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Community composition of bacteria associated with cold-water coral *Madrepora oculata*: within and between colony variability

Lina Hansson[1,2], Martin Agis[1,2], Cornelia Maier[1,3], Markus G. Weinbauer[1,2,*]

[1]Microbial Ecology & Biogeochemistry Group, Université Pierre et Marie Curie-Paris 06, Laboratoire d'Océanographie de Villefranche, 06234 Villefranche-sur-Mer, France

[2]Centre National de la Recherche Scientifique (CNRS), Laboratoire d'Océanographie de Villefranche, 06234 Villefranche-sur-Mer, France

[3]Department of Biological Oceanography, Royal Netherlands Institute for Sea Research (NIOZ), PO Box 59, 1790 AB Den Burg, Texel, The Netherlands

ABSTRACT: Cold-water coral ecosystems are more widespread, diverse and productive than previously thought. However, little is known about the interaction of deep-water corals with microorganisms. To understand whether coral species have specific prokaryotic communities, it is necessary to assess the within and between colony variability. This was studied based on 16S rRNA gene and denaturing gradient gel electrophoresis (DGGE) for one of the main cold-water corals *Madrepora oculata* at Rockall Bank off the coast of Ireland. We successfully applied a rapid, non-toxic and inexpensive method for extracting DNA for 16S rRNA gene fingerprinting of marine prokaryotic communities based on a heat and salt lysis with simultaneous salt extraction (HEATSALT). The within and between colony variability of the community composition of bacteria associated to the mucus and ectodermal tissue of *M. oculata* was then evaluated using a 16S rRNA gene PCR and DGGE approach. Bacterial community composition (BCC) clearly differed between living coral and reference samples (dead coral and surrounding water; 80% dissimilarity). A large within (35–40% dissimilarity between polyps) and between colony variability (ca. 50% dissimilarity) of BCC was detected. We also found preliminary evidence that BCC differed between *M. oculata* and *Lophelia pertusa*. The high intraspecific variability found has consequences for selecting sampling strategies when assessing bacterial diversity and refines the question of controlling mechanisms of bacterial diversity on corals. Sequencing of DGGE bands showed that *Spongiobacter* type phylotypes (STP) dominated the DGGE bands. STP of *M. oculata* were grouped together and were different from those detected in other corals and sponges. In addition, the high sequence diversity of STP suggests specific ecological roles and adaptations of this group in *M. oculata*.

KEYWORDS: DNA extraction · Diversity · *Spongiobacter* · *Lophelia pertusa*

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INTRODUCTION

Tropical corals have been intensively investigated, e.g. with respect to their associations with algae, their ecology and the ecosystem they populate. However, it is not until recently that attention has also focussed on corals inhabiting dark cold-water ecosystems (Roberts et al. 2006). *Lophelia pertusa* and *Madrepora oculata* are important species as main frame-builders of such ecosystems, which sustain a large biodiversity and biomass of associated fauna (Rogers 1999). These habitats are partially threatened by trawl fisheries. A further and potentially more substantial threat is the decrease in pH due to the increased atmospheric pCO_2, since a drop in pH might impair calcification (Turley et al. 2007). Deep-water coral ecosystems are abundant

*Corresponding author. Email: wein@obs-vlfr.fr

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above the aragonite saturation level, i.e. the depth where aragonite dissolves. As predictions indicate that the saturation level will become shallower due to ocean acidification (Orr et al. 2005), the cold-water coral ecosystems could be negatively affected within the next decades. A negative effect of reduced pH on calcification rates has been confirmed for *L. pertusa* (Maier et al. 2009).

Madrepora oculata Linneaus, 1758 is a cosmopolitan scleractinian coral (family *Oculinidae*) with a depth range of ca. 50 to >1500 m. Its geographical and depth distribution is similar to that of *Lophelia pertusa*, although there are also *L. pertusa* reefs without *M. oculata* colonies. *M. oculata* has a weaker and more fragile skeleton compared to *L. pertusa*. Isotope analysis suggests that zooplankton and phytodetritus are the main food sources of these coral species (Duineveld et al. 2004, Kiriakoulakis et al. 2005). There is also evidence that the seasonal spawning is triggered by phytodetrital food fall (Waller & Tyler 2005). In addition, *M. oculata* has an enormous potential for mucus production (Reitner 2005).

Although it has long been known that the coral mucus can harbour large numbers of bacteria, only recently have bacteria been investigated in more detail. For example, a large diversity of *Bacteria* and *Archaea* in the surface mucus layer of corals have been demonstrated (Rohwer et al. 2002, Kellogg 2004, Ritchie & Smith 2004, Bourne & Munn 2005). There is evidence that bacteria associated with coral mucus produce antibiotics as defence mechanisms against free-living bacteria, including potential pathogens (Ritchie 2006). It was recently suggested that coral-associated bacteria (including those in the tissue) function as an equivalent to an immune system (coral probiotic hypothesis) (Reshef et al. 2006). In addition, it has been argued that the prokaryotic community could be a way to counteract environmental disturbances such as those caused by climate change (Rosenberg et al. 2007). Bacteria have been investigated in *Lophelia pertusa* ecosystems (Jensen et al. 2008a, Jensen et al. 2008b), and studies on bacterial diversity are now available for this species (Yakimov et al. 2006, Neulinger et al. 2008, Neulinger et al. 2009, Kellogg et al. 2009, Schöttner et al. 2009). Mucus release has also been demonstrated for cold-water corals (Wild et al. 2008).

To our best knowledge, there is no study on the prokaryotic community composition associated with *Madrepora oculata*. To understand whether coral species have specific prokaryotic communities, it is necessary to assess the within and between colony variability. This was studied based on 16S rRNA gene and denaturing gradient gel electrophoresis (DGGE) for one of the main cold-water corals, *M. oculata* at Rockall Bank off the coast of Ireland. We found that (1) *M. oculata* harbours a specific bacterial community, which is different from the surrounding water or dead corals, (2) there is a large between and within colony variation and (3) *M. oculata* prokaryotic communities are likely different from those of *Lophelia pertusa*.

MATERIALS AND METHODS

Study site and sampling. The sites for sampling of corals were located on the southeastern Rockall Banks (Logachev Mounds). Two complexes were visited, the Clan mounds (Artur mound: 55.44° N, 16.08° W) and the Haas mounds (center: 55.49° N, 15.79° W), which are ca. 20 km apart. The coral mounds at the Rockall Bank slope are found between ca. 550 and 900 m depth (van Duyl et al. 2008). Sampling was performed during the BIOSYS/HERMES cruise (June 21 to July 21, 2005) with the RV 'Pelagia'. Specimens of *Lophelia pertusa* and *Madrepora oculata* were collected from 573 to 781 m. Corals from 6 box cores were finally used in this study (Table 1).

