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Morphological and molecular analyses of microorganisms in Caribbean reef adult sponges and in corresponding reproductive material

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Abstract: Caribbean reef sponges were surveyed for the presence of microorganisms in the mesohyl tissue of adult sponges and the respective reproductive material (embryos, larvae). A clear correlation was found in that high microbial abundance (HMA) sponges always contained microorganisms in their reproductive stages. In contrast, low microbial abundance (LMA) sponges did not contain microorganisms in their reproductive stages. Based on these data, *Ircinia felix* Duchassaing and Michelotti, 1864 was chosen as a model organism for the molecular analysis of microorganisms within the adult sponge and its larvae and juveniles. Denaturing gradient gel electrophoresis (DGGE) of eubacterial 16S rDNA sequences revealed similar banding patterns for the adult individual and its reproductive stages. However, resolution of the DGGE gel was found to be limited. Selected DGGE bands (n=21) were excised and sequenced. The majority of sequences were most similar to sequences obtained from other HMA sponges indicating the presence of members of the previously identified, sponge-specific community in the adult sponge and its reproductive stages.

Keywords: larvae, microbial diversity, Porifera, reproductive stages, sponge

Introduction

Sponges are filter-feeders that pump large volumes of seawater through their aquiferous system and take up food particles like organic particles and microorganisms by phagocytosis (Brusca and Brusca 1990). In addition to these food bacteria, many so called "bacteriosponges" (Reiswig 1981) can permanently harbor large amounts of extracellular microorganisms in their mesohyl that make up 40-60% of the sponge biomass and exceed seawater concentrations by 2-4 orders of magnitude (Friedrich *et al.* 2001, Webster and Hill 2001). These sponge-associated microorganisms are morphologically diverse and often show unusual membrane structures like additional sheaths, slime layers or putative nuclear membranes (Vacelet and Donadey 1977, Wilkinson 1978, Fuerst *et al.* 1998, Friedrich *et al.* 1999, Fieseler *et al.* 2004).

16S rRNA gene based studies revealed phylogenetically complex, sponge-specific microbial consortia that are present in different sponges and that are remarkably different from seawater bacterioplankton, both in terms of concentration and diversity (Hentschel *et al.* 2003, 2006, Taylor *et al.* 2007). In total, representatives of the eubacterial phyla Proteobacteria (Alpha-, Gamma- and Deltaproteobacteria), Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Nitrospira and Gemmatimonadetes (Friedrich *et al.* 1999, Hentschel *et al.* 2002, Taylor *et al.* 2005, Schmitt *et al.* 2007) and of the candidate phylum 'Poribacteria' (Fieseler *et al.* 2004) as well as of the archaeal phylum Crenarchaeota (Preston *et al.* 1996, Margot *et al.* 2002) were detected as specific members of the sponge microbiota. Moreover, it could be shown that the sponge-specific microbial consortia are stable over time and space (Hentschel *et al.* 2002). The microbial community profiles of *Cymbastela concentrica* von Lendenfeld, 1887 were stable over large distances in temperate waters but differed between temperate and tropical seas (Taylor *et al*. 2005). The microbial consortia of verongid sponges were also stable after experimental perturbation (Friedrich *et al.* 2001, Thoms *et al.* 2003); however, microbial community variability was observed after copper exposure in *Rhopaloeides odorabile* Thompson, Murphy, Bergquist and Evans, 1987 (Webster *et al*. 2001).

Vertical transmission has been proposed as a potential mechanism for the establishment and maintenance of specific sponge-microbe-associations. Microorganisms have already been detected by electron microscopy in oocytes of several oviparous sponges (e.g. *Aplysina* spp. (Gallissian and Vacelet 1976), *Stelletta grubii* Schmidt, 1862 (Sciscioli *et al.* 1991), *Geodia cydonium* Jameson, 1811 (Sciscioli *et al.* 1994), *Chondrilla* spp. sponges (Maldonado *et al.* 2005)) and

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in embryos and larvae of several viviparous sponges (e.g. *Spongia* spp. and *Hippospongia* spp. (Kaye 1991, Kaye and Reiswig 1991), *Chondrosia reniformis* Nardo, 1847 (Lévi and Lévi 1976), *Astrosclera willeyana* Lister 1900 (Wörheide 1998)). Usher *et al.* (2001) and Ereskovsky *et al.* (2005) reported in detail on the incorporation and transmission of Cyanobacteria in *Chondrilla australiensis* Carter, 1873 and of a spiral bacterium in *Halisarca dujardini* Johnston, 1842, respectively. Furthermore, Enticknap *et al.* (2006) succeeded in cultivating several alphaproteobacterial strains from larvae of the sponge *Mycale laxissima* Duchassaing and Michelotti, 1864. These bacteria were closely related to each other and also to the strain NW001 isolated from the sponge *R. odorabile* (Webster and Hill 2001).

To obtain a better understanding of the establishment and maintenance of this unique association between sponges and complex microbial consortia we performed a general electronmicroscopical survey for the presence of microorganisms in adult sponges as well as in the respective reproductive stages. In total, ten Caribbean species representing five orders and two different modes of reproduction (ovipary, vivipary) were included. Based on these results, *Ircinia felix* Duchassaing and Michelotti, 1864 was chosen as a model system for the detailed molecular study of vertical transmission. *I. felix* is a viviparous species with internal fertilization. Embryos are brooded in the sponge mesohyl and free swimming larvae spend a few hours in the water column before they settle on a suitable substrate and metamorphose into juveniles. We performed settlement experiments on reefs offshore Florida and applied DGGE analysis and subsequent sequencing of excised bands to characterize and compare the associated microbiota of three different developmental stages (adult, larvae, and juveniles).

