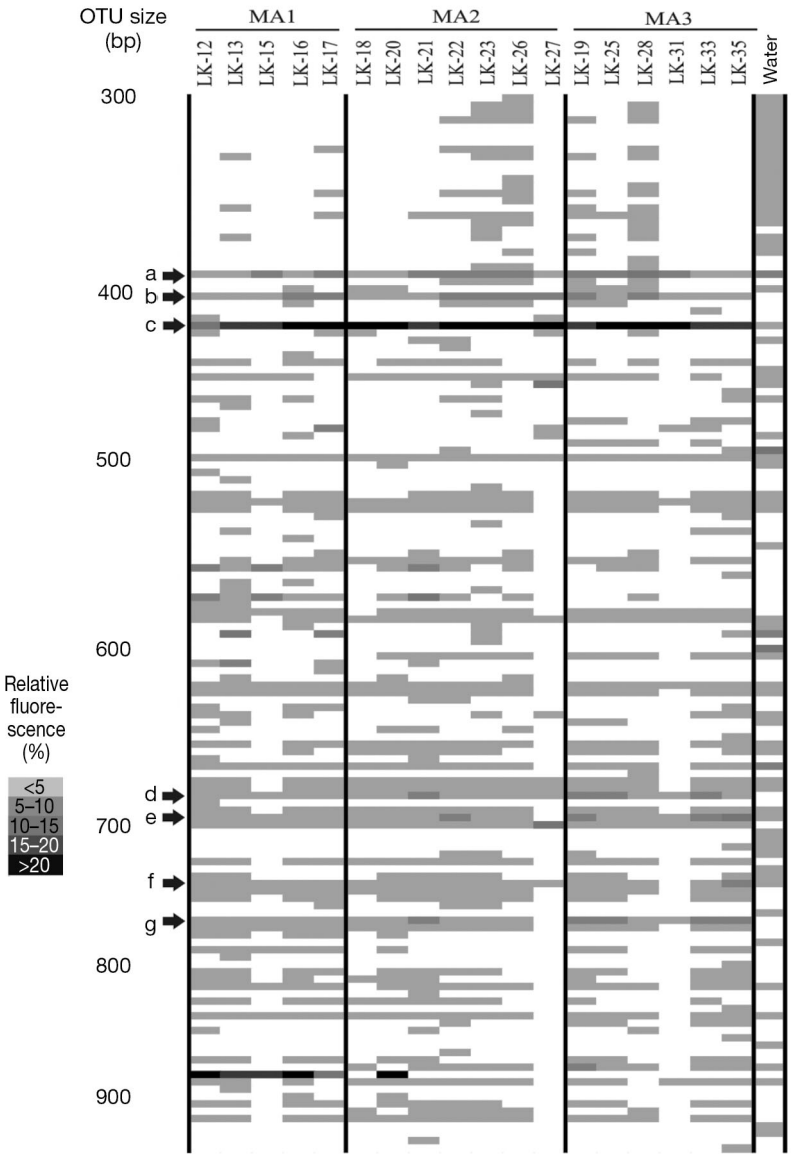

Fig. 2. Total bacterial community operational taxonomic units (OTUs) from ARISA profiles of individual samples across 3 *Montastraea annularis* colonies. The letter designations represent the ARISA OTUs selected for cloning and sequencing and correspond to letter designations in Table 1. Relative fluorescence values are displayed as percentage contributions of individual OTUs to total profile fluorescence

cence, were not detected in the majority of the coral mucus samples (Fig. 2).

Average total community profiles from the 3 *Montastraea annularis* colonies at Looe Key Reef were significantly different from each other (1-way ANOSIM, R = 0.41, $p < 0.001$ and PERMANOVA, $F = 2.94$, $p < 0.001$). A low R value demonstrated overlap in total bacterial community composition, but an average dissimilarity of 31% was observed between the 3 colonies (Fig. 3). Pairwise comparisons of colonies demonstrated that MA1 was significantly different from MA2 (1-way ANOSIM, R = 0.36, $p < 0.001$ and PERMANOVA, $F = 1.64$, $p = 0.01$) and MA3 (1-way ANOSIM, R = 0.51, $p < 0.001$ and PERMANOVA, $F = 1.90$, $p < 0.01$).