A box corer (BCK 18; 50 cm inner diameter, 55 cm high), which closes with a metal blade upon reaching the sea floor, was used to collect bottom samples, i.e. corals and surrounding water (van Duyl et al. 2008). Care was taken to collect samples only from healthy looking tissue without detectable epigrowth or impurities such as sediment grains. Under such conditions, DGGE profiles do differ significantly from corals rinsed with sterile seawater (Großkurth 2007). Coral samples from the box cores were immediately placed in a plastic tray covered with aluminum foil, transported to a laminar flow hood, and mucus bacteria were collected by gently rolling a sterile cotton stick on the coral piece. In addition, the tissue of single polyps was stripped off with cotton sticks. Dead corals were swabbed to collect the bacteria in the biofilm. The cotton tips of the swabs were cut off into sterile 2 ml Eppendorf tubes and were stored at –80°C until analysis.

Table 1. Type and number of samples collected at the Clan and Haas mounds

Sample	Mound	Number of samples analysed		
		Corals	Dead corals	Water
Box core 35	Clan	3	1	1
Box core 46	Haas	1	0	1
Box core 65	Haas	4	0	0
Box core 75	Clan	1	0	0
Box core 153	Haas	1	0	0
Box core 170	Clan	1	0	0

Table 2. Sampling stations, characteristics and number of denaturing gradient gel electrophoresis (DGGE) bands of *Bacteria* and *Archaea* for samples, where heat and salt lysis with simultaneous salt extraction (HEATSALT) protocol was applied. Samples are from the Mediterranean Sea, except for box core and water crate samples, which are from the North Atlantic. *T*, temperature; S, salinity; C, coral ecosystem; W, water; DCM, deep chlorophyll maximum; ND, not detected; NEC, no extraction comparison. Note: all box core and water crate samples are from cold-water coral ecosystems

Sample	Depth (m)	Type of environment	Sampling date	*T* (°)[a]	S	No. of bands[b] *Bacteria*	*Archaea*
Box core 35	781	C	28 Jun 2005	7.7	35.25	9 (6)	ND
Box core 46	580	C, W	29 Jun 2005	9.0	35.34	10 (11)	ND
Box core 65	600	C	01 Jul 2005	9.0	35.34	NEC	NEC
Box core 67	617	W	26 Jun 2005	9.0	35.34	ND	9 (9)
Box core 75	767	C	04 Jul 2005	7.6	35.25	9 (10)	ND
Box core 153	573	C	11 Jul 2005	9.0	35.34	10 (10)	ND
Box core 170	754	C	11 Jul 2005	7.6	35.25	10 (8)	ND
Water crate	767	W	24 Jun 2005	7.6	35.25	ND	8 (8)
Point B	5	W	12 Mar 2008	12.1	38.12	17(17)	ND
Pier	0.5	W	10 Mar 2008	13.1	–	24 (24)	ND
DYFAMED	5	W	3 Mar 2005	13.9	38.35	26 (26)	10
DYFAMED	5	W	31 Mar 2004	13.2	38.35	11 (11)	5 (5)
DYFAMED	110	W, DCM	31 Mar 2004	13.1	38.42	11 (11)	6 (6)
DYFAMED	2000	W	31 Mar 2004	13.1	38.44	ND	7 (7)
DYFAMED	5	W	30 Nov 2003	16.0	38.42	17 (17)	ND
DYFAMED	30	W, DCM	30 Nov 2003	13.5	38.42	23 (23)	12 (12)
DYFAMED	2000	W	30 Nov 2003	13.1	38.43	ND	8 (8)

[a] *T* was not measured in the water crate or box cores; however, CTD casts from the same area (Haas vs. Clan mounds) and depth during the cruise were used to determine *T* and S of ambient seawater
[b] Data are no. of bands in HEATSALT protocol; data in brackets are from reference protocol. Note: same no. of bands corresponds to identical bands (i.e. same position on different lanes)

Water samples from the cold-water coral ecosystems were obtained from the box cores. Additional samples were collected with a 1000 l water-crate, which closes ca. 50 cm above the bottom, when a mechanical release trigger touches the bottom (van Duyl et al. 2008) (Table 2). Cells from 1.5 to 3 l samples were collected on 0.2 µm polycarbonate membrane filters (GTTP 47 mm; Millipore) by low pressure (ca. 100 mm Hg) vacuum filtration. An additional water sample from a box core, which contained sediment, was pre-filtered through 0.8 µm polycarbonate filters (ATTP 47 mm, Millipore) to reduce the sediment load before 0.2 µm filtration. Filters were stored at –80°C until further analysis.

In order to test a new DNA extraction method, additional samples were obtained from coastal, offshore and deep marine waters of the Mediterranean Sea to cover a variety of environments (Table 2). Coastal surface water was collected at the pier of the institute in Villefranche-sur-Mer (France) and at station Point B (43.68° N, 7.32° E) at the entrance of the Bay of Villefranche (water depth ca. 100 m). Offshore and deep-water samples were collected at the station DYFAMED (43.42° N, 7.85° E; 30 nautical miles off the NW French Mediterranean coast) along depth profiles down to 2000 m (water depth ca. 2300 m).

DNA extraction. ***Reference protocol:*** We used an extraction with repeated freeze–thaw cycles, a proteinase K-lysozyme digestion and phenol-chloroform purification as reference (Moeseneder et al. 1999, Winter et al. 2001). Briefly, cell lysis was achieved by 4 freeze (liquid nitrogen) and thaw (37°C) cycles and subsequent enzymatic digestion with lysozyme (final concentration 1.25 mg ml^{-1}) for 30 min at 37°C, and with proteinase K (final concentration 100 µg ml^{-1}) for 120 min at 55°C. DNA was then extracted with phenol-chloroform-isoamylalcohol (25:24:1) and chloroform-isoamylalcohol (24:1) (Moeseneder et al. 1999, Winter et al. 2001). Precipitation of DNA was performed using 100% isopropanol (2-propanol). Nucleic acids were then washed with 500 µl 70% ethanol using centrifugation, and after removal of the supernatant the pellets were dried in a vacuum concentrator (Concentrator 5301; Eppendorf). Finally, the dried pellets were resuspended in autoclaved MilliQ water (typically in 20–60 µl). This protocol should allow for high extraction efficiency, since it combines mechanical and chemical disintegration of cells.

HEATSALT protocol: We also tested a simplified extraction protocol on filter halves or halves of cotton swabs against the other halves, which were processed using the reference protocol. The simplification of the protocol occurred in several steps and the final protocol basically combines a heat/salt lysis with a simultaneous salt extraction step (Miller et al. 1988). This HEATSALT protocol was used as follows: 600 µl of TE* (50 mM Tris, 20 mM EDTA; pH 8; note slight differences in Tris/EDTA to normal TE) and 300 µl of a ca. 5 M NaCl solution were added to the filter or cotton stick pieces in a 2 ml microcentrifuge tube. The samples were incubated in a Thermomixer Comfort

(Eppendorf) at 80°C for 30 min while shaking at 850 rpm. After incubation the liquid was transferred into a new tube and then centrifuged at 16 000 × *g* for 20 min to pellet the salt-protein precipitate. Subsequently, the supernatant was transferred into a new tube. Nucleic acids were then precipitated with 1 volume of 100% isopropanol (2-propanol) while inverting the tubes several times. The samples were centrifuged again (as above), and the supernatant was carefully removed. Nucleic acids were then washed with 500 µl 70% ethanol using centrifugation, and after removal of the supernatant the pellets were dried in a vacuum concentrator (Concentrator 5301; Eppendorf). Finally, the dried pellets were resuspended in autoclaved MilliQ water (typically in 20–60 µl). DNA extracted for corals with the reference and the HEATSALT protocol was further purified using a desalting and DNA concentration kit (QiaExII, Qiagen) according to the protocol of the manufacturer.