Materials and methods

Transmission Electron Microscopy (TEM)

Adult and reproductive material of ten sponge species was collected by SCUBA diving offshore off Key Largo, Florida in June 2002 and June and August 2004 using the NOAA´s National Undersea Research Center (NURC) facilities and vessels (Table 1). The adult sponge samples were cut into small pieces of about $1mm^3$. All samples (adult, larvae) were preserved in 2.5% glutaraldehyde/H_2O_{dd} and washed five times in cacodylate buffer (50 mM, pH 7.2), fixed in 2% osmium tetroxide for 90 min, washed again five times in H_2O_{dd} and incubated overnight in 0.5% uranyl acetate. After dehydration in an ethanol series (30, 50, 70, 90, 96, and three times in 100% for 30 min, respectively), samples were incubated three times for 30 min in 1x propylene oxide, overnight in 1:1 (v/v) propylene oxide/Epon 812 (Serva), two times for 2 h in Epon 812 and finally embedded in Epon 812 for 48 hours at 60°C. The samples were sectioned with an ultramicrotom (OM U3, C. Reichert, Austria) and pieces of three different individuals of each sponge developmental stage (except for *A. coralliphagum* embryos where only one sample was available) were examined by transmission electron microscopy (Zeiss EM 10, Zeiss, Germany).

Denaturing Gradient Gel Electrophoresis (DGGE)

Larvae settlement experiments were performed with the sponge *I. felix* as described by Schmitt *et al.* (2007). Briefly, larvae released by one adult individual were caught using the methodology of Lindquist *et al.* (1997), transferred into sealed plastic containers (~ 75 ml volume) and returned to the reef where they were cable tied to racks at 9 m depth. After settlement, pieces of the Nylon with settled juvenile sponges as well as pieces of Nylon without sponge tissue (control) were collected. DGGE was performed with larvae, juveniles and the respective adult sample. DNA of the adult sponge was extracted using the Fast DNA Spin Kit for Soil (Q-Biogene, Heidelberg, Germany) whereas DNA of three pooled larvae, three pooled juveniles and the control was extracted by heating (100°C) the samples for 10 min. Following PCR amplification with universal, eubacterial 16S rDNA-targeted primers 341f with GC clamp and 907r (Muyzer *et al.* 1998), two independent PCR reactions of each sample (adult, larvae, juveniles, control) were run on a 10% (w/v) polyacrylamide gel in 1x TAE using a 0–90% denaturing gradient; 100% denaturant corresponded to 7 M urea and 40% (v/v) formamide. Electrophoresis was performed for 6 h at 150 V and 60°C. Gels were stained for 30 min in SYBR Gold (Molecular Probes) and scanned on a Typhoon 8600 scanner (Amersham Biosciences). DGGE banding pattern similarities were determined by cluster analysis using the software Quantity One (Bio-Rad, München, Germany). Selected bands were excised with an EtOH sterilized scalpel and incubated in 25µl H_2O_{dd} overnight at 4°C. 4 µl of eluted DNA was subsequently used for reamplification with primers 341f and 907r. PCR products were ligated into the pGEM-T-easy vector (Promega) and transformed by electroporation into competent *E. coli* XL 1-Blue cells. Plasmid DNA of up to three different clones per excised band was isolated by standard miniprep procedures and the correct insert size was verified by using agarose gel electrophoresis following restriction digestion. Sequencing was performed on an ABI 377XL automated sequencer (Applied Biosystems). 16S rRNA gene sequences were deposited in the EMBL/GenBank/DDBJ database under accession numbers DQ661773-DQ661787 and EU095956-EU095961.

Results

Transmission Electron Microscopy

High microbial abundance sponges

In adult samples of *Agelas wiedenmayeri* Alcolado, 1984, *Aka coralliphagum* Ruetzler, 1971, *Ectyoplasia ferox* Duchassaing and Michelotti, 1864, *I. felix* and *Smenospongia aurea* Hyatt, 1875 large numbers of extracellular microorganisms were scattered throughout the sponge mesohyl (Table 1, Fig. 1A, C, E, G, I). These species are therefore regarded as high microbial abundance (HMA) sponges. The microorganisms showed a high variety of morphotypes, such as rods, cocci and other, irregular forms. Many microorganisms possessed additional membrane structures similar to those that were described previously from other HMA sponges (Vacelet and Donadey 1977, Wilkinson

Table 1: Detection of microorganisms in adult sponges and reproductive stages using electron microscopy.

