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Monitoring microbial diversity and natural product profiles of the sponge *Aplysina cavernicola* following transplantation

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Abstract In order to assess the stability of the microbial community of the sponge *Aplysina cavernicola* under in situ conditions, sponges were transplanted from their original location (>40 m depth) to shallower, more light-exposed sites (7–15 m depth). Transmission electron microscopy revealed that the microbial community remained visually unchanged and free of cyanobacteria over the experimental time period of 3 months. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified partial 16S rRNA gene sequences allowed a distinction between the variable and permanent fraction of the bacterial community. Comparative sequence analysis of four variable DGGE bands revealed high sequence similarity to representatives of the Alpha- and Gammaproteobacteria and the phylum Bacteroidetes, which have been recovered previously from Mediterranean seawater as clone sequences or by cultivation. Seven (out of 12) permanent DGGE bands showed high sequence similarity to a sponge-specific, monophyletic 16S rRNA gene sequence cluster within the Acidobacteria division, and to a sequence cluster of uncertain affiliation. These sequence clusters represent members of a common microbial community that is shared among distantly related sponges from different, non-overlapping geographic regions. Four additional permanent DGGE bands showed high sequence similarity to a Betaproteobacterium, *Burkholderia cepacia*, which is not typically known as a marine bacterium. High-performance liquid chromatography analyses of sponge tissues revealed no changes in metabolite pattern, indicating that these compounds are expressed constitutively irrespective of the variations resulting from the transplantation experiment.

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Introduction

Microorganisms play an important role in the diet of sponges (Porifera). As filter feeders, they pump large volumes of seawater (up to 24 m^3 kg^{-1} sponge day^{-1}) through a specialized canal system that permeates the sponge body (Vogel 1977). Microorganisms are filtered out in the choanocyte chambers and transferred into the sponge matrix, termed the mesohyl. The filtration capacities of sponges are remarkable considering that typical seawater contains $1–5\times10^6$ bacteria ml^{-1} and that the expelled seawater is essentially sterile (Reiswig 1974; Pile 1997; Wehrl 2001). Once inside the mesohyl matrix, microorganisms are physically separated from the surrounding seawater by contiguous host membranes. Bacteria are digested via phagocytosis by amoeboid sponge cells, termed archaeocytes (van Soest 1996). There is, however, compelling evidence that a large fraction of mesohyl-associated bacteria may be resistant to digestion. Electron microscopical observations reveal that the abundant bacterial morphotypes in sponges contain slime capsules, enlarged periplasms and additional membranes, which probably serve as shields and barriers to prevent digestion (Wilkinson et al. 1981; Friedrich et al. 1999).

The occurrence of large amounts of bacteria is typical for some demosponges (Vacelet and Donadey 1977; Wilkinson 1987; Willenz and Hartman 1989). In fact, sponges often profit from bacteria-specific traits, such as autotrophy, nitrogen fixation and nitrification

(Wilkinson and Fay 1979; Rützler 1981). By intracellular digestion and translocation of metabolites bacteria may additionally provide nutrition to their hosts (Wilkinson and Garrone 1980; Borowitzka et al. 1988). Furthermore, they have been reported to protect their hosts against UV-radiation (Sara 1971; Regoli et al. 2000) and to synthesize bioactive secondary metabolites that may serve as a chemical defense of the sponges against predators (Unson et al. 1994; Bewley et al. 1996; Althoff et al. 1998). Even though symbiotic interactions between sponges and microorganisms may generally be anticipated, understanding the nature of the interaction between host and associated bacteria still is a challenging task.

Sponges of the Aplysinidae family are known to be associated with large amounts of bacteria that may contribute up to 40% of their biovolume and that exceed the numbers of bacteria in seawater by two to three orders of magnitude (Vacelet 1975). The vast majority of bacteria resides in the mesohyl tissue. Recent studies indicate that the microbial community of the mediterranean species, *A. aerophoba*, could not be cleared upon starvation or upon antibiotics exposure (Friedrich et al. 2001). From these data it was concluded that a major fraction of the microbial community may be permanently associated with *A. aerophoba*, possibly over long periods of time. In contrast, a study by Webster et al. (2001) showed that the microbial community of the Australian sponge *Rhopaloeides odorabile* could be significantly reduced following exposure to sublethal concentrations of cupric ion (Cu^{2+}). Clearly, there is still much to be learned about the stability of the sponge–bacteria interactions and the factors that cause perturbation.