PCR, DGGE and sequencing. For an assessment of the relative amount and quality of the extracted DNA, extracted DNA samples were run on agarose gels beside a DNA mass ladder. PCR and DGGE were used to determine whether the 2 protocols yielded identical fingerprints. Amplification of DNA fragments was performed using a touchdown PCR approach as described in Schäfer & Muyzer (2001). Between 0.5 and 5 µl of nucleic acid extract were used as template in a 50 µl PCR reaction (2 mM $MgCl_2$, 0.25 µM of each primer, 0.25 mM dNTP and 1.25 U Taq polymerase; Fermentas) together with a positive and a negative control. A fragment of the 16S rRNA gene was amplified using the primer pairs 341F-GC/907R and 344F-GC/915R for *Bacteria* and *Archaea* respectively (Casamayor et al. 2000, Schäfer & Muyzer 2001).

DGGE procedures followed those described by Schäfer & Muyzer (2001). The same amount of PCR product per sample, i.e. ca. 500 ng (as quantified on agarose gels using a known amount of a DNA ladder on a parallel lane), was loaded onto DGGE gels. Electrophoresis was run for 18 h at 100 V using 6% acrylamide/bis-acrylamide gels with a denaturing gradient of 30 to 70% (urea and formamide) using an INGENYphorU DNA Mutation Detection System (Ingeny International). DGGE gels were photographed with a gel documentation system (GelDoc EQ; Bio-Rad) after 20 min staining with a 10× SYBR Gold solution (Molecular Probes; # S11494) using a variety of exposure times. Analysis of band patterns between lanes of the same gel was performed with the Quantity One Software (Bio-Rad). Relative band intensities were also assessed. Visual and software based inspection suggested a reproducible detectability when relative band intensity exceeded 2.5% of total band intensity within a lane. Bands were excised from gels using a sterile scalpel. DNA was eluted from the gel overnight at 4°C in RNase free H_2O. Sequencing was performed by MWG-Biotech using the 907R primer.

Phylogenetic analysis. BLAST (Altschul et al. 1990) was used to compare sequences against the GenBank database. Sequences derived from DGGE bands were aligned with the ClustalW2 2.0.10 software (Larkin et al. 2007). The FindModel program (www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html), which is based on ModelTest (Posada & Crandall 1998), was used to find the best evolution model that fits the data set. A maximum-likelihood tree was calculated with PhyML v3.0 (Guindon & Gascuel 2003) based on the sequence evolution model from Tamura & Nei (1993) plus gamma distribution with 100 bootstrap replications. The phylogram was displayed with TREEVIEW 1.6.6 (Page 1996) and edited manually to add name or description, accession number and source for the sequences used for tree calculation. The tree was rooted with *Escherichia coli* (Gammaproteobacteria) as an outgroup.

Statistics. Potential differences in the relative amount of extracted DNA and PCR product yield by the 2 extraction methods was tested using a Wilcoxon test for paired samples. A probability (p) of <0.05 was considered significant.

Statistical analysis of gels was carried out using R (R Project for Statistical Computing, R 2.6.1). Statistical analysis of band patterns was performed using the relative band intensity within a lane as well as a binary matrix of presence vs. absence data. Cluster diagrams were created using the unweighted pair group method with arithmetic mean (UPGMA). The Jaccard similarity coefficient was used for binary data (presence vs. absence of bands), whereas the Bray-Curtis similarity was used when the relative intensity of each band was also taken into account. In addition, multidimensional scaling (MDS) analysis was done in parallel to further elucidate the differences in DGGE profiles. Analysis of similarities (ANOSIM) were also performed using the R package Vegan. The program returns an R-value between –1 and +1 and the significance level; a probability (p) of <0.05 was considered significant. The generated R value indicates the differences between DGGE profiles, a value of zero representing the null hypothesis H_0 (there is no difference between groups).

RESULTS AND DISCUSSION

Yield of genomic DNA, PCR yield and HEATSALT extraction

Filter or cotton halves from several sites (Table 2) were extracted with the reference and the HEATSALT protocol. For both protocols, genomic DNA was intact

for free-living communities (data not shown). Some smear could be observed for the coral extracts — on these gels we cannot distinguish prokaryotic DNA from eukaryotic coral DNA, since it is not possible to sample mucus by swabbing without contamination by coral tissue. Sheared or partially degraded genomic DNA does not necessarily impair prokaryotic fingerprint techniques and it has to be noted that shearing or degradation could not be detected for the free-living community. Other extraction techniques such as using bead beating always results in sheared DNA (Weinbauer et al. 2002).

For communities from deep-marine waters, genomic DNA was not always visible, probably due to the low number of prokaryotes present. For example, prokaryotic abundance in the investigated water column environment is typically 10^8 to 10^9 cells l^{-1} except for samples from 2000 m, where abundance is around 10^7 cells l^{-1} (e.g. Tanaka & Rassoulzadegan 2002, van Duyl et al. 2008). The reference protocol often showed a slightly higher extraction yield, however, there were also occasions where the extraction yield was slightly higher for the HEATSALT protocol (e.g. surface water) or where the yield was similar for both extraction protocols. Across samples, the extraction efficiency did not differ significantly (Wilcoxon, $p > 0.1$) between the 2 extraction methods. This suggests that the HEATSALT protocol yields quantities of DNA roughly comparable to standard protocols.

Amplicons for *Bacteria* could be obtained for all coral samples and all water column samples except the box core and the water crate and 2 deep (2000 m) DYFAMED samples. For *Archaea*, a PCR product could be obtained for all water samples except the 2 coastal samples and 1 DYFAMED sample. For the *Archaea* surface sample from DYFAMED 2003, we could not amplify enough PCR product for DGGE analysis. For the coral swabs, no archaeal PCR product was obtained.

Co-extracted substances, which are inhibitory to PCR, can be a potential problem for genetic fingerprints (von Wintzingerode et al. 1997). Thus, we tested the yield of the PCR amplification for the HEATSALT protocol using equivalent volumes of DNA extract (data not shown). In most cases, PCR product yield was similar for the 2 protocols. In some samples, such as for *Bacteria* in the deep chlorophyll maximum (DCM) at DYFAMED and *Archaea* in surface waters, the PCR product yield was higher for the reference protocol. However, for other samples such as *Bacteria* in surface water at DYFAMED and for *Archaea* in the DCM layer, the product yield was higher with the HEATSALT protocol. Overall, comparison with the reference protocol showed no systematic differences between the 2 protocols with respect to PCR product yield. Across samples, the PCR product yield did not differ significantly (Wilcoxon, $p > 0.2$) between the 2 extraction methods. This suggests that the HEATSALT protocol yields quantities of PCR amplicons roughly comparable to standard protocols.