Species	Order	Detection of microorganisms by TEM	
		Adult	Reproductive stages
High microbial abundance sponges			
Agelas wiedenmayeri Alcolado, 1984	Agelasida	+	+
Aka coralliphagum Ruetzler, 1971	Haplosclerida	+	+
Ectyoplasia ferox Duchassaing and Michelotti, 1864	Poecilosclerida	+	+
Ircinia felix Duchassaing and Michelotti, 1864	Dictyoceratida	+	+
Smenospongia aurea Hyatt, 1875	Verongida	+	+
Low microbial abundance sponges			
Callyspongia vaginalis Lamarck, 1814	Haplosclerida	–	–
Mycale laxissima Duchassaing and Michelotti, 1864	Poecilosclerida	(+)[1]	–
Niphates digitalis Lamarck, 1814	Haplosclerida	–	–
Tedania ignis Duchassaing and Michelotti, 1864	Poecilosclerida	–	–
Ulosa ruetzleri Wiedenmayer, 1977	Poecilosclerida	–	–

[1] Low microbial abundance and diversity in adult mesohyl

1978, Friedrich *et al.* 1999). Morphotype C is characterized by several additional sheaths, type D by a copious, irregular slime layer, and type E by a putative nuclear membrane. Cyanobacteria could be identified by their typical thylacoid membranes and were particularly dominant in the *I. felix* mesohyl (Fig. 1G). Some loosely scattered sponge cells were also present in the mesohyl. Most of these cells were amoeboid-like and contained large nuclei and often vesicles and phagosomes showing their phagocytotic activity. A layer of pinacocytes and/or choanocytes always separated the mesohyl of these sponges from seawater.

Aka coralliphagum, I. felix and *S. aurea* have a viviparous mode of reproduction and release free swimming parenchymella-type larvae into the water column. Many microorganisms were predominantly located in the central region of the larvae (Fig. 1D, H, J). These morphologically diverse microorganisms were extracellular and similar in shape to the microorganisms present in the respective adult tissues including the morphotypes C, D and E. Few amoeboid-like sponge cells, that contain large amounts of lipids, were also present in the center of the larvae. *Agelas wiedenmayeri* and *E. ferox* have an oviparous mode of reproduction. They release oocytes or zygotes, which are embedded in a gelatinous sheath. These early reproductive stages were densely filled with lipids and electron-dense vesicles (Fig. 1B, F). Microorganisms that resembled the adult microbial community were predominantly found in the outer regions of the reproductive stages of *A. wiedenmayeri* and *E. ferox*. (Fig. 1B, F).

Low microbial abundance sponges

EM inspection of *Callyspongia vaginalis* Lamarck, 1814*, M. laxissima, Niphates digitalis* Lamarck, 1814*, Tedania ignis* Duchassaing and Michelotti, 1864 and *Ulosa ruetzleri* Wiedenmayer, 1977 adult samples revealed a low abundance and diversity of microorganisms (Table 1, Fig. 2C) or the complete absence in the mesohyl matrix (Table 1, Fig. 2A, E, G, I) and are therefore classified as low microbial abundance (LMA) sponges. The mesohyl contained few sponge cells that were embedded in a voluminous extracellular matrix. All investigated species are viviparous. The larvae contained many morphological structures that could not be identified unambiguously. Noticable are high numbers and sometimes very large vesicles and lipids. However, no microorganisms could be detected in any of these larvae in this study (Fig. 2B, D, F, H, J).

Denaturing Gradient Gel Electrophoresis (DGGE)

Figure 3A represents the bacterial profiles of *I. felix* adult, larvae and juvenile as well as the control (piece of Nylon without sponge tissue). The DGGE banding patterns of each of two adult, larvae, and juvenile PCR reactions differed in only one, four, and two band positions, respectively, indicating that a PCR bias is negligible. The number of bands in *I. felix* adult was higher than in the larvae (adult n =20.5; larvae n=16), but the DGGE banding patterns appeared highly similar. Overall, adult and larvae samples shared more than 70% of all bands (Fig. 3B). The juvenile sample differed from the adult and larvae samples in that it had generally less bands (n=13) and shared only 53% of all bands with adult and larvae (Fig. 3B). The cluster analysis placed the juvenile sample next to the control (number of bands: n=17), albeit with only 54% similarity (Fig. 3B).

16S rDNA sequence analysis

Twenty four bands were excised from the *I. felix* DGGE gel (Fig. 3A). After removal of 5 sequences as chimaeras (sequences of bands 4, 14, 15, 16, 21), a total of 21 16S rRNA gene sequences were obtained: 9 from adult, 3 from larvae, 8 from juvenile, and 1 additional sequence from the control (Table 2). Three clones of DGGE band 18 revealed different sequences whereas two clones of DGGE bands 1 and 17 each revealed identical sequences. The overall diversity was high with representatives of four different bacterial phyla (Acidobacteria, Chloroflexi, Gemmatimonadetes, and Proteobacteria (Alpha-, Gamma-, Deltaproteobacteria)). In the adult sample, all ten sequences