The aim of the present study was to cause perturbations of the microbial community by transplantation of *A. cavernicola* sponges under natural conditions. Secondly, we asked whether changes of the microbial community may be correlated with changes in secondary metabolite profiles. *A. cavernicola* was chosen as a model system, because: (1) well-characterized metabolites can be used as biomarkers (Ebel et al. 1997), (2) because the microbial composition has been characterized to some extent (Vacelet 1971, 1975; Friedrich et al. 1999), (3) because *A. cavernicola* is restricted to a well-defined habitat of deeper waters and underwater caves of the Mediterranean Sea (Pansini 1997), and (4) because in situ transplantation experiments had previously revealed illumination as an environmental stress factor resulting in reduced growth of these sponges (Wilkinson and Vacelet 1979). In our study, *A. cavernicola* sponges were transplanted from their natural environment (> 40 m) to shallower depth regimes (ranging from 7 to 15 m). The microbial community was documented using denaturing gradient gel electrophoresis (DGGE) of PCR (polymerase chain reaction)-amplified partial 16S rRNA gene (16S rDNA) sequences and transmission electron microscopy (TEM). Natural product profiles were monitored using high-performance liquid chromatography (HPLC).

Materials and methods

Sponge transplantation

Aplysina cavernicola specimens (formerly *Verongia cavernicola*, family Aplysinidae, order Verongida, class Demospongiae) were collected by SCUBA-diving at a depth of 40 m off the coast of Elba in the Mediterranean Sea (42°43′N; 10°09′E) in early May 2001. Sponges of about 10 cm height were removed with their rocky substrate from one large colony and brought to the surface in separate plastic bags. Additionally, *A. cavernicola* specimens were collected and immediately frozen at −20°C, representing the sample 40 $m_{t=0}$. In order to prevent disturbance by recreational diving, the sponges were moved to a more remote location about 10 km away (42°43′N; 10°17′E). In order to provide a solid basis for the sponges, the substrate they grew on was poured into synthetic resin (VIAPAL 223BS + catalyzer MEKP, Behnke) that was contained in a metal bin. The bins were fastened to concrete foundations with wire. They were then transplanted to different depths (7 m, 12 m, 15 m). At each depth, positions were chosen within a radius of about 30 m, that were suitable for sponge survival and that represented variable environmental conditions (Table 1). After 2 months, the condition of the transplanted sponges was inspected visually by SCUBA-diving. After 3 months, the sponges were brought to the surface in separate plastic bags. Additionally, samples from the original sponge colony were taken, representing the sample 40 $m_{t=end}$. Tissue samples were removed from the center of sponges with an EtOH-sterilized cork bohrer (4 mm in diameter) and from the sponge surface with a sterile scalpel. They were rinsed three times in sterile seawater, and samples for DGGE and HPLC were immediately frozen in liquid nitrogen.

Transmission electron microscopy

Tissue samples for electron microscopy were taken from the core and surface tissues of each sponge, cut into small slices with an

Table 1 *Aplysina cavernicola* transplantation experiment

Specimen	Sampling depth and location	Fate following transplantation
7a	7 m, light exposed a large rocky platform	Dead
7b	7 m, light exposed a large rocky platform	Dead
7c	7 m, light exposed between boulders	Disappeared
12a[a]	12 m, within the half shadow of a large rock	Healthy
12b[a]	12 m, within the half shadow of a large rock	Healthy
15a[a]	15 m, exposed on a large boulder	Algalover growth
15b	15 m, shady position under a rocky overhang	Disappeared
40 $m_{t=0}$[a]	40 m, on a stone wall	Healthy
40 $m_{t=end}$[a]	40 m, on a stone wall	Healthy

[a] *A. cavernicola* specimens used for DGGE analysis (Fig. 2)

EtOH-sterilized scalpel, rinsed three times in sterile seawater, fixed in 2.5% glutaraldehyde and stored at 4°C. They were then cut into smaller pieces of several cubic millimeters in size, rinsed 3×10 min in 1× PBS and fixed overnight in 2% osmium tetroxide. After two additional rinses with 1× PBS, the pieces were dehydrated in an ethanol series (30%, 50%, 70%, 100%) and incubated 3×30 min in 1x propylene oxide. Following overnight incubation in 1:1 (v/v) propylene oxide/Epon 812 (Serva) the samples were embedded in Epon 812 at a temperature of 60°C. The embedded samples were subsequently sectioned with an ultramicrotome (OM U3, Reichert, Austria) and examined by TEM (Zeiss EM 10, Zeiss, Germany).