Prokaryotic community structure and HEATSALT extraction

The number of bacterial DGGE bands from *Madrepora oculata* in the comparison of DNA extraction protocols ranged from 6 to 10 (Fig. 1, Table 2). In one of the samples from *M. oculata*, the presence vs. absence pattern of bands was identical for the 2 protocols (Table 2). In 2 others, the number of bands was higher (by 2 to 3 bands) for the HEATSALT protocol than for the reference protocol, while for 2 samples, the opposite trend was observed (by 1 band). Using a cluster analysis based on relative band intensity, band patterns from all specimens clustered correctly together, independent of the extraction method (Fig. 2).

For free-living prokaryotes, between 17 and 26 bands were detected for *Bacteria* and between 5 and 12 for *Archaea*. Some slight differences between protocols seem to exist in relative band intensities of iden-

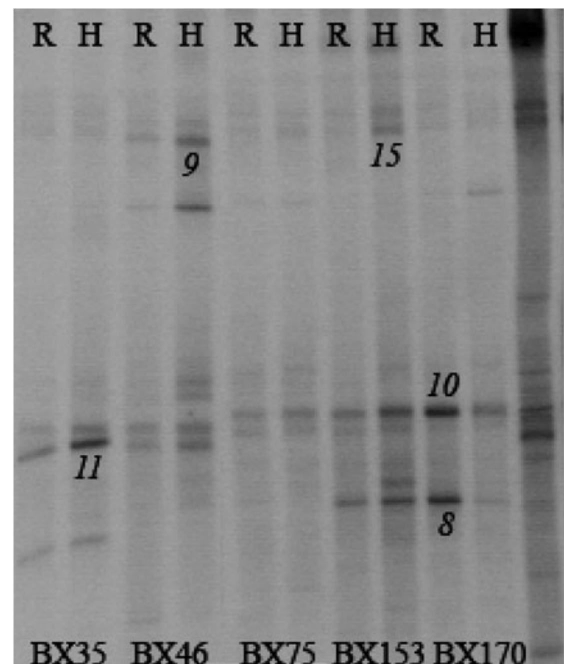


Fig. 1. *Madrepora oculata.* DGGE gel of bacterial community composition from reference and HEATSALT protocols. Numbers in italics are excised bands (for identification see Table 3). Unlabelled lane on the right is a positive control, i.e. a reference sample from the Bay of Villefranche; R, enzymatic reference extraction protocol; H, HEATSALT protocol; BX, box core (for sample identity see Table 2)

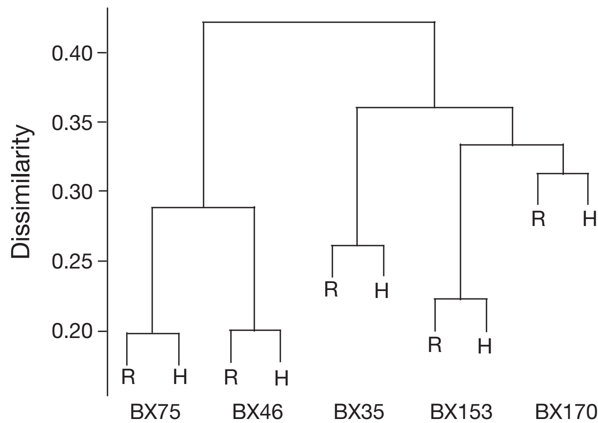


Fig. 2. *Madrepora oculata.* Bacterial community composition from reference and HEATSALT protocols. Data from Fig. 1 were used to calculate an UPGMA cluster dendrogram (see 'Materials and methods—Statistics') based on the Bray-Curtis similarity coefficient (including relative band intensity). R, reference extraction protocol; H, HEATSALT extraction protocol; BX, box core (for sample identity see Table 1)

tical bands, however, as samples were not run routinely in replicates, this was not further explored. More importantly, there was no difference in band pattern for bacterial and archaeal fingerprints from water column samples between the reference and the HEATSALT protocol in the sense that there was no difference in the presence of specific bands (Table 2).

The higher variability of the band patterns between the protocols for corals than for water samples could be due to the sampling strategy. Cotton sticks were rolled over the entire colony. This might have resulted in the selective collection on the different halves of the cotton stick of microbial communities from polyps, tissue connecting polyps (coenosarc) or different growth regions of the colony. Such a non-homogeneous distribution of the bacteria on the cotton sticks could have resulted in the slight differences of the DGGE patterns, since the community composition can vary strongly within colonies of *Madrepora oculata*. However, discrete samples clustered together, irrespective of the extraction methods applied, thus suggesting only a minor influence of the sampling or extraction method.

For the water column communities, the band patterns for the HEATSALT and the reference protocol were identical for *Bacteria* and *Archaea* and differences for coral-associated bacteria were not systematic. We tested the protocol for a range of marine environments such as offshore surface waters, the DCM layer, which is often enriched in cells, deep-marine waters, waters containing resuspended sediments, and the mucus of corals. The deep sea contains a significant abundance of *Archaea* (Karner et al. 2001) and archaeal bands could be obtained in almost all deep water column samples (Table 2). This suggests that the HEATSALT protocol, which is more rapid, less toxic and less expensive than traditional methods, can be used in a wide range of marine environments.

Archaeal DNA in corals

No archaeal DNA could be amplified from the extracted mucus samples of the cold-water corals, contrary to several studies on tropical corals (Kellogg 2004, Wegley et al. 2004). This might be due to a problem with the archaeal DGGE primers, since these primers seem to be more efficient for *Euryarchaeota* than for the *Crenarchaeota* (Winter et al. 2009). However, a cloning approach did not reveal archaeal sequences for living and dead colonies of *Lophelia pertusa* (Yakimov et al. 2006), whereas archaeal DNA could be amplified with the DGGE primers from sponges and gorgonian and antipatharian corals co-occurring with *L. pertusa* and *Madrepora oculata* at Rockall Banks (C. Maier unpubl. data) as well as in water samples from 1 box core (Table 2). This suggests specific but only poorly understood archaeal associations in corals.

Phylogenetic analysis

Sixteen bands from DGGE gels could be successfully sequenced (Table 3). The majority of the sequenced bands were related most closely to sequences from uncultured *Gammaproteobacteria*. Two other sequences belonged to *Actinobacteria* (Uncultured clone 78; accession number AM748253) and were not found in dead corals. However, *Actinobacteria* were found in tropical corals (Rohwer et al. 2001, Rohwer et al. 2002, Lampert et al. 2008) and *Lophelia pertusa* (Neulinger et al. 2008) using culture-independent techniques. From a survey of corals and sponges inhabiting the cold-water coral ecosystems of the Rockall Bank, 2 actinobacterial isolates were obtained (data not shown). One of the isolates was from *Madrepora oculata*. A sequence type specific for dead corals was most closely (99% similarity) related to an alphaproteobacterial sequence (Uncultured *L. pertusa* clone H04_W02; accession number AM911490) from seawater of a *L. pertusa* ecosystem in Trondheimfjord (Norway) (Neulinger et al. 2008) suggesting some common or related (but not coral-associated) phylotypes in cold-water coral ecosystems from the eastern North Atlantic.