Overall, ARISA profiles from individual locations within a colony were more likely to be grouped together than ARISA profiles chosen at random from the 3 different colonies; however, there were several exceptions to this pattern (e.g. LK-27 and LK-31; Fig. 3). A focus on the intracolony profiles revealed a high level of variability (9 to 61% dissimilarity) for samples taken only 10s of cm apart. For example, the total bacterial community profile from LK-31 is 50% dissimilar to most other samples from MA3 (Fig. 3). Several individual samples (i.e. LK-16, LK-20, and LK-28) were more similar to samples taken from different colonies than to other samples from their original colony (Fig. 3).

Although the ARISA profiles from each of the coral mucus samples shared many OTUs, 8 OTUs were consistently detected in every coral mucus sample (Fig. 2). SIMPER analysis indicated that differences in the abundance of 3 of these 8 OTUs (P393, P403, and P419) contributed 20 to 25% of the variation of samples within a colony. Sequencing of P393 revealed that this OTU was 92% identical to an uncultured marine bacterial clone from the San Pedro Ocean Time Series (SPOTS) (Brown et al. 2005), and comparison to the Ribosomal Database Project (RDP) further resolved P393 as a gammaproteobacterium (Table 1). P419 was abundant in all the *Montastraea annularis* mucus samples (10.8 to 69.8% of total fluorescence in individual profiles), but was also found in the water sample at lower abundance (4.2% of total fluorescence). P419 was 99% identical to an uncultured marine bacterial clone from SPOTS (Brown et al. 2005), and further resolved as an actinobacterium based on comparison to the RDP (Table 1). Many OTUs (e.g. P533, P577, P617, P693, P697, P740, P742, P766, P770, and P841) were found in the vast majority of the coral mucus samples across all colonies, but absent in one or more of the individual coral samples (Fig. 2). Several of these OTUs were sequenced and found to have <94% identity to previously sequenced bacteria (Table 1). P892 was predominantly found on only one coral colony and SIMPER analysis showed this OTU was responsible for 10% of the observed variation, demonstrating intercolony variability in bacterial community composition. Other OTUs were only detected sporadically amongst the coral samples.

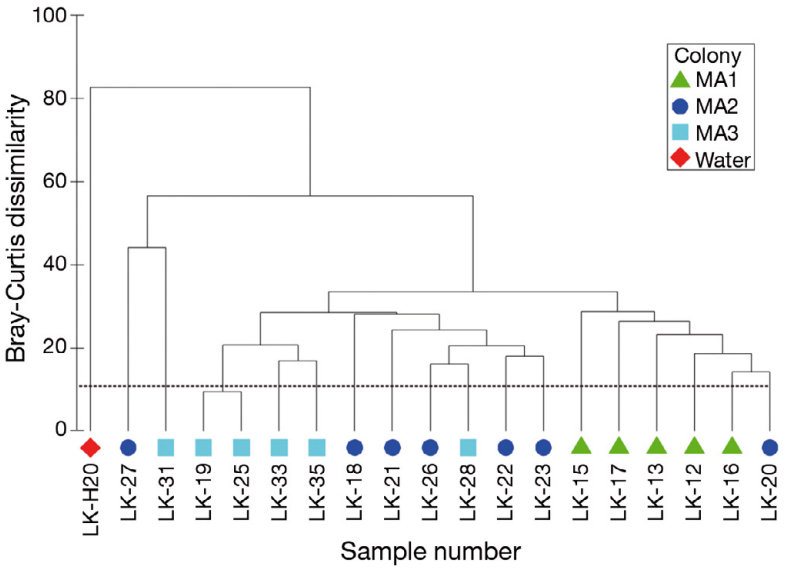

Fig. 3. Total bacteria community ARISA profiles on 3 *Montastraea annularis* colonies (see Fig. 1). The dotted horizontal line represents the average Bray-Curtis dissimilarity among triplicates to demonstrate methodological reproducibility

TCBS counts of culturable *Vibrio* spp.