Denaturing gradient gel electrophoresis

For DNA extraction, the tissue was ground using a mortar and pestle while being submerged in liquid nitrogen. Genomic DNA was extracted using the Fast DNA Spin Kit for soil (Q-Biogene, Heidelberg, Germany) according to the manufacturer's instructions and stored at 4°C. The universal primers 341F with the GC-clamp [30] spanning *Escherichia coli* positions 341–357 and 518R spanning *E. coli* positions 518–534 were used for 16S rDNA amplification (Muyzer et al. 1998). PCR was performed using a Mastercycler Gradient (Eppendorf, Hamburg, Germany) as follows: one initial denaturation step for 2 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 54°C and 1.5 min at 72°C; and one final elongation step for 10 min at 72°C. The PCR mix consisted of 45 µl 1× reaction buffer, 1 µl of each primer (100 pmol final concentration), 1 µl of deoxyribonucleoside triphosphates (10 µmol), 2.5 U MasterTaq DNA polymerase (Eppendorf) and 1 µl DNA template. Three independent PCR reactions were performed for each specimen. DGGE was performed using a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad, München, Germany) on a 10% (w/v) polyacrylamide gel in 0.5× TAE and using a 0–100% denaturing gradient; 100% denaturant corresponded to 7 M urea and 40% (v/v) formamide. Gradient optimization was performed using 20–80% and 30–70% denaturing gradients; however, the overall resolution was not improved because of increased smearing effects. Electrophoresis was performed for 6 h at 150 V and 60°C. Gels were stained for 30 min in aqueous ethidium bromide solution (0.5 µg ml^{-1}) and photographed with a GelDoc system (GelDoc 2000, BioRad).

Sequencing and phylogenetic analysis

DNA bands were excised from DGGE gels and stored in 20 µl of sterilized distilled water overnight at 4°C. Subsequently, 4 µl of eluted PCR product was used as above, except that the primer 341f did not contain a GC-clamp (Perkin Elmer, Foster City, Calif., USA). The size and quality of the PCR product was verified on 2% agarose gels. Sequencing was performed on an ABI 377XL automated sequencer (Applied Biosystems) using the 341f and 907r primers. The obtained 16S rRNA gene sequences were aligned, and a consensus sequence was assembled using the ABI Prism Autoassembler v. 2.1 software (Perkin Elmer, Foster City, Calif., USA). Sequences were added to the ARB 16S rDNA sequence database (release June 2002; Strunk and Ludwig 1997) along with additional 16S rRNA gene sequences from sponge-associated microbial communities that had been published previously (Webster et al. 2001; Hentschel et al. 2002). Phylogenetic analyses were performed using the ARB software package. Initially, trees were calculated with near full-length sequences only, and the obtained tree topologies were validated using the distance matrix (Jukes–Cantor correction), maximum-parsimony and maximum-likelihood (fast DNAml: Olsen et al. 1994) methods implemented in ARB. Partial sequences were added subsequently to the respective trees by use of the ARB "parsimony interactive" method. The recovered 16S rDNA sequences were deposited in GenBank under accession numbers AY180076–AY180091.

High-performance liquid chromatography

Sponge tissue frozen with liquid nitrogen was lyophilized (Lyovac GT-2, Steris, Hürth, Germany). A total of 10 g dry tissue weight was homogenized and extracted twice with 50 ml methanol (MeOH) for 1 h at room temperature. The crude extract was dried with a rotary evaporator (Rotavapor R200, Büchi, Switzerland) and dissolved in 35 ml MeOH + 15 ml Nanopure water. For HPLC analysis, 20 µl of this solution was injected into a HPLC system coupled to a photodiode-array detector (Dionex, München, Germany). The separation column (125×4 mm) was prefilled with Eurospher C_{18} (Knauer, Berlin, Germany). Alkaloids were detected by their online-recorded UV-spectra and by direct comparison with previously isolated standards (Ebel et al. 1997).