One type of gammaproteobacterial sequences (98% similarity compared to the uncultured bacterium clone H10_CW02; accession number AM911426) was most closely related to sequences obtained from *Lophelia pertusa* off Norway (Neulinger et al. 2008) and was related

Table 3. Phylogenetic affiliation of sequences from DGGE bands with closest uncultured matches. No. of bases used to calculate sequence similarity is in parentheses in third column. Note: sequences (e.g. band number 16) were also obtained from parts of gels not shown in this study

Bands	Closest match	GenBank acc. no.	Sequence similarity in % (no. of bases)	Taxonomic group
5	Uncultured *Spongiobacter* sp. clone ME19	DQ917863	97 (447)	*Gammaproteobacteria*
6	Uncultured *Spongiobacter* sp. clone ME19	DQ917863	97 (378)	*Gammaproteobacteria*
24	Uncultured bacterium clone CD207A05	DQ200562	97 (530)	*Gammaproteobacteria*
25	Uncultured *Spongiobacter* sp. clone ME19	DQ917863	97 (476)	*Gammaproteobacteria*
7	Uncultured *Spongiobacter* sp. clone ME19	DQ917863	95 (389)	*Gammaproteobacteria*
10	Uncultured bacterium clone CD207F08	DQ200624	97 (466)	*Gammaproteobacteria*
27	Uncultured bacterium clone CD207F08	DQ200624	97 (469)	*Gammaproteobacteria*
8	Uncultured clone 78	AM748253	99 (455)	*Actinobacteria*
16	Uncultured bacterium clone H10_CW02	AM911426	98 (285)	*Gammaproteobacteria*
11	Uncultured bacterium clone CD207F08	DQ200624	97 (466)	*Gammaproteobacteria*
23	Uncultured gamma proteobacterium clone CD207F08	DQ200624	97 (466)	*Gammaproteobacteria*
9	Uncultured *Spongiobacter* sp. clone ME19		97 (481)	*Gammaproteobacteria*
31	Uncultured bacterium clone CD207F08	DQ200624	97 (502)	*Gammaproteobacteria*
30	Uncultured clone 78	AM748253	95 (428)	*Actinobacteria*
15	Uncultured bacterium clone L4-B08	FJ930535	95 (367)	*Gammaproteobacteria*
28	Uncultured bacterium clone H04_W02	AM911490	99 (441)	*Alphaproteobacteria*

to the genus *Acinetobacter*. All other *Gammaproteobacteria* sequences were most closely related to a specific sequence type obtained from several tropical coral species (Klaus et al. 2007, Lampert et al. 2008, Raina et al. 2009) and sponge species from the Mediterranean Sea (Thiel et al. 2007). The closest isolates were *Spongiobacter nickelotolerans* and *Endozoicomonas elysicola* (Fig. 3). These *Spongiobacter* type phylotypes (STP) had a similarity of 95 to 97% compared to published sequences and grouped together in a phylogenetic tree (Table 3, Fig. 3). This could suggest that specific STP were present, e.g. as consequence of adaptation to the environments (Rockall Bank) or to the coral *Madrepora oculata*. While it is difficult to compare bands between gels, we found up to 4 different STP bands per gel (Band nos. 9, 10, 11 and 15 in Fig. 1). The closest relatives were: uncultured bacterium clone CD207A05 (DQ200562), uncultured *Spongiobacter* sp. clone ME19 (DQ917863), uncultured bacterium clone CD207F08 (DQ200624) and uncultured bacterium clone L4-B08 (FJ930535) (Table 3). Sequences of bands considered identical per gel based on the position on the lane were also identical or almost identical (Bands 5, 6 and Bands 24, 25 in Table 3). The dominance of STP in our fingerprints indicates that this group could be important in association with *M. oculata*. The high diversity of 16S rRNA gene sequences (Fig. 3) suggests adaptive radiation of STP in *M. oculata*. Interestingly, STP were not or infrequently found in *L. pertusa* from the Mediterranean Sea (Yakimov et al. 2006), the Gulf of Mexico (Kellogg et al. 2009), the North Atlantic off Norway (Neulinger et al. 2008) and the Mingulay reef (Scotland; M. Weinbauer unpubl. data). We detected STP in *L. pertusa* only once (uncultured *Spongiobacter* sp. clone ME19 [DQ-917863]) (Band 7, Table 3; see Fig. 6) assuming that bands with identical position on a gel (see Fig. 6) also are identical in sequence. This could suggest that specific communities populate these 2 cold-water corals (see also 'Comparison of *Madrepora oculata* and *Lophelia pertusa*' below). A close association of *Spongiobacter* related sequences with healthy (i.e. non-bleached) colonies was found for the tropical coral *Acropora millepora* by using clone libraries and DGGE (Bourne et al. 2008). The coverage in this clone library was 41% for the pre-bleaching and 33% for the post-bleaching period. The high representation in some species and during specific ecological situations could indicate that *Spongiobacter* are involved in excluding invading microorganisms as suggested by Bourne et al. (2008).

Methodological considerations

For free-living microorganisms, the idea has been developed that 'everything is everywhere' but 'the environment selects' (e.g. de Wit & Bouvier 2006). This concept could also be applicable to mucus-associated bacteria as long as they are not species-specific symbionts or mutualists (Weinbauer & Rassoulzadegan 2007), since universally dispersed microorganisms will also reach corals. However, the concept is strongly debated for prokaryotes (Martiny et al. 2006), and the technologies available so far do not allow us to draw definitive conclusions on the first part of the hypothesis, i.e. on the proof of the absence of phylotypes. Recently, more focus has been put onto the second part of the concept, which deals with relative abundances, e.g. the biogeographical variation in relative abun-