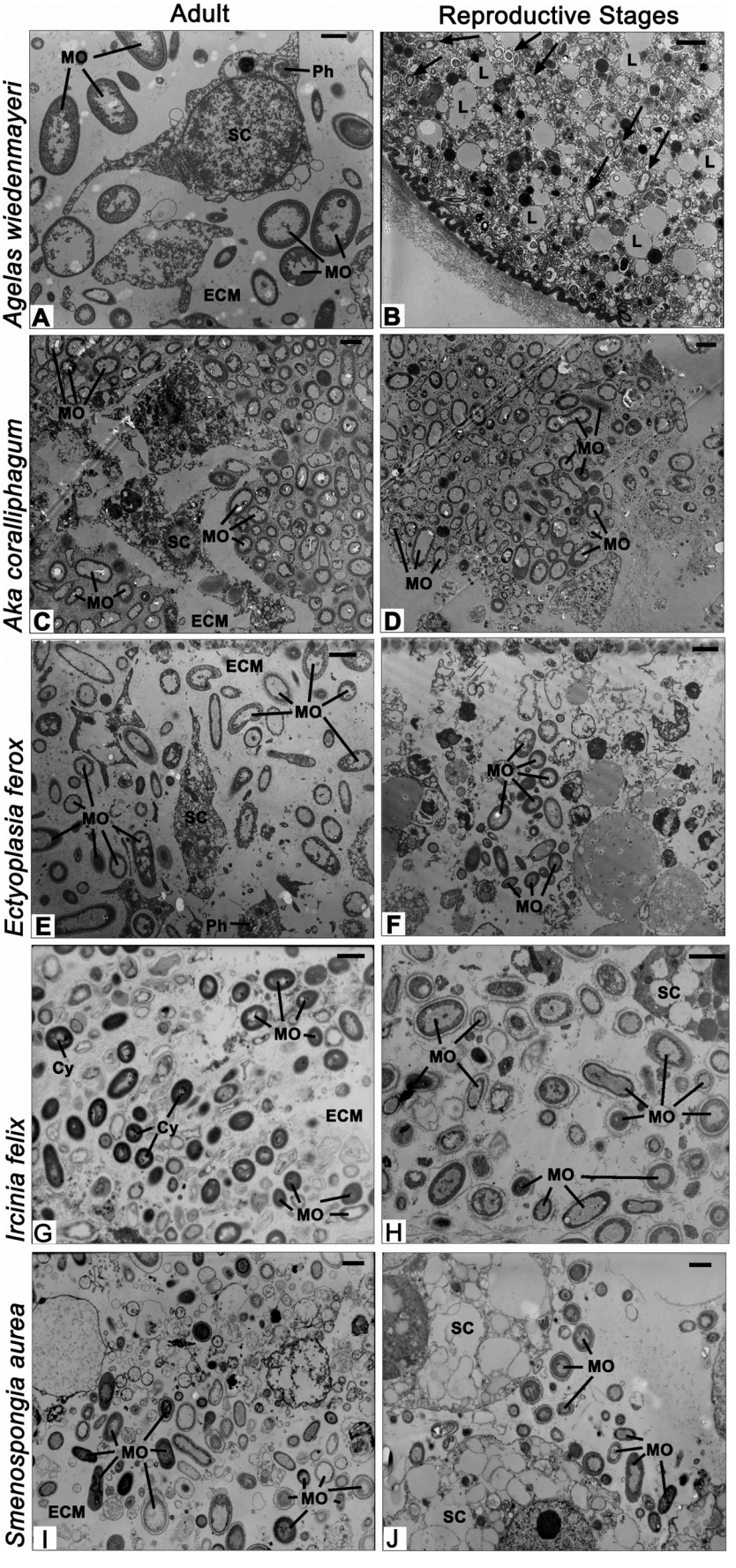

Fig. 1: Transmission electron microscopy of the HMA sponges *A. wiedenmayeri* adult (**A**) and embryo (**B**), *A. coralliphagum* adult (**C**) and larva (**D**), *E. ferox* adult (**E**) and embryo (**F**), *I. felix* adult (**G**) and larva (**H**) and *S. aurea* adult (**I**) and larvae (**J**). Lines indicate microorganisms. Cy: cyanobacteria, ECM: extracellular matrix, L: lipids, MO: microorganisms, Ph: phagosome, SC: sponge cell. Scale bar: 1 µm (A, C, D, G, H, I, J), 2 µm (B, E, F).

were most similar to sequences derived from other sponges: *Aplysina aerophoba* Schmidt, 1862, *Aplysina cavernicola* Vacelet, 1959, *Theonella swinhoei* Gray, 1868, *Agelas dilatata* Duchassaing and Michelotti, 1864 and *Plakortis* sp. In the larvae, two sequences were most similar to a 16S rRNA gene sequence from *A. cavernicola*, whereas clone B13-1 was related to a seawater clone. The juvenile sample contained four 16S rRNA gene sequences most similar to sequences derived from the sponges *A. aerophoba* and *A. dilatata*, two sequences most similar to *Alcanivorax* sp., and two sequences most similar to a cold seep and a hot spring clone, respectively. The 16S rRNA gene sequence obtained from the control was related to a marine *Pseudoalteromonas* sp. sequence (Table 2).

Discussion

The EM survey for the presence of microorganisms in the sponge mesohyl yielded two different groups of sponges. One group contained large numbers of morphologically diverse microorganisms whereas the mesohyl of the second group was almost devoid of microorganisms. These data expand early observations on patterns of microbial abundances in sponges by Vacelet (1975) and Vacelet and Donadey (1977). Whenever microorganisms were present in the adult sponge, microorganisms were also contained in the respective reproductive stages (Table 1, Fig. 1). Whenever microorganisms were present in low numbers or absent in the adult sample, microorganisms were also missing in the reproductive stages (Table 1, Fig. 2). This correlation suggests that HMA sponges transfer microorganisms vertically through their reproductive stages. Morphotypes C, D and E that were found to be abundant and consistently associated with other sponges (Vacelet and Donadey 1977, Wilkinson 1978, Friedrich *et al.* 1999) could also frequently be detected in adult mesohyl