Results and discussion

The Mediterranean sponges *Aplysina cavernicola* and *A. aerophoba* populate the same geographic region; however, a co-existence of the two species has, to our knowledge, not been reported. While *A. cavernicola* is restricted to deeper waters (> 40 m) and underwater caves, *A. aerophoba* is found in shallow locations (5–15 m), where they prefer light-exposed positions. In the present study, *A. cavernicola* was transplanted from its natural habitat (40 m) to shallower locations (15 m, 12 m, 7 m) with different levels of light exposure. *A. cavernicola* specimens were collected 3 months later and inspected with respect to changes in microbial diversity and natural product profiles (Table 1). Already after 2 months, all sponges at 7 m depth showed signs of tissue disintegration, and after 3 months they had degraded entirely. This indicates that the upper limits of the transplantation experiment have been reached. In contrast, the sponges that had been placed within the half shadow at the base of a large rock at 12 m depth appeared to be in a good healthy condition by visual judgement and tissue consistency. From the sponges that had been placed at 15 m depth, one had disappeared, while the other one was overgrown by algae and displayed a color change from sulfidic-yellow to ochre-yellow. The individual that had been placed at 15 m exposed to sunlight was in worse condition than the two individuals placed in the shade at 12 m depth. Therefore, light exposure appears to be the most restrictive parameter for the survival of the transplanted sponges. These results are consistent with the observations by Wilkinson and Vacelet (1979), who showed that growth of *A. cavernicola* was significantly reduced following increased illumination. Because of the low level of replication, a statistical analysis is not possible, making it difficult to interpret the results with respect to environmental variability.

The sponges *A. cavernicola* and *A. aerophoba* contain strikingly similar microbial communities as judged by morphological inspection and molecular techniques, such as fluorescence in situ hybridization (Friedrich 1998). To investigate whether the microbial community of *A. cavernicola* specimens had changed over time, the tissues were examined by TEM (Fig. 1). A high density

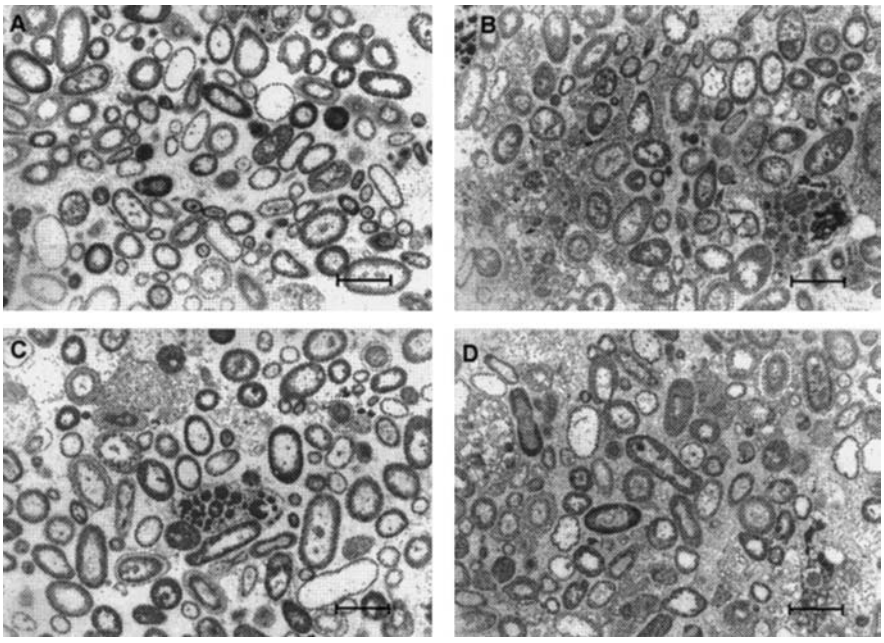

Fig. 1a–d *Aplysina cavernicola*. Transmission electron micrographs of the mesohyl. Tissues were analyzed from the original colony at 40 m depth at the beginning (**a**) and the end of the experiment (**b**), as well as from sponges transplanted to 15 m (**c**) and 12 m (**d**), each taken at the end of the experiment. The pictures are representative of the other sponge tissues analyzed (data not shown). *Scale bars*: 2 μm

of bacteria within the mesohyl tissue of all remaining sponges was noted. To compare the microbial diversity between different sponge tissues, bacterial outer membrane features were used as a visual indicator (Friedrich et al. 1999). Inspection of sponge tissue electron micrographs revealed no differences in bacterial numbers or in morphotype diversity (Fig. 1). Similarly, no differences were observed in surface tissues (data not shown). Occasionally, dividing bacteria were found, indicating their metabolic activity.

