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SHORT COMMUNICATION

The diversity of the bacterial communities associated with the azooxanthellate hexacoral *Cirrhipathes lutkeni*

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This study examined the symbiotic microbiota of the hexacoral *Cirrhipathes lutkeni* using traditional plate culture, fluorescence *in situ* hybridization (FISH) and 16S rDNA characterization. FISH counts for the whole coral (holobiont) showed a major presence of γ-Proteobacteria (22%) and Actinobacteria (19%), followed by α-Proteobacteria (14%), Firmicutes (9%), *Cytophaga-Flavobacterium* (7%), β-Proteobacteria (6%) and Chloroflexi (2%). In contrast to the diversity observed by FISH, plate cultures were found to be selective for γ-Proteobacteria (22 cultures) with the exception of an Actinobacterium. The methods employed in this study detected 76% of all microbes estimated by DAPI staining of *C. lutkeni* homogenates. The absence of zooxanthellae in this particular hexacoral was confirmed by PCR and spectrophotometry using fresh tissue isolated from the holobiont. This is the first study describing the microbial associations of shallow-water hexacorallia, which opens further insight into coral microbial ecology and may enhance the search for novel natural products in the near future.

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Introduction

In contrast to studies on sponge microbiota (Webster and Hill, 2001; Hentschel *et al.*, 2003; Imhoff and Stöhr, 2003), studies on the bacterial diversity associated with non-diseased corals are rare. Most studies focusing on coral microbiota compare the microbe associations of healthy versus diseased scleractinians (Bourne and Munn, 2005; Yokouchi *et al.*, 2006; Klaus *et al.*, 2007). By contrast, little is known about the bacterial associations of soft corals, although many bioactive metabolites have been isolated from these organisms and symbiotic bacteria are thought to be source of these compounds (Fenical 1987; Schmidt *et al.,* 2000; Harder *et al.*, 2003; Penn *et al.*, 2006; Brück *et al.*, 2007; Webster and Bourne, 2007). As soft corals are also sensitive to pathogenic processes, it is important to define microbial associations in healthy individuals.

This study focuses on the bacteria associated with the Western Atlantic black wire-coral *Cirrhipathes lutkeni* (*Order: Hexacorallia*, Genus: *Antipatharia*). *Hexacorallia* include approximately 200 recognized species of black corals (Echevarría, 2002). Research on these organisms, which mostly inhabit deep water, is limited due to complications associated with collecting in these environments. Compared to other black corals, *C. lutkeni* is found at shallower depths (below 30 m); yet very little is known about the biology of this particular coral genus. While many shallow-water hard and soft corals are known to harbor endosymbiotic dinoflagellates (zooxanthellae, *Symbiodinium* sp.), it is unknown whether *C. lutkeni* also requires endophytic symbionts for long-term survival (Rosenberg *et al.*, 2007).

To analyze the bacterial communities of *C. lutkeni*, we used species-specific fluorescent *in situ* hybridization (FISH) oligonucleotide probes and traditional plate culture along with PCR-based 16S rRNA gene identification of bacterial isolates (Brück

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et al., 2007). The presence of endophytic symbionts was assessed using dinoflagellate-specific PCR probes and spectrophotometric methods detecting dinoflagellate chlorophyll absorbance patterns.

Materials and methods

All chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA). Preformulated bacterial media were supplied by Difco Laboratories (Detroit, MI, USA).

Several colonies of *C. lutkeni* were collected by scuba in June 2005 at the Jim Atria wreck site, Pompano Beach, Florida at a depth of 45 m (27 °C). Live coral specimens were handled aseptically and homogenized as previously described (Brück *et al.*, 2007). Plate cultures were prepared by spreading dilute coral homogenate on Nutrient (NA) and diluted Marine Agar (MA, 9.2 g Marine Agar 2216, 12.5 g Bacto agar, 1 l 83% artificial sea water) and incubated aerobically at 27 °C (Webster and Hill, 2001). Pure cultures were isolated over a 3-month period and sequenced (Brück *et al.*, 2007). All isolates were assessed using Gram stain and motility (hanging drop technique) to confirm 16S rRNA gene sequence identities. Testing for the presence of dinoflagellate symbionts in coral tissue was carried out using standard PCR and spectrophotometric techniques (Brück *et al.*, 2007).