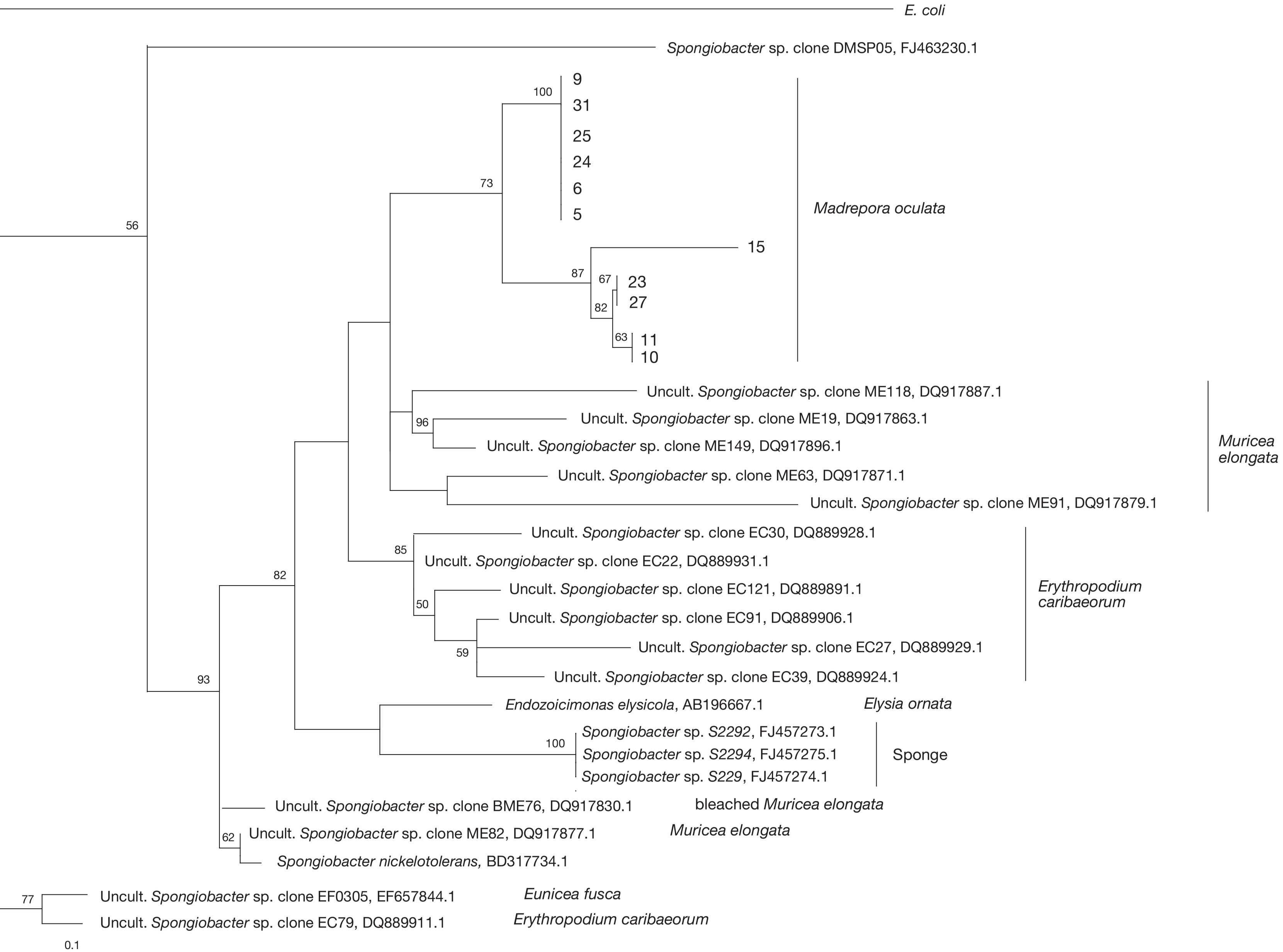


Fig. 3. Maximum-likelihood (ML) tree with sequences from the *Spongiobacter* (*Gammaproteobacteria*) group and sequences derived from DGGE bands from our study. The ML tree was calculated according to the Tamura & Nei (1993) substitution model plus gamma distribution with 100 bootstrap replications. The phylogram was rooted with *Escherichia coli* (*Gammaproteobacteria*) as an outgroup. Only bootstrap values above 50 are shown

dance of phylotypes (Dolan 2005). Such biogeographical patterns have been found e.g. for *Lophelia pertusa* (Neulinger et al. 2008, Kellogg et al. 2009).

16S rRNA gene PCR-based bacterial community fingerprints for specific environments will be influenced by a variety of factors (von Wintzingerode et al. 1997) such as the primers used, the relationship between a partial sequence and species identity, the sequence difference of multiple operons and the copy number of the 16S rRNA gene per cell. For example, some primers select against *Gammaproteobacteria*. However, an improved primer set was used that also detects the SAR11 cluster and some *Gammaproteobacteria* (Sanchez et al. 2007). Indeed, the majority of the detected sequences were *Gammaproteobacteria*. The cell number of phylotypes with an identical partial sequence has a strong influence on the fingerprint. In this sense, differences in community fingerprints as discussed in the following can be seen as a proxy for the differences of the relative abundance of bacterial phylotypes in different coral environments. A difference in a genetic community fingerprint does not allow for assessing 'who is where' in the sense of proof of absence, but it means that the community composition is different e.g. in the sense of relative abundances of phylotypes. Thus, fingerprints such as those obtained by DGGE are suitable to determine whether the bacterial community composition (BCC) differs between species and evaluate the potential variability in BCC within a coral species. It is sometimes difficult to compare band patterns from different gels. Thus, only per gel comparisons are made in the following.

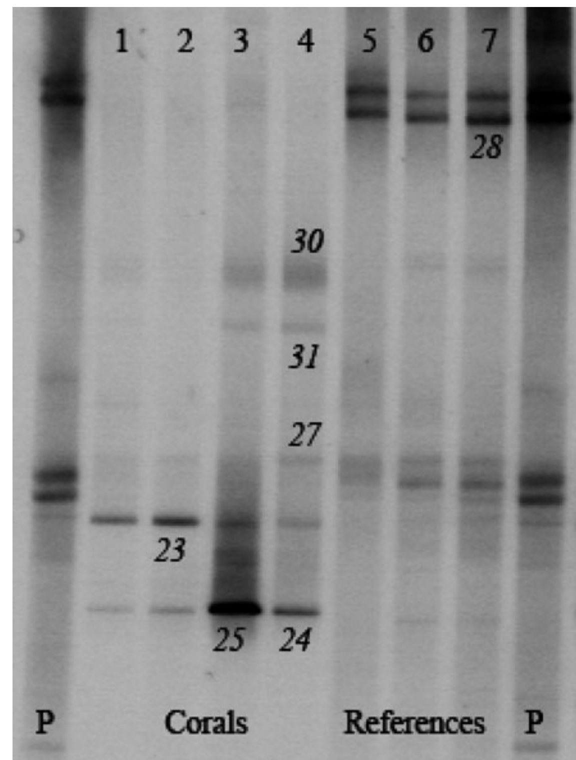


Fig. 4. *Madrepora oculata.* DGGE gel of bacterial community composition and reference samples (biofilm from dead corals and water samples). Lane numbering: 1, surface swab of first colony from BX35; 2, surface swab of second colony from BX35; 3, tissue sample of first colony from BX65; 4, tissue sample of second colony from BX65; 5, dead colony from BX35; 6, water sample from BX35; 7, water sample from BX46. For more details of sample identity see Table 1. Numbers in italics are excised bands (for identification see Table 1). P, positive control, i.e. a reference water sample from the Bay of Villefranche

Comparison of BCC on *Madrepora oculata* with dead corals and surrounding water

Four *Madrepora oculata* samples (2 surface mucus layer and 2 tissue samples) and 3 reference samples (one dead *M. oculata* and 2 box core water samples) from 2 different sites (BX35 and BX65; Table 1) were compared on a single DGGE gel (Fig. 4). A total of 15 discernible different bands were obtained for this gel. On average, 5 bands (range 4 to 6) were detected in coral samples whereas 7 bands (range 5 to 9) were found in reference samples. Four intense bands in the reference samples were absent or less intense in the coral samples. Two bands were present in all *M. oculata* samples (both STP) and absent in the reference samples.

The live coral samples showed a band pattern different from the reference samples. The coral samples clustered apart from the reference samples in UPGMA dendrograms (less than 20% similarity) and MDS analysis based on band intensity pattern confirmed that the 2 groups were well separated (Fig. 5). The dead coral was somewhat different from the water samples, which were nearly identical (approx. 90% similarity), but clustered clearly together with the reference samples. The dendrograms based on presence vs. absence data also showed the same difference in BCC between coral and reference samples (data not shown). An ANOSIM based on binary data and on intensity data ($R = 1$; $p < 0.05$) confirmed these findings.