The sponge species *A. aerophoba* and *A. cavernicola* differ with respect to the presence of cyanobacteria. *A. aerophoba* contains high numbers in its surface tissues that likely protect their host against UV-radiation (Sara 1971; Vacelet 1971; Rützler 1985; Regoli et al. 2000). In contrast, the tissues of *A. cavernicola* are notably devoid of this conspicuous morphotype, with one noticeable exception (Vacelet 1971). Interestingly, there was no TEM evidence for cyanobacterial invasion into the surface or core tissues of *A. cavernicola*, not even in sponge 15a, which was overgrown by algae and had changed color towards ochre-yellow. These results are consistent with previous reports by Vacelet (1959), who found that transplantation of both species into the same habitat did not result in an exchange of cyanobacteria. Possibly, the tissue of *A. cavernicola* may not be amenable to cyanobacteria because of physical barriers or chemical defense, because the appropriate strain was not present in the seawater, or because the exposure time was too short. Moreover, in adult sponges the distances between the choanocyte chambers as sites of bacterial uptake and the surface tissues as their final destination might be located too far away. However, it cannot be excluded that juvenile *A. cavernicola* could accommodate cyanobacteria while the sponges are still small in size.

The bacterial diversity within the sponge mesohyl was examined using DGGE analysis of PCR-amplified partial 16S rDNA sequences (Fig. 2). This molecular approach allows the fingerprinting of microbial

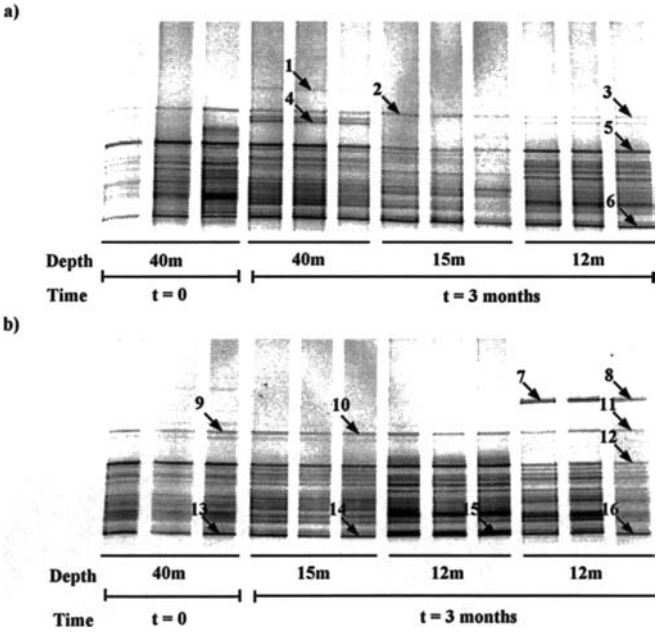

Fig. 2a, b *Aplysina cavernicola*. Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments from individual *A. cavernicola* sponges following transplantation (see Table 1). Three independent polymerase chain reactions were performed for each specimen. In order to show the variability within the original sponge colony over time, the $t = 0$ and $t = 3$ month time points were run (**a**). In order to show the variability following transplantation within the same depth regime, samples from two different sponges were run (**b**). *Arrows* indicate DGGE bands for which sequence information was obtained. DGGE analysis was performed from center core (**a**) and surface layer tissues (**b**)

communities without the need to culture the respective microorganisms (Muyzer et al. 1998). In the ideal case, the DGGE bands represent single 16S rDNA gene sequences that are separated on an increasing denaturing gradient gel based on the melting properties of the respective DNA hybrids (which are directly correlated to the G+C content of the 16S rDNA sequences). Thus, each lane represents a fingerprint of a microbial community at a given time. DGGE analysis is particularly useful for the characterization of the sponge-associated microbial community, as it allows us to distinguish between the permanent and the variable bacterial fraction.

