REPORT

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Diversity of bacteria associated with the Caribbean coral *Montastraea franksi*

Abstract In this study we characterized the prokaryotic microbiota associated with the reef-building coral *Montastraea franksi* in a culture-independent manner by sequencing 16S rDNAs. The majority of the bacteria identified by this method were novel species and belonged to a wide variety of microbial groups, with cyanobacteria and α-proteobacteria being the most abundant. In contrast, the bacteria cultured from the same *M. franksi* samples were closely related to previously described bacteria and consisted mostly of γ-proteobacteria. These results show that the microbial diversity associated with corals can be greatly underestimated when relying exclusively on culture-based methods. Our analyses also showed that one α-proteobacteria species was present in all *M. franksi* samples isolated from five reefs separated by up to 10 km. This finding is suggestive of a specific microbe–coral association.

Keywords Coral · Bacteria · Microbes · Denaturing gradient gel electrophoresis (DGGE) · 16S rDNA

Introduction

Numerous investigators (DiSalvo 1971a, 1971b; DiSalvo and Gundersen 1971; Sorokin 1973; Lewis and Price 1975; Mitchell and Chet 1975; Ducklow and Mitchell 1979; Rublee et al. 1980; Segel and Ducklow 1982; Herndl and Velimirov 1986; Paul et al. 1986; Rogers 1990; Ritchie and Smith 1995; Santavy 1995; Santavy et al. 1995; Kushmaro et al. 1996; Koh 1997; Kushmaro et al. 1997; Ritchie and Smith 1997; Santavy and Peters 1997; Torreton et al. 1997; Gast et al. 1998; Gili and Coma 1998; Lyons et al. 1998; Richardson 1998; Toren et al. 1998) have examined the interactions between corals and microbes. These studies have shown that there is a dynamic microbiota living on the surface, and possibly within the tissue, of corals and in the surrounding reef waters. However, it is still not known whether microbes play specific roles in coral biology or if the observed associations are merely opportunistic interactions of the coral animal with water-column bacteria.

A few studies have suggested that corals may associate with specific microbes. Santavy (1995) observed that *Porites astreoides* samples harvested from throughout the Caribbean harbored bacteria-filled ovoids. It has also been shown that some corals harbor nitrogen-fixing bacteria, suggesting that corals may obtain fixed nitrogen from associated microbes that are fed and protected in an anaerobic environment within the colony (Wilkinson and Fay 1979; Williams et al. 1987; Shashar et al. 1994; Kuhl et al. 1995). Ritchie and Smith (1995) showed that mucus-associated microbes had characteristic carbon source utilization patterns that were consistently associated with certain coral species and varied among different species of coral. These authors speculated that differences in the composition of the surface mucus produced by specific corals resulted in different populations of associated microbes.

In order to study coral–microbial interactions, it is first necessary to determine which microbes are actually living on or within corals. All previous studies of coral-associated microbial diversity have been based on culturing techniques. However, it is well established that the majority of microbes cannot be cultured using standard methodologies (Fuhrman and Campbell 1998),

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making it possible that the important relationships between corals and microbes have been overlooked due to technological limitations. With the advent of molecular techniques (e.g. 16S rDNA sequencing), it is now possible to identify microbes without the initial bias of culturing. This paper describes the characterization of bacteria associated with a reef-building coral using both culture-based and culture-independent methods. We show that the bacterial diversity associated with the coral genus *Montastraea* is greater than previously reported (Ritchie and Smith 1995). We also show that a specific α-proteobacteria is associated with *M. franksi* samples taken from separate reefs, which is suggestive of a specific relationship between this important reef-building coral and a bacteria.