In conjunction with surveying the total bacterial community, the culturable *Vibrio* fraction was examined in detail. Group-specific culturing for *Vibrio* spp., a Gram-negative bacterial group, was selected due to ease of cultivability, and the documented association of culturable *Vibrio* spp. with both healthy and bleached/diseased corals (Ritchie et al. 1994, Ben-Haim et al. 1999, 2003b, Cervino et al. 2004, Breitbart et al. 2005, Ritchie 2006, Ainsworth et al. 2007, Hall-Spencer et al. 2007, Bourne et al. 2008). Plate counts and ARISA profiling were used to determine if the culturable *Vibrio* community displayed spatial heterogeneity across a coral colony.

Spatial variability in the abundance of culturable *Vibrio* spp. was observed across all 3 *Montastraea annularis* colonies (range: 27 to 533 CFU ml^{-1}; Fig. 1). Mucus samples from different locations on the same coral colony produced highly variable numbers of culturable *Vibrio* spp. (up to 10-fold difference), with no discernable patterns based on the location of the sample on the coral colony (Fig. 1).

Vibrio community profiles

To determine if the composition of the *Vibrio* community demonstrated spatial heterogeneity across coral colonies, ARISA profiles were produced for the *Vibrio* spp. cultured from each mucus sample and the water column. The culturable *Vibrio* fraction contained an average of 37 OTUs. Only a single OTU (VP841) was present in every sample, where it only contributed an average of 1.6% of the *Vibrio* profile fluorescence. Four other OTUs (VP579, VP590, VP598, VP787) were conserved across most *Montas-*

Table 1. Phylogenetic assignments of common ARISA clones detected in *Montastraea annularis* total community profiles. Letters correspond to ARISA OTUs in Fig. 2. Bacterial group classification based on analysis of sequence in Ribosomal Database Project CLASSIFIER. OTU: operational taxonomic unit. See 'Materials and methods: Identification of ARISA OTUs' for details. nr database: non-redundant database number

OTU length (bp)	Letter	Accession no.	Closest NBCI match (nr database)	Nucleotide identity (%)	Bacterial group
393	a	HM209393	Uncultured marine bacterium clone SPOTSAPR01_5m146 DQ009152.1	92	*Gammaproteobacteria*
405	b	HM209394	Uncultured marine bacterium clone SPOTSAUG01_5m6 DQ009149.1	94	*Gammaproteobacteria*
419	c	HM209395	Uncultured marine bacterium clone SPOTSOCT00_5m60 DQ009123.1	99	*Actinobacteria*
681	d	HM209396	Uncultured bacterium GRIST23 genomic sequence EU795161.1	92	*Actinobacteria*
697	e	HM209397	Uncultured marine bacterium clone SPOTSAPR01_5m18 DQ009159.1	85	*Gammaproteobacteria*
740	f	HM209398	Uncultured marine bacterium clone SPOTSAUG01_5m55 DQ009099.1	83	*Bacteroidetes*
767	g	HM209399	Uncultured marine bacterium clone SPOTSAPR01_5m146 DQ009084.1	73	*Bacteroidetes*

traea annularis mucus samples, with these 4 OTUs totaling between 27 to 57% of the *Vibrio* community profile fluorescence for each sample. Three *Vibrio* OTUs (VP598, VP654, VP841) were also detected in the total community data. ANOSIM and PERMANOVA results for culturable *Vibrio* community profiles revealed no significant difference in *Vibrio* community profiles between colonies overall (1-way ANOSIM, R = 0.14, p = 0.09; PERMANOVA, F = 1.15, p = 0.25). Unlike the total community profiles, cluster analysis of *Vibrio* community data showed that the water column profile was similar to the mucus samples and did not result in grouping of mucus samples from the same coral colony (Fig. 4, VLK represents *Vibrio* Looe Key simple number). The average Bray-Curtis dissimilarity value was 61%, demonstrating that the structure of the *Vibrio* community was not determined by the colony from which the samples originated. *Vibrio* community samples collected from different locations on the same coral colony were as dissimilar to each other as samples from different coral colonies.