Bacterial counts per sample were estimated with DAPI staining as well as with a general eubacterial and several species-specific FISH probes according to established protocols (Brück *et al.*, 2007).

Seawater (3×10 l) was collected and passed through 0.2 μm Nylon filters (Millipore, Bedford, MA, USA). The retentate was fixed on the filter matrix with 100% (v/v) ethanol and used for FISH analysis using the same probes used to analyze the coral.

Phylogenetic trees of the 16S rRNA data were constructed using ARB (Ludwig *et al.*, 2004). Novel sequences were paired with known sequences from the NCBI database to show their relatedness. Sequences are available in GenBank (DQ857736 through DQ857758).

Results and discussion

FISH analysis of *C. lutkeni* showed that species-specific probes covered 76% of the total microbial diversity visualized by DAPI staining (Figure 1). The γ- (22%), α-Proteobacteria (14%) and Actinobacteria (19%) were the most abundant groups. γ-Proteobacteria are the most common group in other tropical soft-corals (Harder *et al.*, 2003; Brück *et al.*, 2007; Webster and Bourne 2007), whereas many shallow-water scleractinians feature α-Proteobacteria as the major group (Yokouchi *et al.*, 2006; Klaus *et al.,* 2007).

The remaining bacterial groups in *C. lutkeni* are Firmicutes (9%), *Cytophaga-Flavobacterium* (7%), β-Proteobacteria (6%) and Chloroflexi (2%), which were also a minor fraction of the bacterial microbiota

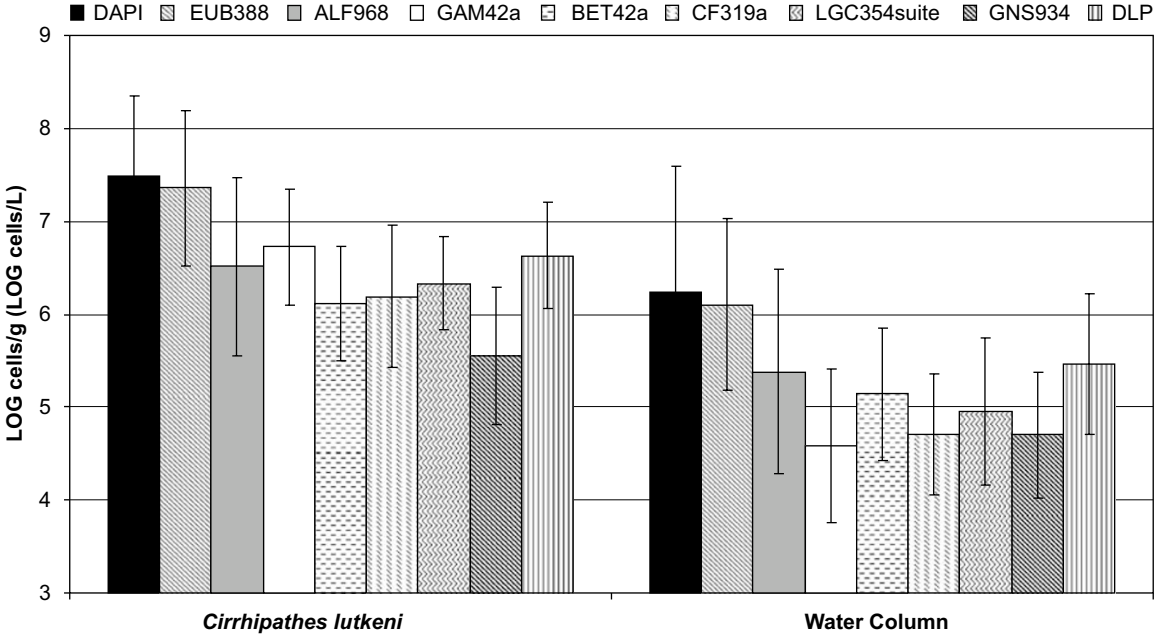

Figure 1 Log average populations of bacteria associated with *C. leutkeni* and the surrounding water column estimated by means of FISH and DAPI counting. Error bars are ±1 standard deviation. LOG cells per gram of coral is the logarithmic (base 10) cell count (bacteria) per gram of coral (wet weight). LOG cells per liter is the logarithmic (base 10) cell count (bacteria) per liter of water column (wet weight). Probes stated in legend are specific for the following organisms: DAPI: total counts (all DNA present in sample), EUB338: total counts (most bacterial genera), ALF968: α-Proteobacteria, BET42a: β-Proteobacteria, GAM42a: γ-Proteobacteria, CF319a: *Cytophaga-Flavobacterium*, LGC354suite: Firmicutes, GNS934: Chloroflexi, DLP: Actinobacteria.