Materials and methods

Sample collections

Samples of *M. franksi* were harvested from five different sites separated by 0.2 to 10 km around Bocas del Toro, Panama (Fig. 1). All corals were collected between 5 and 10 m in depth. A punch and hammer were used to remove 1.3-cm-diameter cores from five *M. franksi* colonies at each site. The samples were immediately placed in a plastic bag. At the surface, each sample was washed twice with 0.2 μm filtered and autoclaved seawater to remove any loosely associated microbes and then placed on ice. The samples were transported back to the field station (< 2 h), and each core was airbrushed (80 psi) with 10X TE [100 mM Tris(hydroxymethyl)aminomethane hydrochloride (pH 8.0)/10 mM ethylenediaminetetraacetic acid (EDTA)] to remove the tissue and associated microbes. The slurry was aliquoted into cryovials and frozen in liquid nitrogen.

For one sample collected at site #1, approximately 50% of the core was airbrushed with sterile seawater and 100-μl aliquots were spread onto glycerol artificial seawater medium (GASW) agar plates (Smith and Hayasaka 1982). The rest of the core was then airbrushed with the 10X TE solution and frozen. To determine if bacteria in the water column were contaminating the coral samples, a 5-l water sample was harvested from the water column immediately above the same colony at site #1. As an additional control, a
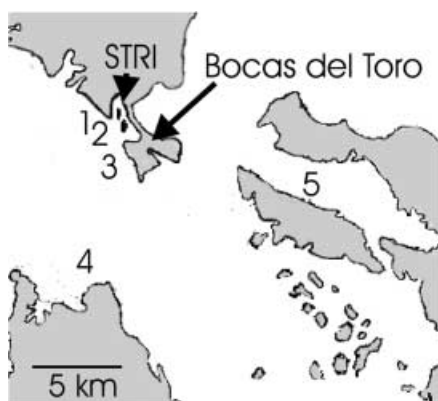
collection bag was opened underwater at site #1 and the inside of the bag was treated as if it were a sample.

DNA isolations from coral/TE slurries

To determine whether coral-associated bacterial populations characterized by culturing differed in species composition from those obtained without culturing (i.e., by isolation and analysis of the 16S rDNAs), it was first necessary to develop a method of isolating DNA from coral samples. This required a DNA isolation protocol that would: (1) yield DNA suitable for analysis with the polymerase chain reaction (PCR), and (2) include DNA from the majority of the bacterial species in the coral-associated community. Three different DNA isolation protocols were tested on the coral/TE slurry (S), and on a pellet obtained by centrifuging the slurry (P): (1) SOIL – UltraClean Soil DNA Kit (Mo Bio, Solana Beach, CA), (2) GITC – a guanidinium isothiocyanate (GITC)-based nucleic acid extraction method (RNA/DNA Mini Kit; Qiagen, Valencia, CA), and (3) CTAB – a cetyltrimethylammonium bromide(CTAB)-lysis of the tissue followed by a phenol extraction (Ausubel et al. 1995). For the SOIL extraction, 250 μl of the coral/TE slurry was prepared as recommended by the manufacturer. In addition, 1 ml of the slurry was pelleted for 30 min at $20,000 \times g$, resuspended in 250 μl 10X TE, and prepared according to the manufacturer's protocol. The GITC DNA preparation was carried out on 50 μl of the coral/TE slurry and on a pellet derived from 1 ml of the slurry (see above) according to the manufacturer's directions. CTAB extractions (Ausubel et al. 1995) were performed on 2 ml of the coral/TE slurry and on a pellet derived from 1 ml of the coral/TE slurry and then resuspended into 2 ml of fresh 10X TE.

Denaturing gradient gel electrophoresis (DGGE)

Nested PCR was used to amplify ∼200 bp of the third hypervariable region (V3) of the bacterial 16S rDNA. Initially, 15 cycles of PCR with the 27F bacterial-specific primer and the 1492R Universal primer were performed on approximately 1 μg of the total DNA. A 5-μl aliquot of this reaction was transferred to a second PCR utilizing the 358F primer with a GC clamp and the 517R primer (Riemann et al. 1999). The DNA concentration of the products resulting from the second PCR was determined, and 500 ng DNA of each sample was loaded onto 30–70% denaturing gradient gels. Denaturing gradient gel electrophoresis (DGGE) separates 16S rDNA PCR products of the same size based on their nucleotide composition. In theory, each band on a DGGE gel represents a single bacterial species. After electrophoresis, the gels were stained with SYBR-Green (Molecular Probes) and photographed using a digital camera.