To help interpret the results from the *Vibrio* community profiles, ARISA profiles were produced for 10 individual *Vibrio* isolates cultured from a single location on each of the 3 *Montastraea annularis* colonies. An average of 16 peaks (size range 502–998 bp) were produced for each isolate, demonstrating that multiple OTUs observed in the community profiles could be attributed to a single isolate.

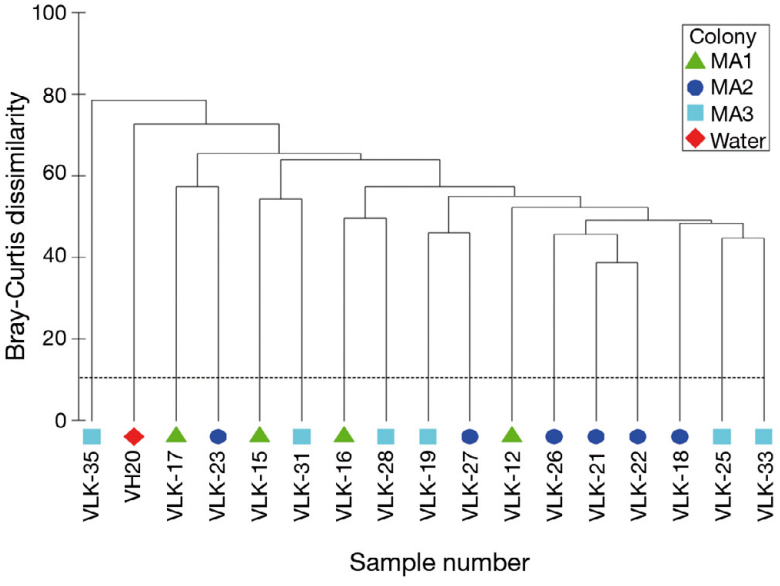

Fig. 4. *Vibrio* community ARISA profiles on 3 *Montastraea annularis* colonies (see Fig. 1). The dotted horizontal line represents the average Bray-Curtis dissimilarity among triplicates to demonstrate methodological repro-ducibility. VLK: *Vibrio* Looe Key (LK) sample number, location corresponds to LK sample number

DISCUSSION

Using a combination of culture-independent and culture-based analyses, this study documented within- and between-colony variability in the composition of the bacterial community in the mucus of healthy *Montastraea annularis* colonies in the Florida Keys. Total bacterial community composition between mucus samples and the water column were significantly different (Figs. 2 & 3), which is consistent with previous reports that a coral's mucus layer contains bacterial communities that are distinct from the surrounding seawater (Rohwer et al. 2001). Some OTUs were shared between the coral and seawater samples, which was likely due to small amounts of seawater taken up when coral mucus samples were collected *in situ* by syringe. Total community ARISA data indicated significant variation between colonies within the same reef site. Most profiles grouped together with other samples from the same colony, however several exceptions were noted. Fig. 2 displays the abundance of a unique OTU (P451) in LK-27 and absence of many shared OTUs in LK-27 and LK-31, which could explain why these 2 samples are exceptions to the general observed grouping of samples from the same colony (Fig. 3). Several bacterial OTUs were detected in most or all of the coral samples, hence the low level of dissimilarity (31%) in most profiles. However, total community profiles also revealed OTUs unique to individual locations within a coral colony. The disparate patterns observed for the *Vibrio* spp. as compared to the total community demonstrates that bacterial groups can exhibit different spatial structure across a coral colony.

The degree of spatial heterogeneity observed for coral-associated microbes in this study is similar to patterns observed for a variety of other host-associated microbial communities. Sponges are another example of benthic, sessile metazoa that contain a diverse bacterial assemblage throughout their ultrastructure (Thiel et al. 2007). A denaturing gradient gel electrophoresis (DGGE) survey of bacterial communities in multiple samples from 2 marine sponges *Cymbastela concentric* and *Callyspongia* sp. contained levels of within-individual heterogeneity (≤30% dissimilarity) (Taylor et al. 2004) comparable to total community profile results in this study. In terrestrial systems, bacteria on individual plant leaves also display high levels of spatial heterogeneity (Beattie & Lindow 1999, Andrews & Harris 2000). Furthermore, 16S rDNA pyro-

sequencing studies have recently uncovered remarkable spatial heterogeneity of bacteria associated with human hosts. Less than 1% of all OTUs were shared among 27 different locations on the human body (Costello et al. 2009).