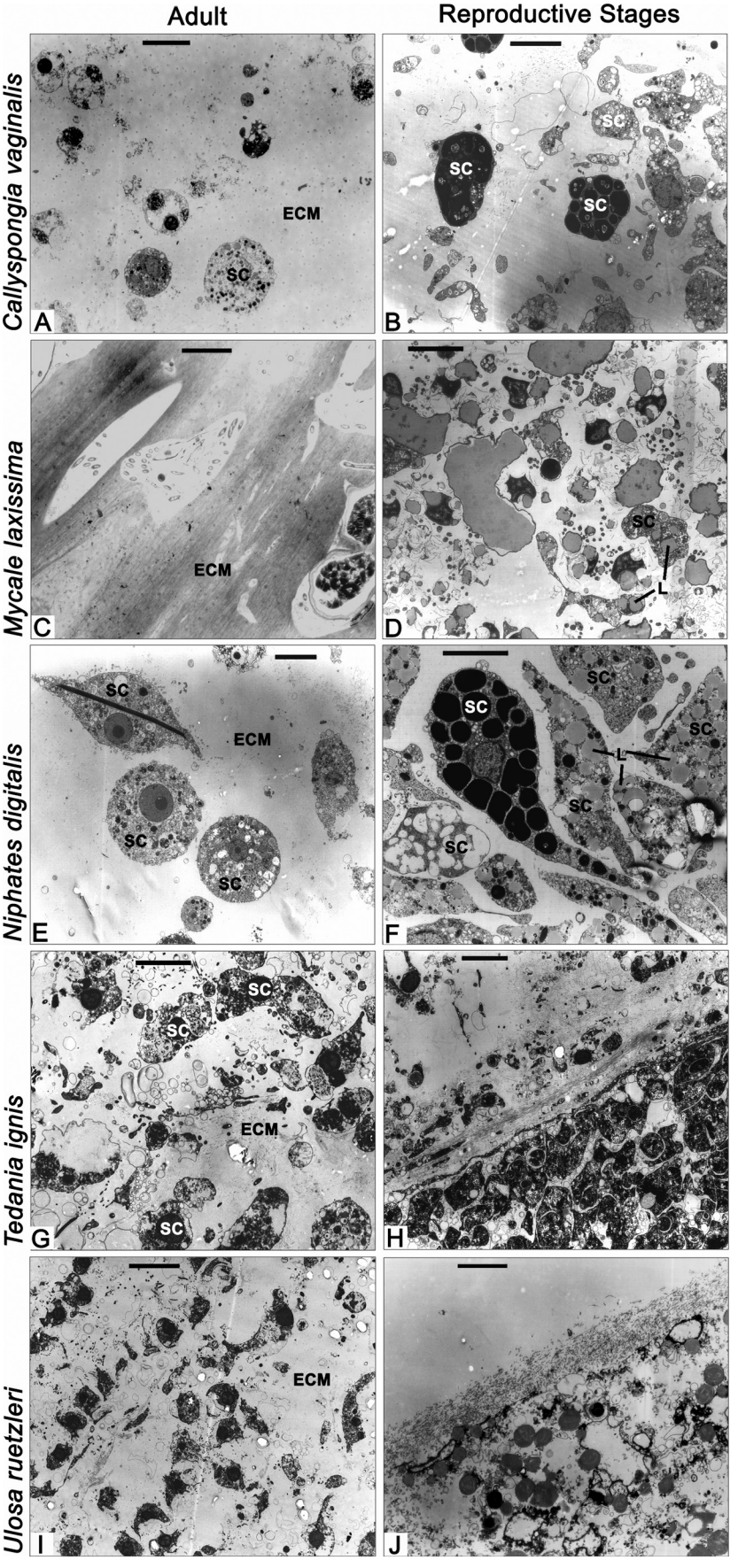

Fig. 2: Transmission electron microscopy of the LMA sponges *C. vaginalis* adult (**A**) and larva (**B**), *M. laxissima* adult (**C**) and larva (**D**), *N. digitalis* adult (**E**) and larva (**F**), *T. ignis* adult (**G**) and larva (**H**) and *U. ruetzleri* adult (**I**) and larva (**J**). ECM: extracellular matrix, L: lipids, SC: sponge cell. Scale bar: 3 µm (J), 4 µm (F), 5 µm (A, B, C, D, E, G, H), 8 µm (I).

and reproductive material of the sponges of this study. Moreover, these types were also present in juvenile sponges of *I. felix* (data not shown, Schmitt *et al.* 2007). This indicates that several similar microbial morphotypes are vertically transmitted in different sponges. Apparently, vertical transmission is common and widespread among HMA sponges.

The mode of reproduction seems not to be a determining factor for vertical transmission as both oviparous (*A. wiedenmayeri, E. ferox*) and viviparous (*A. coralliphagum*, *I. felix, S. aurea*) species belong to the HMA sponge group (Table 1). This is consistent with previous electron microscopy studies that also documented the presence of microorganisms in oocytes of oviparous species (Gallissian and Vacelet 1976, Usher *et al.* 2001) and in oocytes and embryos of viviparous species (Kaye 1991, Ereskovsky *et al.* 2005). This further supports the general character of the microbial transfer through larvae in sponges.

Based on these microscopic results the HMA sponge *I. felix* was chosen for a molecular comparison of the bacterial profile of the adult sponge and its developmental stages (larvae, juveniles) using DGGE. Ideally, DGGE bands represent single 16S rDNA sequence fragments that are separated by their GC content on an increasing denaturing gradient gel. The number of bands and the banding pattern correspond to the microbial numbers and diversity of a certain sample. In previous studies DGGE was found to be useful to describe the total microbial profile of sponges as well as the profile of specific microbial groups (Diaz *et al.* 2004, Taylor *et al.* 2005, Wehrl *et al.* 2007).