Construction and analysis of a 16S rDNA library from uncultured bacterial communities

Total DNA was prepared from the coral samples using the SOIL method. The 16S rDNA loci were PCR-amplified using the 27F bacterial-specific primer and the 1492R Universal primer (1X REDTaq Buffer, 1U REDTaq, 200 μM each dNTP, 1 μM each primer, ∼1 μg target DNA; 5 min at 94 °C, 30 cycles of [1 min at 94 °C, 1 min at 62 °C, 3 min at 72 °C], 10 min at 72 °C). The PCR products were TA cloned into pCR2.1- or pCR4-TOPO as recommended by the manufacturer (Invitrogen, Carlsbad, CA). Transformants were selected on Luria-Bertani (LB)/kanamycin plates with X-gal (5-bromo-4-chloro-indoly β-D-galactopyranoside). White colonies were picked, streaked into 96 well plates, and subjected to PCR with M13F and M13R primers. The resulting products were isolated using Qiagen's 96-well PCR clean-up kit and sequenced twice using the 27F primer. Consensus sequences were constructed by aligning the two sequences from each clone with ContigExpress (InforMax, North Bethesda, MD). The first ∼550 bp of each consensus sequence were analyzed by BLAST (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) to identify the nearest previously described bacteria.

Fig. 1 Map of collection sites at Bocas del Toro, Panama (*STRI* Smithsonian Tropical Research Institute's field station). Five *M. franksi* samples were collected from each site

Characterization of cultured bacteria

After inoculation (see above), the GASW plates were incubated at room temperature for 5 days and ~30 colonies were picked at days 2, 3, and 5 and restreaked onto fresh GASW plates. This step was repeated an additional time to ensure that the clones represented only one species of bacteria. DNA was prepared from the clones using Qiagen's Tissue DNeasy Kit and subjected to PCR with bacterial-specific primers (1X REDTaq Buffer, 1U REDTaq, 200 µM each dNTP, 1 µM 27F and 1492R primers, and target DNA; 5 min at 94 °C, 30 cycles of [1 min at 94 °C, 1 min at 62 °C, 3 min at 72 °C], 10 min at 72 °C). The resulting products were isolated, sequenced twice using the 27F primer, and analyzed by BLAST (as described above).

Results

As shown in Fig. 2, either pelleting or direct extraction with the SOIL method produced the greatest number of bands when analyzed by DGGE. Since each band theoretically represents one bacterial species, this protocol appeared to be the most efficient at extracting DNA from various members of the coral-associated bacterial community.
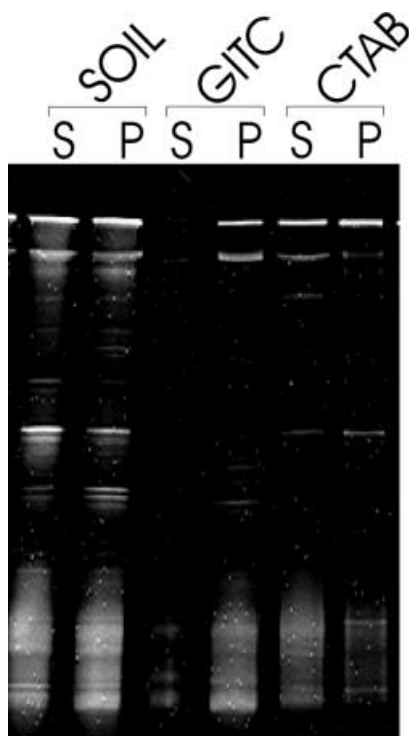

Fig. 2 Comparison of coral-associated bacterial 16S rDNA sequences obtained using different DNA isolation protocols. DNA was extracted directly from the coral/TE slurry (*S*) or from a pellet (*P*) obtained by centrifuging the slurry. DNA extraction protocols tested were: (1) Mo Bio's SOIL DNA Kit (*SOIL*), (2) Qiagen's RNA/DNA Mini Kit (*GITC*), and (3) CTAB lyses/phenol extraction (*CTAB*) (Ausubel et al. 1995). The resulting DNAs were subjected to PCR to amplify bacterial 16S rDNAs, and the PCR products were analyzed by DGGE