The application of DGGE to *A. cavernicola* following transplantation revealed a large number of permanent bands that remained unchanged throughout the experiment. Consistent with the TEM data, there was no evidence for the occurrence of novel, cyanobacterial DGGE bands. These are visually easily identified as thick bands that run in the top half of the gel (Hentschel, unpublished data). Several bands could not be well separated even on a 30–70% gradient, because of increased smearing, and thus eluded sequencing (data not shown). Moreover, bands appear in the same location in the gel that gives rise to different sequences (DGGE bands 9–11 and 13–16). Because the DGGE method separates based on the melting behavior of the DNA fragment, which depends on its GC-content rather than on the sequence per se, it is not always possible to separate the fragments despite sequence variation (Muyzer et al. 1998). Accordingly, as the GC-contents vary only by 0.5% (bands 9–11) and 1.01% (bands 6, 13–16), it is possible that they were not separated accurately on the gradient. Alternatively, more than one band might have been cut out, due to the high number of bands on the gradient (for example, band 15). While gradient optimization was attempted, the resolution of individual bands could not be improved (data not shown). Two of the variable bands (1, 4) appeared in the original sponge colony at 40 m after 3 months, indicating that changes in the microbial community were not due to transplantation (Fig. 2a). Two additional variable bands (7, 8) occurred in one of the two sponges at 12 m depth, suggesting also that microbial community changes were not due to transplantation (Fig. 2b).

The variable bacterial community is represented by bands 1, 4, 7 and 8. Sequencing and phylogenetic analyses revealed that the 16S rDNA sequence represented by DGGE band 1 is most closely related to an uncultured vent bacterium within the phylum Bacteroidetes, and that DGGE bands 7 and 8 represent 16S rDNA sequences affiliated with a sulfur-oxidizing bacterium OAII2 within the Gammaproteobacteria (Figs. 2, 3; Table 2). Both clone sequences were recovered from Mediterranean hydrothermal vents near Milos (Greece) (Sievert et al. 2000; S.M. Sievert and J. Kuever, GenBank, unpublished). The 16S rDNA sequence represented by DGGE band 4 is most closely related to the Alphaproteobacterium MBIC3368 that was originally isolated from a marine sponge (T. Hamada, unpublished, GenBank accession number AB012864). Phylogenetically highly similar bacterial strains have also been isolated from the Australian sponge *Rhopaloeides odorabile* (isolate NWCu001) (Webster and Hill 2001), from the Mediterranean sponge *A. aerophoba* (isolate SB89) (Hentschel et al. 2001), from the Adriatic sponge *Suberites domuncula* (isolates SB1, SB2) (Thakur et al. 2003) and from tropical deep-water sponges (Ohlson et al. 2002). Apparently, this group of closely related Alphaproteobacteria is frequently associated with diverse marine sponges.