In this study, some bands represented single 16S rDNA sequence fragments (e.g. bands 1 and 17 each revealed two identical sequences) whereas other bands represented more than one sequence (e.g. band 18 revealed three different sequences). Therefore, the total microbial diversity in each sponge developmental stage is probably higher than indicated by the number of bands per sample. The banding patterns of the *I. felix* adult sponge and its larvae and juveniles appeared

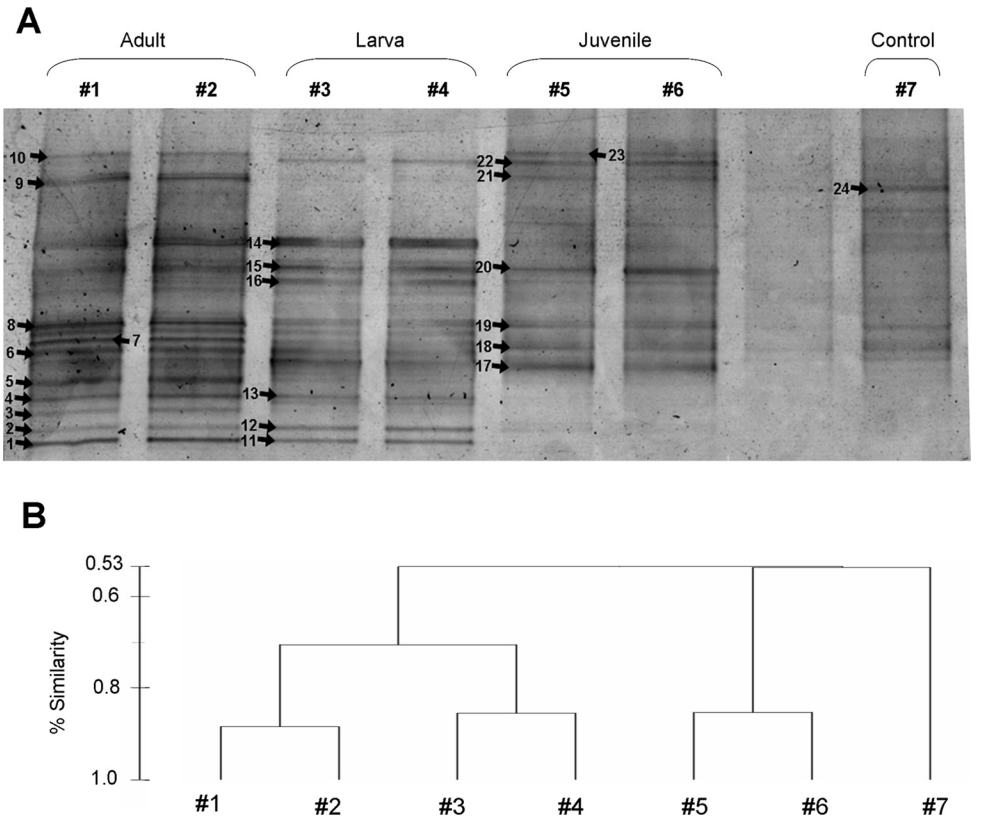

Fig. 3: A. 16S rDNA-DGGE gel of *I. felix* adult, larvae and juvenile samples as well as a Nylon-control sample. Two independent PCR reactions were run for each sample. Arrows mark excised bands. **B.** Cluster analysis of the DGGE gel showing percentage similarity of banding patterns.

Table 2: 16S rDNA sequence analysis of bands excised from the DGGE gel of *Ircinia felix*.

	Clone	Closest sequence match in GenBank	Similarity (%)	Length (bp)	Taxonomic affiliation
adult	B1-1	sponge clone PAUC43f (AF186415)	99	585/589	Gemmatimonadetes
	B2-1	sponge DGGE Band 6 (AY180081)	97	487/499	Acidobacteria
	B3-1	sponge clone TK19 (AJ347028)	96	567/589	Gemmatimonadetes
	B5-1	sponge clone TK13 (AJ347034)	98	577/588	Deltaproteobacteria
	B6-1	sponge clone AD040 (EF076132)	96	566/589	Deltaproteobacteria
	B7-2	sponge clone PK035 (EF076097)	95	554/579	Gammaproteobacteria
	B8-1	sponge clone PK035 (EF076097)	99	582/587	Gammaproteobacteria
	B9-1	sponge clone TK16 (AJ347035)	97	548/564	Chloroflexi
	B10-1	sponge clone AD015 (EF076136)	93	524/561	Alphaproteobacteria
larvae	B11-1	sponge DGGE Band 6 (AY180081)	97	487/499	Acidobacteria
	B12-1	sponge DGGE Band 6 (AY180081)	97	486/499	Acidobacteria
	B13-1	seawater clone (AY592226)	96	569/589	Acidobacteria
juvenile	B17-1	sponge clone TK34 (AJ347030)	98	548/559	Alphaproteobacteria
	B18-1	cold seep clone (AB015247)	94	530/560	Alphaproteobacteria
	B18-3	sponge clone TK97 (AJ347054)	96	529/547	Alphaproteobacteria
	B18-4	sponge clone TK34 (AJ347030)	98	551/559	Alphaproteobacteria
	B19-2	marine *Alcanivorax* sp. (AY726812)	89	502/561	Alphaproteobacteria
	B20-1	hot spring clone pItb-vmat-60 (AB294961)	95	567/594	Gammaproteobacteria
	B22-1	sponge clone AD015 (EF076136)	92	522/562	Alphaproteobacteria
	B23-1	*Alcanivorax* sp. Mho1 (AB053124)	99	586/587	Gammaproteobacteria
control	B24-1	*Pseudoalteromonas* sp. (AM111085)	96	472/489	Gammaproteobacteria