The bacterial community associated with one *M. franksi* sample harvested from site #1 was characterized by sequencing 16S rDNA obtained from DNA directly extracted from the coral sample. Of the 75 sequences characterized without culturing (Table 1), 36% were cyanobacteria and 27% were various α-proteobacteria. Two species of β-proteobacteria were also identified. Three-fourths of the sequences were observed only once. The average identity of this library to previously characterized bacteria was 93% over the first 550 bp of the 16S rDNA. In comparison, for isolates obtained by culturing from the same *M. franksi* sample and characterized by 16S rDNA sequencing, the most commonly isolated bacteria were closely related to *Pseudoalteromonas* (39%) and *Vibrio* (38%) (Table 2). Of the 71 16S rDNA sequences obtained from the cultured isolates, 83% belonged to the γ-proteobacteria group. Most of the isolates were closely related to previously described bacterial species, with an average identity of 97% over the first 550 bp of the 16S rDNA. There were no 16S rDNA sequences in common between the cultured isolates and the 16S rDNA sequences obtained without culturing. Thus the two approaches yielded dramatically different profiles of the coral-associated microbial community.

To determine if the source of coral-associated bacteria was the surrounding water, a 5-l water sample was harvested from the water column immediately above the *M. franksi* colony at site #1. DNA was prepared from the water sample and compared against the coral-associated bacterial community by DGGE. Only two or three bands were shared between the two samples (Fig. 3A). Therefore, the surrounding water did not appear to be the source of coral-associated bacteria.

Since the surrounding water column did not appear to be the source of coral-associated bacteria, we hypothesized that there may be bacteria specifically adapted to live on coral. If this hypothesis is true, it would be expected that corals from different reefs would have similar bacterial communities. *M. franksi* samples were harvested from five different reefs and the bacterial communities were compared using DGGE. As shown in Fig. 3B, one bacterial species was associated with *M. franksi* samples collected from all five reefs. This band was found in a total of 25 samples, five from each reef (only one representative sample from each reef is shown in Fig. 3B). This band could be explained in two ways: (1) there was a systematic contamination of the samples, or (2) this band represented a bacteria associated with *M. franksi* located in each of the five different areas. This band was not associated with the negative control (bag opened and closed above coral; see Methods), nor was it seen in the DNA from the water samples (Fig. 3A), suggesting that contamination was not responsible. Sequence analysis of this band revealed that it represented an α-proteobacteria closely related to the

Table 1 Uncultured bacterial 16S rDNA sequences from *M. franksi*. A bacterial 16S rDNA library was constructed by isolation of DNA directly from the coral sample, PCR amplification with bacterial-specific primers, and cloning. The first 550 bp at the 5′ end of the 16S rDNA sequences were submitted to BLAST and nearest hit and percent identities to nearest neighbor were determined. The percentage of the total isolate population was calculated by dividing the number of times a certain 16S rDNA sequence appeared in the library by the total number of sequences analyzed