of other tropical soft corals. Approximately 24% of the organisms detected by DAPI and EUB338 were not covered by the species-specific FISH probes used here. Other studies using different types samples (human and rhesus monkey feces) reported comparable levels of species coverage (approximately 31%–98% detection with probes used) (Brück *et al.*, 2006, 2003). It may have been possible to observe a larger number of the total microbiota by FISH with the use of additional probes specific for archaea and other organisms.

However, as was previously observed in other studies, detection of all unknown organisms contained in the sample may be impossible by existing FISH methods due to the relatively high detection limit of approximately 10^3–10^4 target cells per ml (Loy *et al.*, 2002).

In comparison, previously examined octocoral homogenates indicated that α-, β- and γ-Proteobacteria were the most common bacterial genera observed (Brück *et al.*, 2007). Actinobacteria were only a minor component of the microbiota (1%–3%). Again, a large number of the bacteria detected by either DAPI or EUB338 were not covered by the species-specific FISH probes. This was particularly prevalent in homogenates of *Iciligorgia schrammi* where 51% of the bacteria were not covered by FISH probes used in the study. The prevalence of γ-Proteobacteria in both *C. lutkeni* and octocoral samples suggests that they may have a symbiotic function related to nutrient uptake as was previously observed in *Rhopaloeides odorabile* and *Pocillopora damicornis* (Bourne and Munn, 2005). The bacterioplankton background from the water column is a problem when assessing the resident microbiota of marine species (Hentschel *et al.*, 2003). FISH analysis of the surrounding water column showed that Actinobacteria (23%) and α-Proteobacteria (19%) were the major groups while γ-Proteobacteria (11%) was less prominent, indicating a different species distribution in the water sample compared to the *C. lutkeni* sample set (Figure 1).

Bacterial cultures resulted in 23 distinct bacterial isolates, almost exclusively γ-Proteobacteria (Figure 2), suggesting high selectivity of culture conditions for this group. Culture conditions can influence the distribution of phylogenetic groups in microbial isolates (Imhoff and Stöhr, 2003). Additional information on bacterial characterization can be found in Table 1. The species distribution of *C. lutkeni* bacterial isolates resembled those of