similar (Fig. 3A, B). However, sequencing of excised bands that showed the same migration distance revealed only once the same sequence (bands 2 and 12). Overall, there appears to be little overlap among the sequences obtained from the adult individual, its larvae and the juvenile sponges. This might also be the result of a lack of resolution of the DGGE gel.

The total phylogenetic diversity of *I. felix* is high and encompasses at least four bacterial phyla. Interestingly, most of the sequences (15 out of 20) obtained from the three sponge developmental stages show highest homology to sequences derived from other HMA sponges whereas the sequence from the control is related to the seawater bacterium *Pseudoalteromonas* sp. (Table 2). Apparently, the sponge-specific microbial consortium is present in the *I. felix* adult sponge as well as in its reproductive stages although the phylogenetic diversity seems reduced in the latter one. However, this might be due to the smaller number of excised and sequenced bands in larvae and juvenile samples compared to the adult sponge. Similarly, the presence of the sponge-specific microbial community was previously documented in embryos of the HMA sponge *Corticium* sp. (Sharp *et al.* 2007). Furthermore, three selected phylotypes were consistently found in adult sponges and throughout the embryonic development indicating vertical transmission of these microbes. In a recent study, a large set of sequences including 15 sequences of this study were used to compare the microbial diversity of *I. felix* adult sponges and reproductive stages (larvae and juveniles) (Schmitt *et al.* 2007). Phylogenetic tree construction revealed vertical transmission clusters (IF-clusters) that contained sequences of both adult sponges and reproductive material. Therefore, this study in conjunction with the larger sequence dataset (Schmitt *et al.* 2007) clearly showed an overlap among the microbial communities of *I. felix* adult sponges and reproductive stages suggesting vertical transmission of the sponge specific microbial community in *I. felix*.

In summary, the TEM survey revealed that the Caribbean sponges *A. wiedenmayeri*, *A. coralliphagum*, *E. ferox*, *I. felix* and *S. aurea* are associated with large amounts of microorganisms and that these microorganisms are most likely transferred vertically via the sponge reproductive stages. Other sponges that coexist in the same habitat (*C. vaginalis*, *M. laxissima*, *N. digitalis*, *T. ignis* and *U. ruetzleri*) contain few or no microorganisms in the adult mesohyl and the corresponding larvae. DGGE sequence analysis of adult, larvae and juvenile samples of *I. felix* revealed that representatives of the previously described sponge specific microbial consortium (Hentschel *et al.* 2002) are present in *I. felix* and its reproductive stages. Vertical transmission might be important to establish and maintain the phylogenetically complex yet highly sponge-specific microbial community in many other marine HMA sponges.

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References

Brusca RC, Brusca GJ (1990) *Invertebrates*. Sinauer, Sunderland. pp. 181-210

Diaz CM, Akob D, Cary CS (2004) Denaturing gradient gel electrophoresis of nitrifying microbes associated with tropical sponges. *In:* Pansini M, Pronzato R, Bavestrello G, Manconi R (eds). *Sponge science in the new millennium. Boll Mus Ist Biol Univ Genova* 68: 279-289

Enticknap JJ, Kelly M, Peraud O, Hill RT (2006) Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Appl Environ Microb* 72: 3724-3732

Ereskovsky AV, Gonobobleva E, Vishnyakov A (2005) Morphological evidence for vertical transmission of symbiotic bacteria in the viviparous sponge *Halisarca dujardini* Johnston (Porifera, Demospongiae, Halisarcida). *Mar Biol* 146: 869-875

Fieseler L, Horn M, Wagner M, Hentschel U (2004) Discovery of the novel candidate phylum "Poribacteria" in marine sponges. *Appl Environ Microb* 70: 3724-3732

Friedrich AB, Fischer I, Proksch P, Hacker J, Hentschel U (2001) Temporal variation of the microbial community associated with the Mediterranean sponge *Aplysina aerophoba*. *FEMS Microbiol Ecol* 38: 105-113

Friedrich AB, Merkert H, Fendert T, Hacker J, Proksch P, Hentschel U (1999) Microbial diversity in the marine sponge *Aplysina cavernicola* (formerly *Verongia cavernicola*) analyzed by fluorescence *in situ* hybridisation (FISH). *Mar Biol* 134: 461-470

Fuerst JA, Webb RI, Garson MJ, Hardy L, Reiswig HM (1998) Membrane-bounded nucleoids in microbial symbionts of marine sponges. *FEMS Microbiol Lett* 166: 29-34

Gallissian MF, Vacelet J (1976) Ultrastructure de quelques stades de l`ovogénèse des spongiaires du genre *Verongia* (Dictyoceratida). *Ann Sci Nat Zool Biol Anim* 18: 381-404