Group	Most closely related hit in GenBank	Accession	Identities over 550 bp (%)	(%) of total 16S rDNAs sequenced
α	*Silicibacter lacuscaerulensis*	SLU77644	94	21
α	*Paracoccus* sp. B8B2	PSP012068	92	1
α	Alpha proteobacterium MBIC1876	AB026194	95	1
α	Uncultured marine eubacterium HstpL28	AF159650	95	1
α	*Rhodobacter apigmentum*	AF035433	87	1
α	*Allorhizobium undicola*	AUY17047	91	1
α	*Roseobacter* sp. ISM	AF098495	95	1
β	*Comamonas acidovorans* strain MC1	AF149849	100	4
β	*Alcaligenes* sp. R4	ASPAJ2815	96	3
γ	Endosymbiont of *Pachypsylla venusta*	AF077606	87	1
γ	*Acinetobacter lwoffii*	ALZ93440	98	1
γ	*Pseudomonas putida* IFO 3738	D85999	99	1
γ	Unidentified gamma proteobacterium JTB148	AB015252	92	1
γ	Uncultured marine eubacterium HstpL43	AF159674	93	3
γ	*Vibrio carchiariae*	VCH16SRRA	98	1
γ	*Silicibacter* sp. DSS-3	AF098491	90	1
γ	Marine eubacterial sp.	MABRRJ	90	1
Bacillus/Clostridium	*Clostridium paradoxum*	CPRPARA25	89	1
Cyano	*Gloeocapsa* PCC73106	AF132784	91	25
Cyano	*Synechococcus* PCC7942	AF132930	92	3
Cyano	Unidentified cyanobacterium, clone LD27	UCAJ7876	97	3
Cyano	*Spirulina* sp. P7	AF091109	90	1
Cyano	*Dermocarpella incrassata* PCC 7326	DIZ82807	93	1
Cyano	*Spirulina subsalsa*	AB003166	92	1
Cyano	*Prochlorothrix hollandica*	AF132792	94	1
Cyano	Unidentified cyanobacterium, clone LD27	UCAJ7876	97	1
Plastid(?)	*Flintiella sanguinaria* plastid	AF170715	92	1
Plastid(?)	*Ochrosphaera* sp. 181 plastid	OSSSRRNA	95	1
Plastid(?)	*Glaucocystis nostochinearum* plastid	GN16SRRNA	85	1
Plastid(?)	Uncultured eukaryote clone CR-PA43	AF141429	92	1
Unknown	Uncultured marine eubacterium HstpL29	AF159634	98	7
Protozoa	Uncultured *Treponema*, clone RFS8	AF068343	90	1
		Average	93	

Silicibacter lacuscaerulensis found in the 16S rDNA library.

Discussion

DNA extraction protocols and contamination

The SOIL DNA extraction protocol yielded the greatest number of species during DGGE analysis, proving it to be a more efficient extraction method than either the CTAB or GITC lyses. Moreover, a Gram-positive sequence was isolated in the 16S rDNA library made from the SOIL-extracted DNA, showing that this procedure can lyse these often problematic bacteria. The SOIL method consistently produced PCR quality DNA and it would be possible to process 50 + coral samples per day using this approach. Of the other protocols tested, CTAB worked the least well and was the most time consuming. Although the GITC protocol was slower than the SOIL method and probably did not result in DNA release from all of the bacterial species present, we were able to extract high-quality RNA from the coral/TE slurries with this protocol. Obtaining RNA may be important in future studies, and it should be possible to get more effective bacterial lysis with the GITC buffer by sonicating or shaking with glass beads. Pelleting the bacteria before isolating the DNA did not have an observable effect on the number of species observed on the DGGE gel. This may be explained by the fact that coral-associated bacteria are relatively large and therefore more amendable to pelleting (Paul et al. 1986).

Contamination is a major point of concern when working with environmental samples. Our procedures for isolating coral-associated bacterial DNA resulted in relatively little contamination from the surrounding water column. This is most likely due to the fact that the bacterial concentration on corals is much greater than in the surrounding water (Herndl and Velimirov 1986; Paul et al. 1986, unpublished observations).

Table 2 16S rDNA sequences from bacteria cultured from the same sample of *M. franksi* as presented in Table 1. Bacterial colonies were isolated on GASW plates, purified by restreaking, and the 5′ end of the 16S rDNA was sequenced and analyzed as described in Table 1. *CFB Cytophaga–Flavobacter/Flexibacter–Bacteroides*