The consistent detection of a few bacterial OTUs from *Montastraea annularis* in this study parallels previous studies demonstrating that certain bacteria are specifically associated with some coral species (Rohwer et al. 2002, Casas et al. 2004). Prior studies have shown that some bacterial OTUs can be found throughout a colony, but other OTUs are only detected at certain locations on a colony. For example, Rohwer et al. (2002) detected a specific gammaproteobacteria (PF1) at multiple locations across *Porites furcata* colonies, yet also consistently identified a unique bacterial OTU found on the tips of *P. furcata* branches that was absent from samples taken at the colony bases. *Pocillopora damicornis* also exhibited both intercolony conservation and intracolony patchiness (Bourne & Munn 2005), and similar patterns have recently been detected in the cold water coral, *Madrepora oculata* (Hansson et al. 2009). Guppy & Bythell (2006) observed within-colony spatial variation in the boulder coral, *Montastraea faveolata*, but overall the samples from an individual reef site were not significantly different, leading to the conclusion that a single sample could represent the bacterial community for an entire coral colony. While that study addressed ecosystem-wide scales, this study focused explicitly on centimeter-scale variability within individual colonies. Individual sample profiles within a colony exhibited small-scale variation, where OTUs were reduced in abundance or completely absent (see Figs. 2 & 3; LK-16 vs. LK-17) from locations centimeters apart.

The genus *Vibrio* is a ubiquitous group of marine bacteria containing members found in healthy coral specimens (Alves et al. 2010) and also implicated in a number of coral diseases (Ben-Haim et al. 2003b, Cervino et al. 2008, Sussman et al. 2008). *Vibrio* spp. abundances have been observed to increase in reef communities across gradients of increasing dissolved nutrients, pathogens, and human populations (Dinsdale et al. 2008). Ambient bacterial communities in corals are known to shift in structure and become dominated by *Vibrio* spp. during incidences of coral bleaching or disease (Ritchie & Smith 1995, Bourne & Munn 2005, Koren & Rosenberg 2006, Ritchie 2006, Bourne et al. 2008). However, previous studies have not elucidated whether this component of the community is evenly distributed across individual healthy colonies. In this study, a great deal of spatial heterogeneity in the abundance of culturable *Vibrio* spp. was observed across individual healthy coral colonies (up to 10-fold variation). It is possible that differing amounts of coral mucus were obtained in the 12 ml samples collected by syringe, which could have contributed to the *Vibrio* community variability. In addition, it is also possible that the areas of the coral with higher *Vibrio* spp. abundances may have been experiencing stress or disease conditions despite being visually indistinguishable from the rest of the coral colony. Although prior studies have observed higher abundances of *Vibrio* spp. associated with diseased corals, there is not a standard sample type or volume used for comparing *Vibrio* counts in healthy versus diseased corals. The high degree of spatial heterogeneity in the abundance of culturable *Vibrio* spp. observed on apparently healthy corals in this study demonstrates that *Vibrio* abundance should not be used, independent of other tests, as a diagnostic of coral health.

In contrast to the total community profiles where the coral-associated bacteria were significantly different types than those found in the overlying water column, ARISA profiles of culturable *Vibrio* communities from the water sample clustered with the coral mucus samples (Fig. 4). Despite much focus on pathogenicity, this and previous studies have consistently detected *Vibrio* spp. in healthy coral samples. However, the coral-associated *Vibrio* spp. did not form a distinct cluster from those in the water column, which supports the idea that vibrios are 'visitors' as opposed to 'residents' of the coral microbiome (Ritchie 2006). Composition of the culturable *Vibrio* community was highly variable across individual colonies and samples from the same coral colony did not cluster together. Intracolony heterogeneity in the composition of the *Montastraea annularis* culturable *Vibrio* community rivaled intercolony variation (Fig. 4). Although the *Vibrio* communities across *M. annularis* coral colonies were very different (61% dissimilar), there may still be some conservation of certain *Vibrio* spp. in *M. annularis*, given that 3 *Vibrio* OTUs were shared among all 3 coral colonies.