Hentschel U, Fieseler L, Wehrl M, Gernert C, Steinert M, Horn M, Hacker J (2003) Microbial diversity of marine sponges. *In*: Müller WEG (ed). *Molecular marine biology of sponges*. Springer-Verlag, Heidelberg. pp. 59-88

Hentschel U, Hopke J, Horn M, Friedrich AB, Wagner M, Hacker J, Moore BS (2002) Molecular evidence for a uniform microbial community in sponges from different oceans. *Appl Environ Microb* 68: 4431-4440

Hentschel U, Usher KM, Taylor MW (2006) Marine sponges as microbial fermenters. *FEMS Microbiol Ecol* 55: 167-177

Kaye H (1991) Sexual reproduction in four Caribbean commercial sponges. II. Oogenesis and transfer of bacterial symbionts. *Invertebr Reprod Dev* 19: 13-24

Kaye HR, Reiswig HM (1991) Sexual reproduction in four Caribbean commercial sponges III. Larval behaviour, settlement and metamorphosis. *Invertebr Reprod Dev* 19: 25-35

Lévi C, Lévi P (1976) Embryogénèse de *Chondrosia reniformis* (Nardo), démosponge ovipare, et transmission des bactéries symbiotiques. *Ann Sci Nat Zool* 18: 367-380

Lindquist N, Bolser R, Laing K (1997) Timing of larval release by two Caribbean demosponges. *Mar Ecol Progr Ser* 155: 309-313

Maldonado M, Cortadellas N, Trillas MI, Rützler K (2005) Endosymbiotic yeast maternally transmitted in a marine sponge. *Biol Bull* 209: 94-106

Margot H, Acebal C, Toril E, Amils R, Fernandez Puentes J (2002) Consistent association of crenarchaeal archaea with sponges of the genus *Axinella*. *Mar Biol* 140: 739-745

Muyzer G, Brinkhoff T, Nübel U, Santegoeds C, Schäfer H, Wawer C (1998) Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. *In:* Akkermans ADL, Elsas JD van, Bruijn FJ de (eds). *Molecular microbial ecology*, manual 3.4.4. Kluwer, Dordrecht. pp. 1-27

Preston CM, Wu KY, Molinski TF, DeLong EF (1996) A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov. *Proc Natl Acad Sci USA* 93: 6241-6246

Reiswig HM (1981) Partial carbon and energy budgets of the bacteriosponge *Verongia fistularis* (Porifera: Demospongiae) in Barbados. *PSZN Mar Ecol* 2: 273-293

Sciscioli M, Lepore E, Gherardi M, Scalera-Liaci L (1994) Transfer of symbiotic bacteria in the mature oocyte of *Geodia sydonium* (Porifera, Demospongiae): an ultrastructural study. *Cah Biol Mar* 35: 471-478

Sciscioli M, Scalera-Liaci L, Lepore E, Gherardi M, Simpson TL (1991) Ultrastructural study of the mature egg of the marine sponge *Stelletta grubii* (Porifera, Demospongiae). *Mol Reprod Dev* 28: 346-350

Schmitt S, Weisz JB, Lindquist N, Hentschel U (2007) Vertical transmission of a phylogenetically complex microbial consortium in the viviparous sponge *Ircinia felix*. *Appl Environ Microb* 73: 2067-2078

Sharp KH, Eam B, Faulkner DJ, Haygood MG (2007) Vertical transmission of diverse microbes in the tropical sponge *Corticium* sp. *Appl Environ Microbiol* 73: 622-629

Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiol Mol Biol Rev* 71: 295-347

Taylor MW, Schupp PJ, de Nys R, Kjelleberg S, Steinberg PD (2005) Biogeography of bacteria associated with the marine sponge *Cymbastela concentrica*. *Environ Microbiol* 7: 419-433

Thoms C, Horn M, Wagner M, Hentschel U, Proksch P (2003) Monitoring microbial diversity and natural products profiles of the sponge *Aplysina cavernicola* following transplantation. *Mar Biol* 142: 685-692

Usher KM, Kuo J, Fromont J, Sutton DC (2001) Vertical transmission of cyanobacterial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). *Hydrobiologia* 461: 15-23

Vacelet J (1975) Étude en microscopie électronique de l'association entre bactéries et spongiaires du genre *Verongia* (Dictyoceratida). *J Microsc Biol Cell* 23: 271- 288

Vacelet J, Donadey C (1977) Electron microscope study of the association between some sponges and bacteria. *J Exp Mar Biol Ecol* 30: 301-314

Webster N, Hill RT (2001) The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-proteobacterium. *Mar Biol* 138: 843-851

Webster NS, Webb RI, Ridd MJ, Hill RT, Negri AP (2001) The effects of copper on the microbial community of a coral reef sponge. *Environ Microbiol* 31: 19-31

Wehrl M, Steinert M, and Hentschel U (2007) Bacterial uptake by the marine sponge *Aplysina aerophoba*. *Microb Ecol* 53: 355-365

Wilkinson CR (1978) Microbial associations in sponges. III. Ultrastructure of the *in situ* associations in coral reef sponges. *Mar Biol* 49: 169-176

Wörheide G (1998) The reef cave dwelling ultraconservative coralline demosponge *Astrosclera willeyana* Lister, 1900 from the Indo-Pacific - micromorphology, ultrastructure, biocalcification, isotope record, taxonomy, biogeography, phylogeny. *Facies* 38: 1-88