| Group	Most closely related hit in GenBank	Accession	Identities over 550 bp (%)	(%) of total rDNAs sequenced
α	DSMP-degrading bacterium	L15345	99	1
α	Alpha proteobacterium MBIC1876	AB026194	98	1
α	Alpha proteobacterium MBIC1402	AB019044	92	1
α	Alpha proteobacterium MBIC3368	AB012864	95	3
γ	*Pseudoalteromonas haloplanktis* subsp. *tetraodonis* strain KMM 458	AF214729	97	3
γ	*Moritella viscosa*	MVY17574	98	1
γ	*Pseudoalteromonas haloplanktis* subsp. *tetraodonis* strain IAM 14160	AF214730	99	1
γ	*Pseudoalteromonas piscicida*	AF081498	93	3
γ	*Pseudoalteromonas* sp. A28	AF227238	89	3
γ	*Pseudoalteromonas* sp. S511-1	AB029824	97	3
γ	*Pseudoalteromonas* sp.	PLY15323	95	23
γ	*Pseudoalteromonas* sp. ER72M2	AF155038	99	3
γ	*Pseudomonas elongata* ATCC 10144T	AB021368	97	1
γ	*Pseudomonas plecoglossicida*	AB009457	99	1
γ	*Pseudomonas* sp. isolate S2	PSPAJ2813	96	1
γ	*Pseudomonas alcaligenes*	PAZ76653	100	1
γ	*Shewanella alga*	SH16RRNAB	99	1
γ	*Vibrio* sp. Lu1	AF094701	99	21
γ	*Vibrio tubiashi*	VT16SRRNA	100	17
CFB	Marine psychrophile IC076	MPU85882	93	1
CFB	*Microscilla furvescens*	MIORR16SB	98	1
CFB	*Flexibacter* sp. MED28	AF182015	99	1
CFB	*Cytophaga* sp. F12	AF125325	99	1
Bacillus/Clostridium	*Halobacillus litoralis*	X94558	99	1
Bacillus/Clostridium	*Bacillus megaterium*	BAC16SRR08	96	1
		Average	97	
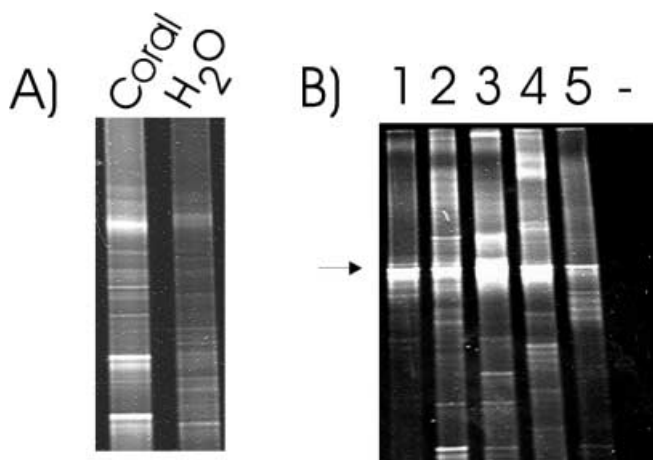
Fig. 3 Analysis of possible contamination sources. **A** Comparison of the bacterial 16S rDNA community associated with *M. franksi* (*Coral*) and surrounding water (*H₂O*); **B** DGGE analysis of coral-associated bacterial communities isolated from different locations (*1–5*) and from a negative control (*–*) (see Methods); *arrow* indicates a band found in all 25 *M. franksi* samples analyzed (only one representative sample from each site is shown). Sequence of band indicated by *arrow* was determined as described by Riemann et al. (1999)
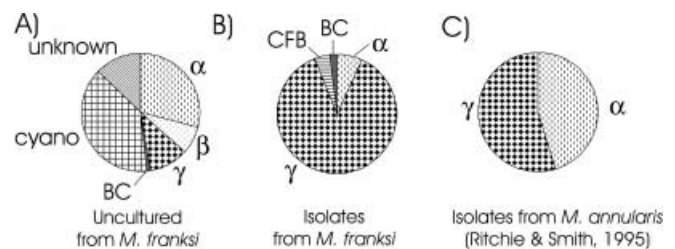

Fig. 4 Differences between coral-associated bacterial communities characterized by **A** harvesting DNA directly from coral samples and analyzing associated 16S rDNAs (this study), **B** collecting isolates and then characterizing them by 16S rDNA analysis or BIOLOG plates (this study), and **C** data derived from Ritchie and Smith (1995). *CFB Cytophaga–Flavobacter/Flexibacter–Bacteroides*; *BC Bacillus–Clostridium*