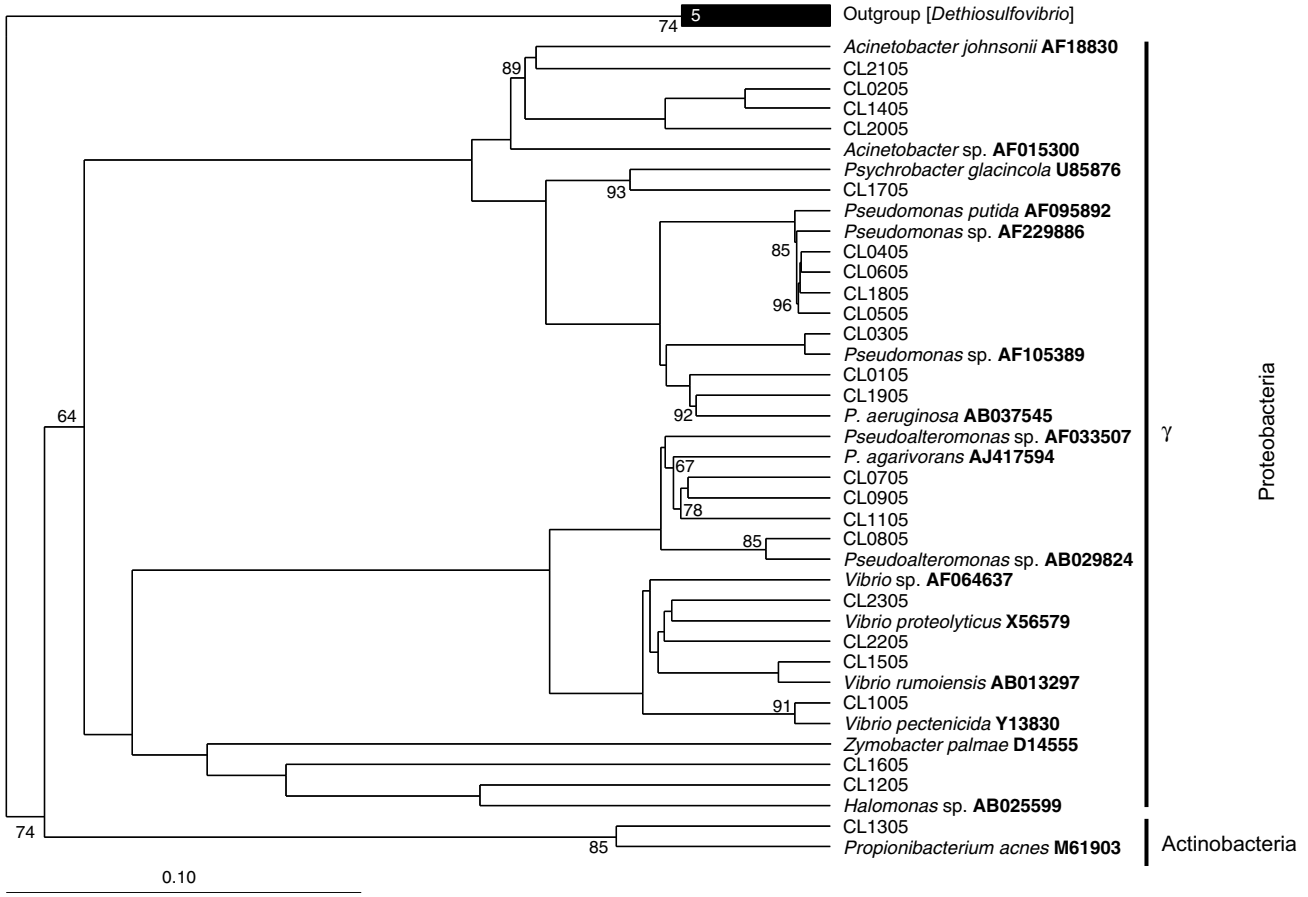

Figure 2 *De novo* phylogenetic tree of culturable bacteria isolated from *C. lutkeni*. Numbers at nodes are percentages indicating levels of bootstrap support, based on neighbor-joining analysis of 1000 re-sampled data sets. Reference sequences derived from previous studies and GenBank entries are described in the text or are written with their corresponding accession numbers.

Table 1 *Cirrhipathes lutkeni* isolates

Isolate	*GenBank no.*	*Cell morphology*	*Gram stain*	*Motility*	*Phylogenetic association*	*Closest GenBank match*	*Similarity (%)*	*Accession no.*
CL0105	DQ857736	Rods, single	–	+	γ-Proteobacteria	*Pseudomonas resinovorans*	99	AB021373
CL0205	DQ857737	Coccobacilli, pairs	–	–	γ-Proteobacteria	*Acinetobacter johnsonii*	99	EF114343
CL0305	DQ857738	Rods, single	–	+	γ-Proteobacteria	*Pseudomonas* sp. R-25343	99	AM084028
CL0405	DQ857739	Rods, single	–	+	γ-Proteobacteria	*Pseudomonas* sp.	99	AM410621
CL0505	DQ857740	Rods, single	–	+	γ-Proteobacteria	*Pseudomonas* sp.	99	AM411620
CL0605	DQ857741	Rods, single	–	+	γ-Proteobacteria	*Pseudomonas* putida S18	99	DQ387442
CL0705	DQ857742	Rods, single	–	+	γ-Proteobacteria	*Pseudoalteromonas* sp.	99	AB106189
CL0805	DQ857743	Rods, pairs	–	+	γ-Proteobacteria	*Pseudoalteromonas* sp.	98	EF648109
CL0905	DQ857744	Rods, single	–	+	γ-Proteobacteria	*Pseudoalteromonas tetraodonis*	99	AB257325
CL1005	DQ857745	Rods, single	–	+	γ-Proteobacteria	*Vibrio* sp.	99	AF500207
CL1105	DQ857746	Rods, single	–	+	γ-Proteobacteria	*Pseudoalteromonas* sp.	99	EF648126
CL1205	DQ857747	Rods, single	–	+	γ-Proteobacteria	*Halomonas* sp.	94	AY745837
CL1305	DQ857748	Rods, single	+	–	Actinobacteria	*Propionibacterium acnes*	92	AY642053
CL1405	DQ857749	Short rods, pairs	–	–	γ-Proteobacteria	*Acinetobacter* sp.	99	AJ244764
CL1505	DQ857750	Rods, single	–	+	γ-Proteobacteria	*Vibrio* sp.	99	DQ480135
CL1605	DQ857751	Rods, single	–	+	γ-Proteobacteria	*Cobetia* sp.	96	EF198244
CL1705	DQ857752	Coccobacilli, pairs	–	–	γ-Proteobacteria	*Psychrobacter pacificensis*	99	EF179615
CL1805	DQ857753	Rods, single	–	+	γ-Proteobacteria	*Pseudomonas* sp.	99	DQ229316
CL1905	DQ857754	Short rods, pairs	–	–	γ-Proteobacteria	*Acinetobacter* sp.	95	AY177359
CL2005	DQ857755	Short rods, clusters	–	–	γ-Proteobacteria	*Acinetobacter* sp.	97	AY576723
CL2105	DQ857756	Coccobacilli, single	–	–	γ-Proteobacteria	*Acinetobacter* sp.	96	AF417863
CL2205	DQ857757	Rods, single, curved	–	+	γ-Proteobacteria	*Vibrio* sp.	98	AF410778
CL2305	DQ857758	Rods, single	–	+	γ-Proteobacteria	*Vibrio* sp.	97	AF500207