The difference in BCC between coral samples and surrounding water or biofilms on dead corals indicates the presence of specific bacterial associations with the coral. Similar data were found for *Lophelia pertusa* from different environments, the Trondheimfjord (Neulinger et al. 2008) and the Mingulay Reef (Großkurth 2007). It was surprising that the water column community was closer to the biofilm than to the mucus community. This suggests a strong biological influence of *Madrepora oculata* on the BCC. These findings sup-

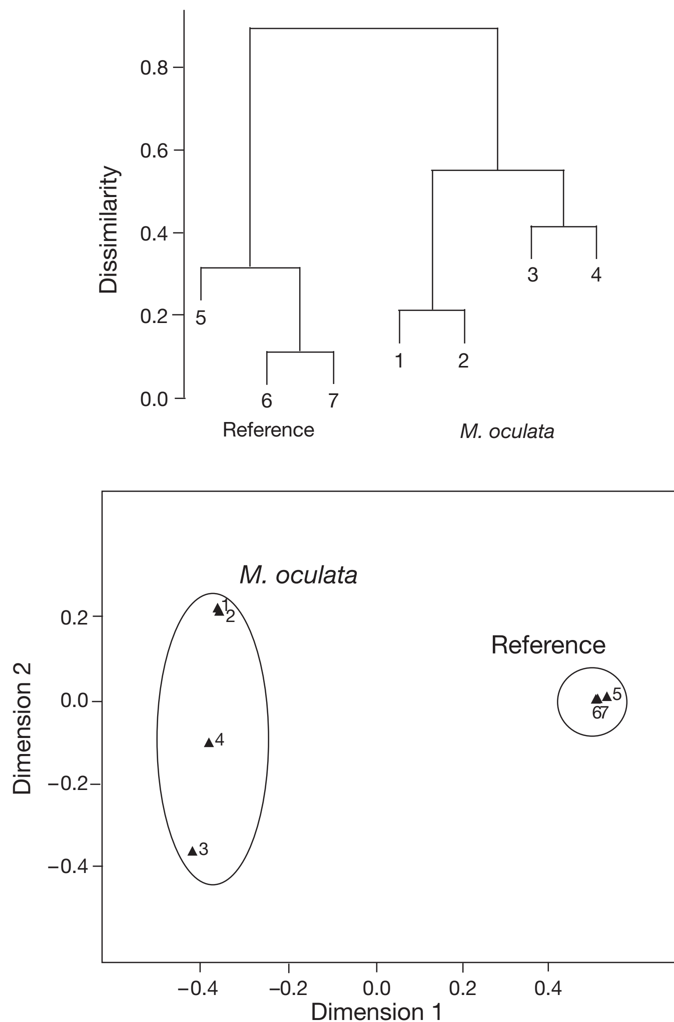


Fig. 5. *Madrepora oculata*. Bacterial community composition and reference samples (biofilm from dead corals and water samples). Data from Fig. 4 were used to calculate similarities. Numbers refer to lanes in Fig. 4. (a) UPGMA cluster dendrogram based on the Bray-Curtis similarity coefficient (considering also band intensity); (b) MDS analysis

port the emerging view from tropical corals that the associated prokaryotic community is under strong control of the coral host and that differences between species exist (e.g. Rohwer et al. 2001, Rohwer et al. 2002).

Comparison of *Madrepora oculata* and *Lophelia pertusa*

In general, samples of *Madrepora oculata* yielded more extracted DNA, higher amounts of PCR products and more bands on a DGGE gel than *Lophelia pertusa* samples (data not shown). Fig. 6 shows DGGE patterns from several polyps of a single colony of *M. oculata* and several polyps of a single colony of *L. pertusa* from the same box core (BX65). A total of 12 different bands were observed, on average 8 bands for *M. oculata* (range 7 to 11) and 6 (range 5 to 7) for *L. pertusa.* One intense band (uncultured *Spongiobacter* sp. clone ME19 [DQ917863]) was present in all *M. oculata* samples. This band could also be detected in the other gels and belonged to the STP group. This band could not be found in *L. pertusa* and no specific bands for *L. pertusa* could be detected.

The number of bands detected by DGGE was low for *Madrepora oculata* and *Lophelia pertusa* (this study; Großkurth 2007). A low number of peaks (median 14) was also found in the mucus of *L. pertusa in situ* using automated ribosomal intergenic spacer analysis (ARISA), although seawater samples displayed >100 phylotypes (Schötter et al. 2009). Interestingly, a low number of phylotypes (12) could also be detected in *L. pertusa* using a cDNA clone library used to target the more active members of the community (Yakimov et al. 2006). Rarefaction curves suggested that 83% of the phylotypes were detected. In contrast, rarefaction curves based on DNA clone libraries indicate a much higher bacterial richness for *L. pertusa* (Neulinger et al. 2008,

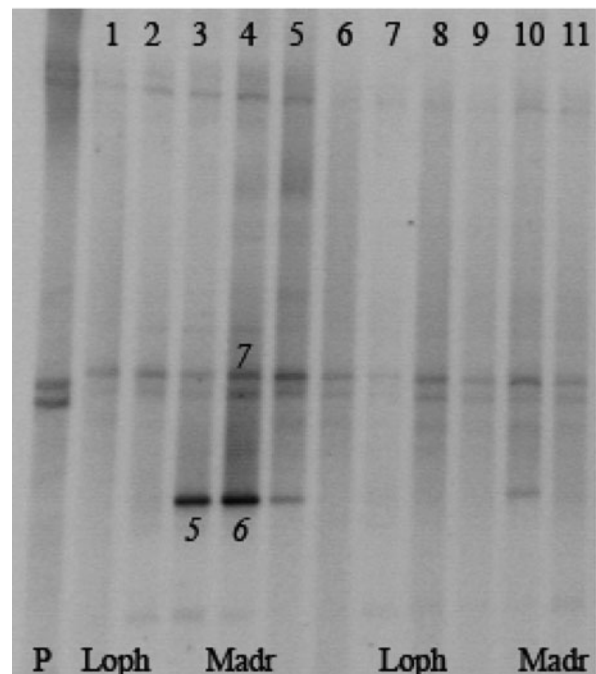
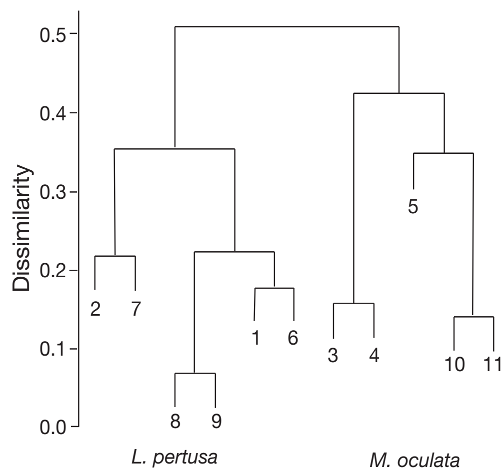


Fig. 6. *Madrepora oculata* and *Lophelia pertusa.* DGGE gel of within colony and between species variability of bacterial community composition. Note that per species several polyps of a single colony were analysed. Both colonies were from the BX65 (see Table 1). Lanes 1–2, 6–9: 6 polyps of *L. pertusa*; lanes 3–5, 10–11: 5 polyps from *M. oculata*. Numbers in italics are excised bands (for identification see Table 1). P, positive control, i.e. a reference water sample from the Bay of Villefranche; Loph, *L. pertusa*; Madr *M. oculata*

Kellogg et al. 2009). Thus, while bacterial richness is potentially high, the low number of phylotypes detected by DGGE, ARISA and an RNA based clone library suggests a limited number of phylotypes dominating the cold-water coral associated bacterial community.