Individual *Vibrio* isolates were shown to produce numerous ARISA peaks, demonstrating that each peak on the ARISA profile does not necessarily represent a different OTU. It is not unusual that individual *Vibrio* isolates produced numerous ARISA peaks, as *Vibrio* spp. are known to contain multiple rRNA copies within their genomes (Crosby & Criddle 2003, Acinas et al. 2004, Stewart & Cavanaugh 2007) and thus do not follow the 'one bacteria, one peak' assumption. The multiple rRNA copy number complicates comparisons of *Vibrio* community profiles between samples, since the differences between profiles may be attributable to one or more isolates. We must also consider that culturing fails to capture most bacteria (Staley & Konopka 1985, Amann et al. 1995).

Many bacteria, including *Vibrio* spp. have been shown to enter viable but non-culturable phases (Xu et al. 1982, Colwell et al. 1985, Oliver et al. 1995), during periods of starvation and environmental stress, and will only re-grow under certain conditions (Gauthier 2000, Vattakaven et al. 2006).

Many different factors might have contributed to the observed heterogeneity in bacterial community composition. Different locations on a coral colony may be impacted by varying degrees of light exposure, nutrient availability, currents, sedimentation, mucus age, and competition with other microbes (Brown & Bythell 2005, Shnit-Orland & Kushmaro 2009, Teplitski & Ritchie 2009, Rypien et al. 2010). Ritchie (2006) observed that mucus plays a strong role in structuring the bacterial community, with antibiotic activity inhibiting the growth of potential pathogens. Furthermore, that study demonstrated that environmental stress altered the regulatory capacity of native mucus bacteria (i.e. loss of antibiotic production), which allowed for the settlement of opportunists (Ritchie 2006). The variation in mucus composition among coral species (Ducklow & Mitchell 1979b) and during times of stress (Ritchie & Smith 1995) has previously been used to explain differences in coral-associated bacterial communities. However, these explanations can be extended to intracolony scales, where bacteria are influenced by physical, chemical, and biological factors when competing to fill microniches. The small-scale variation (centimeters apart) described here may explain why some signs of coral disease initiate at what appear to be random locations on a coral's surface.

The significant spatial heterogeneity in both the composition of the total bacterial community, as well as in the abundance and diversity of culturable *Vibrio* spp., has important implications for experimental design of coral microbiology studies. Often, research on coral-associated bacteria is based on a single point sample collected from the surface of a coral colony. The high degree of spatial variability described in this study demonstrates that a single point sample may not be representative of the total coral-associated bacterial community. In addition, caution is needed when interpreting differences in point samples collected from different coral colonies, since these differences may be attributable to spatial heterogeneity on each colony instead of actual intercolony variation. As suggested by Hansson et al. (2009), an appropriate sample needs to integrate over the entire coral colony, or multiple spatially-distinct samples from each colony need to be obtained. However, caution also must be used when interpreting results from whole-colony surface samples, since these composites average over many highly variable microscales.

In addition to the implications for coral microbiology research, this study also has practical applications for coral restoration projects. Coral aquaculture and transplantation are currently being employed to address worldwide decline of coral reefs and promote reef ecosystem recovery (Abelson 2006). Although the introduction of pathogens from aquaculture to natural reef systems is a significant concern with coral restoration projects, there are currently no microbiological assessments included in health certification procedures prior to returning coral fragments to the wild. The complex nature of the coral-associated microbial community has made it difficult to establish a 'healthy baseline' for these microbes. Examining spatial heterogeneity of microbial communities across individual coral colonies is crucial for coral restoration projects because each of the fragments used for restoration originates from a different location on the original parent coral colony. Overall, significant heterogeneity was observed in the bacterial community within individual coral colonies, demonstrating that fragments originating from the same colony do not contain identical bacterial communities. Until knowledge improves regarding the variability of microbial community composition on healthy corals and the specific pathogens responsible for coral diseases, it will be difficult to incorporate microbial parameters into health assessments for restoration projects.

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