tropical octocorals previously isolated under equivalent culture conditions, which inferred formation of region-specific microbial associations (Brück *et al.*, 2007). Most isolates belonged to the Pseudomonaceae which have been previously associated with marine sediment, octocorallia, scleractinians and the water column (Suzuki *et al.*, 1997; Bourne and Munn, 2005; Penn *et al.*, 2006; Brück *et al.*, 2007). Even though the two isolates of *Halomonas* sp. and *Cobetia* sp. detected here were of low sequence homology (94% and 96%, respectively), it may be inferred that their presence appears to be specific to *C. lutkeni.* The abundance of *Halomonadaceae* was also reported in deep-water black coral (Penn *et al.*, 2006), which may indicate a species-specific microbial association (Hentschel *et al.*, 2003). It is interesting to note that while most organisms found here are common in an marine environment, *Vibrio* sp. have often been associated with a variety of coral diseases (Knowlton and Rohwer, 2003; Cervino *et al.*, 2004; Bourne and Munn, 2005). However, no pathological changes were observed in *C. lutkeni*, indicating that the *Vibrio* sp. may be part of the natural microbiota. The single isolate belonging to the Actinobacteria, *Proprionibacterium* sp., has never been isolated from a marine source and based on its low sequence similarity (92%) to other GenBank deposits, may actually represent a new genus of Actinobacteria. Also of note is the identification of *Acinetobacter johnsonii* in association with this coral. Although this genus is commonly found in terrestrial and oil-polluted marine environments and in association with marine invertebrates (Alvarez *et al.*, 1997; Juni, 2005; Sfanos *et al.*, 2005), *A. johnsonii* is typically found in clinical specimens, activated sludge and foodstuffs (Juni, 2005). There has been one study in which this bacterium was found in association with a marine sponge (Li *et al.*, 2006); however, further work will be needed to show whether this bacterium is a natural part of the bacterial assemblage of marine invertebrates or whether its presence is due to pollution of the marine environment.

The presence of symbiotic dinoflagellates in *C. lutkeni* was assessed by PCR and spectrophotometric methods. Dinoflagellate-specific chlorophyll (a and c2) absorbance patterns and PCR signals could only be detected in the positive control, the zooxanthellate octocoral *Pseudopterogorgia elisabethae*, but not in the *C. lutkeni* sample. The lack of symbiotic dinoflagellates in *C. lutkeni* (data not shown) is consistent with previous observations that certain species of octo- and hexacorals, which thrive in light-deprived environments, do not require algal symbionts for survival (Penn *et al.*, 2006; Brück *et al.*, 2007).

To the best of our knowledge, this is the first study examining the microbial associations of moderate depth hexacorals (40–100 m). This study provides a preliminary assessment of the microbial ecology of hexacoral-specific microbial associations. Evaluation of microbial isolates for the production of novel bioactive metabolites is currently underway in our laboratory. As *Halomonas* sp. are excellent producers of poly-hydroxyalkanoates, the novel isolates may also be of interest for the industrial production of biopolymers.

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