Patterns of microbial diversity

The species composition of the cultured microbial population from *M. franksi* differed dramatically from that of the uncultured population. This finding is consistent with results from other systems (Fuhrman and Campbell 1998), which have shown that the majority of microbes are not easily cultured using standard microbiological techniques. Additionally, the diversity of *Montastraea*-associated microbes is greater than previously realized. Figures 4B and C compare the bacterial groups obtained by culturing (this study; Ritchie and Smith 1995) with the groups determined by analyses of the 16S rDNA libraries obtained without culturing (Fig. 4A; this study). Cultured isolates consisted mostly of γ-proteobacteria (53–86%), while only 10% of the bacteria identified in the uncultured 16S rDNA library were

γ-proteobacteria. It is striking that cyanobacteria were exceptionally prevalent (36%) and diverse (eight species, six of which would be considered novel) in the uncultured library. It is also interesting that two species of β-proteobacteria were identified using our approach, because these bacteria are rarely found in the marine environment (Hagstrom et al. 2000). A total of 32 species were identified in the uncultured 16S rDNA library, compared to only 25 species in the cultured library.

The average relationship between the cultured isolates and previously characterized bacteria was significantly higher than that of the uncultured bacteria (97% versus 93% identity, respectively). Although taxonomic criteria based on sequence divergence are necessarily arbitrary (Ward 1998), bacteria with a 16S rDNA homology of 90–95% to other bacteria are often considered to be different species, and those with ≤ 90% homology are considered different genera. Using these values, 5 out of the 25 isolates analyzed in this study would be considered new species and 1 would be considered a new genus. In contrast, 16 of the 32 16S rDNA sequences in the uncultured library would be considered new species, and 8 of the 32 would be considered new genera.

Both culturing and the 16S rDNA libraries will be biased towards over-representation of certain microbial groups. It is well established, for example, that solid media and/or high nutrients favor the growth of certain bacteria (Fuhrman and Campbell 1998). Similarly, PCR analysis is biased by the number of 16S rDNA loci that a bacterial species has, with a tendency to over-represent the bacteria with the highest number of loci (Wintzingerode et al. 1997). It is interesting that there was no overlap between the isolates and the uncultured 16S rDNA sequences analyzed in this study. This shows that our characterization of coral-associated microbes, by either method, was not exhaustive. Characterizing microbial communities by sequencing alone means that isolates, with which physiology studies can be performed, are not available. However, culture-dependent techniques often overlook the most abundant bacteria, yielding an inaccurate representation of the true microbial community composition. By first sequencing and then optimizing culture conditions to isolate under-represented groups (e.g., cyanobacteria), the short-comings of both methods can be circumvented. Finally, neither method is useful for quantifying microbes. Therefore, an in situ hybridization approach for coral-associated microbes must be developed before detailed examinations of coral-associated microbial community structure can be made.

The finding that *M. franksi* may be involved in a specific association with a bacteria related to *Silicibacter lacuscaerulensis* is intriguing. This species belongs to a group of the α-proteobacteria called the Rhodobacteria, which are often phototrophs capable of fixing nitrogen. Previous studies have implicated nitrogen-fixing bacteria in coral metabolism (Wilkinson and Fay 1979; Williams et al. 1987; Shashar et al. 1994; Kuhl et al. 1995). Therefore, it is possible that the specific association we

have identified involves nitrogen cycling between *M. franksi* and a bacteria. Currently, we do not know the exact basis of this particular coral–microbe interaction, but the maintenance of the association between different reefs suggests that the relationship may be important to the health and growth of *M. franksi*. Identifying coral-associated microbes and characterizing their interactions with the coral host is essential for a complete understanding of the biology of coral reefs. Additionally, identifying members of the coral-associated microbiota in healthy corals and the stresses that disrupt these associations will offer another way of monitoring the condition of reefs. We believe that further investigations of coral–microbe associations will open a new chapter in coral reef ecology.

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