Further assessment of the difference in BCC between *Madrepora oculata* and *Lophelia pertusa* was performed using UPGMA cluster analysis. The 2 coral species clustered separately in UPGMA dendrograms based on band intensity, but the similarity between the 2 species was > 50% (Fig. 7). A dendrogram based on



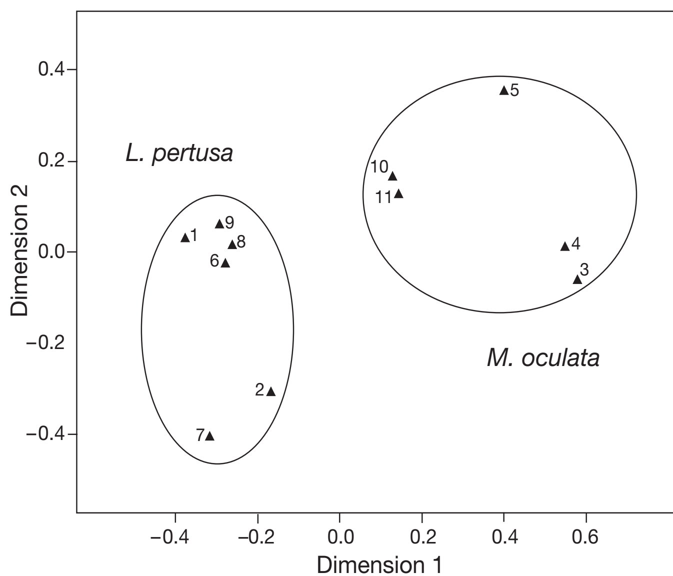


Fig. 7. *Madrepora oculata* and *Lophelia pertusa*. Within colony and between species variability of bacterial community composition. Data from Fig. 6 were used to calculate similarities. Numbers refer to lanes in Fig. 6. (a) UPGMA cluster dendrogram based on the Bray-Curtis similarity coefficient (considering also band intensity); (b) MDS analysis

presence vs. absence data had the same general features as the one based on intensity data (data not shown). Differences between the 2 corals were also confirmed by MDS analysis and ANOSIM based on binary data (R = 0.848, p = 0.03) and band intensity (R = 0.867, p = 0.001). Since coral samples were from the same box core, we have minimized potential between-site variation on BCC. Thus, our data indicate that deep-water coral species from the same site can harbour different communities. Another line of argument comes from a comparison of available sequences. STP dominated the DGGE sequences of *M. oculata* from Rockall Bank (Table 3), whereas no such sequences were found in clone libraries and DGGE analysis from *L. pertusa* from the Tronheimfjord and the Gulf of Mexico (Neulinger et al. 2008, Kellogg et al. 2009). This suggests differences in BCC between *M. oculata* and *L. pertusa*. Differences in BCC between species in the same habitat have been shown before for tropical corals (Klaus et al. 2005).

Variability in bacterial community composition within and between colonies

The surface mucus samples from BX35 and the tissue samples from BX65 clustered separately (Fig. 5). This difference could be due to a difference between surface mucus layer and tissue or to the fact that the samples were collected at different sites. Differences between coral mucus and tissue are known for *Lophelia pertusa* from the Scottish Mingulay Reef (Großkurth 2007).

A strong difference in BCC between corals is suggested for mucus samples from corals from 5 different box cores (only ca. 50% similarity) (Fig. 2). Binary data and relative intensity-based data showed different relations between corals, however, maximum dissimilarity values were similar. Using the band patterns from the 2 extraction protocols as replicates (Figs. 1 & 2), ANOSIM showed for binary data (R = 0.570, p = 0.017) and intensity-based data (R = 0.910, p = 0.001) that the differences in BCC between colonies were significant. Differences between colonies could be due to several reasons such as differences between study sites, physiology or genetic identity. For tropical corals it is known that BCC on coral species can vary within reefs or on a seasonal scale and that the amount of this variability can differ strongly between coral species (Klaus et al. 2005, Guppy & Bythell 2006). Interestingly, these samples did not cluster geographically, i.e. they did not cluster within the Haas or Clan mounds (Table 1, Fig. 2). An ANOSIM based on band intensity (R = 0.175, p = 0.162) and binary data (R = 0,189, p = 0,126) also showed that BCC on *Madrepora oculata*

did not differ significantly between these 2 environments. This suggests that differences of BCC between colonies or box cores can be stronger than differences between environments that are 20 km apart.

The within colony variability of BCC on *Madrepora oculata* based on intensity data was high (ca. 40% dissimilarity), as indicated by cluster analysis (Fig. 7). A cluster analysis based on binary data showed the same general features (ca. 35% dissimilarity, data not shown). A similar variability (ca. 35%) between single polyps of the same colony was obtained for *Lophelia pertusa* (Fig. 7). An ANOSIM analysis could not be applied, since replicate samples for a single polyp could not be collected due to the small size of the polyps. However, the finding that dissimilarity values of BCC between colonies (for which an ANOSIM showed significant differences) (Fig. 2) was similar to dissimilarity values within single colonies (Fig. 7) suggests that the detected within colony variability of BCC is not an artefact. In addition, similar dissimilarity values between polyps were found for 2 colonies further supporting a high within-colony variability.

This high intra-colony variability in BCC has methodological and biological implications. On the methodological side, sampling different parts of colonies could be a reason for the variability between colonies as observed in this (Figs. 2 & 4) and other studies (Klaus et al. 2005). To reduce the potential bias by intra-colony variability, cotton sticks were rolled over entire or at least large parts of colonies for the between-colony assay to harvest the majority of the bacterial cells. Such integrated samples should improve the comparability between colonies. This is important when the variability within and between sites is assessed.

Several factors could result in the observed differences in BCC between coral polyps of the same colony. First, the variability might be explained by random opportunistic associations with the surrounding water. Such 'visitors' (Ritchie 2006) could be trapped into or settle in the mucus e.g. as function of the water flow, which is known to vary within colonies (Chamberlain & Graus 1975) and between polyps (Sponaugle 1991). Second, it is noteworthy that between-polyp variation exists also for physiological parameters of *Lophelia pertusa*, such as calcification rates, and this can be linked to the age of the polyp (Maier et al. 2009). As physiological differences can result in differences of the chemical composition of the mucus (Brown & Bythell 2005), the within-colony variability of BCC could also depend on the bioavailability of the organic matter present in the mucus. Third, following the hypothesis that changing the bacterial community is a means of corals to adjust to changing environmental conditions (Reshef et al. 2006, Rosenberg et al. 2007), the within-colony variability of BCC could also correspond to an active control of bacteria by corals. It remains to be shown whether the intra-colony variability is a random feature or controlled by the coral.

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