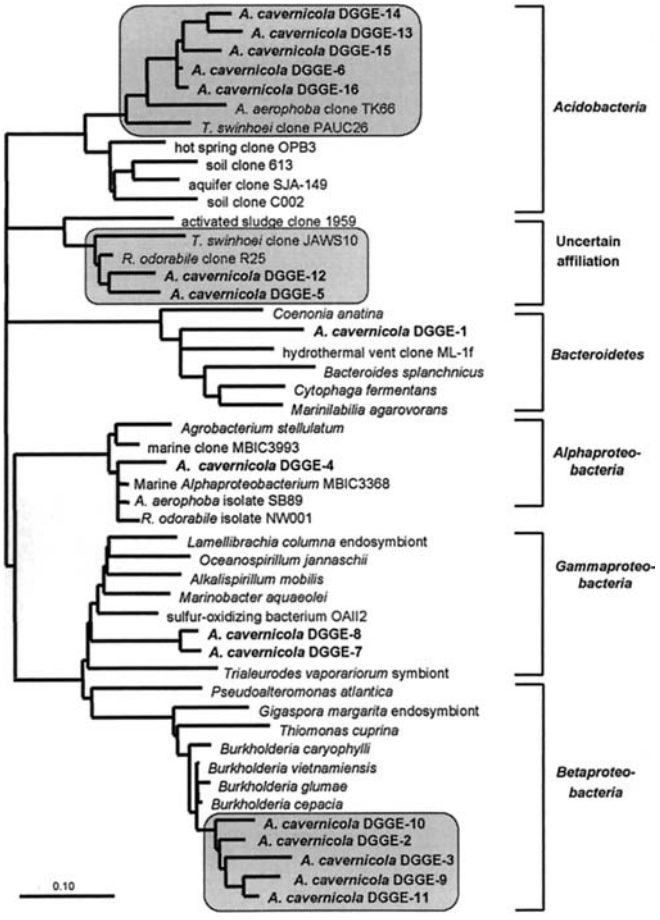

Fig. 3 Neighbor-joining tree showing the affiliation of partial 16S rDNA sequences (approx. 500 bp) representing the respective DGGE bands shown in Fig. 2. Multifurcations indicate that the branching order could not unambiguously be resolved using different treeing methods. *Gray boxes* indicate monophyletic sequence clusters. *Scale bar* indicates 10% sequence divergence

The permanent bacterial fraction revealed at least two distinct phylogenetic lineages (Figs. 2, 3; Table 2). The 16S rDNA sequences represented by DGGE bands 2, 3 and 9–11 show high sequence similarity (on average 97.0–98.4%) to the betaproteobacterial species *Burkholderia cepacia*. *B. cepacia* has been isolated from various terrestrial environments including drinking water (Butler et al. 1995; Zanetti et al. 2000), but, to our knowledge, not yet from the marine environment. Several pathogenic variants of *B. cepacia* exist that cause

Table 2 Denaturing gradient gel electrophoresis (*DGGE*) sequence analysis

DGGE band	GC content	DGGE pattern	Homology	Nearest sequence match in GenBank (BLAST)	Bacterial division
1	48.64%	Variable	89.2%	Hydrothermalvent clone ML-1f (AF208990)	Bacteroidetes
2	52.86%	Constant	97.0%	*Burkholderiacepacia* (AF335494.1)	Betaproteobacteria
3	52.72%	Constant	91.2%	*Burkholderiacepacia* (AF097533)	Betaproteobacteria
4	52.60%	Variable	98.8%	Marine alphaproteobacterium MBIC3368 (AF218241)	Alphaproteobacteria
5	55.99%	Constant	98.6%	*Rhopaloeidesodorabile* clone R25 (AF333537)	Gammaproteobacteria
6	61.19%	Constant	92.8%	*Theonellaswinhoei* clone PAUC26 (AF186410)	Acidobacteria
7	50.76%	Variable	90.6%	Sulfur-oxidizing bacterium OAII2 (AF170423)	Gammaproteobacteria
8	49.81%	Variable	91.0%	Sulfur-oxidizing bacterium OAII2 (AF170423)	Gammaproteobacteria
9	53.35%	Constant	95.8%	*Burkholderia cepacia* (AF335494.1)	Betaproteobacteria
10	53.55%	Constant	98.4%	*Burkholderia cepacia* (AF335494.1)	Betaproteobacteria
11	53.63%	Constant	97.1%	*Burkholderia cepacia* (AF335494.1)	Betaproteobacteria
12	56.39%	Constant	98.2%	*Rhopaloeides odorabile* clone R25 (AF333537)	Gammaproteobacteria
13	60.85%	Constant	91.7%	*Theonellas winhoei* clone PAUC26 (AF186410)	Acidobacteria
14	61.42%	Constant	90.9%	*Theonellas winhoei* clone PAUC26 (AF186410)	Acidobacteria
15	61.99%	Constant	91.1%	*Theonella swinhoei* clone PAUC26 (AF186410)	Acidobacteria
16	61.32%	Constant	91.7%	*Theonella swinhoei* clone PAUC26 (AF186410)	Acidobacteria

opportunistic infections in humans and plants (Butler et al. 1995). *B. cepacia* is also known for its extremely versatile metabolism, being able to use p-nitrophenol as a sole C, N and energy source (Prakash et al. 1996). It is conceivable that closely related marine bacteria with a similar metabolic repertoire may be able to take nutritional advantage of the high concentrations of brominated phenolic compounds that are present in *Aplysina* sponges (Teeyapant et al. 1993).

The permanent bands 6 and 13–16 represent 16S rDNA sequences that group with a 16S rDNA sequence cluster within the phylum Acidobacteria that was previously identified in the marine sponges *A. aerophoba* and *Theonella swinhoei* (Hentschel et al. 2002) (Figs. 2, 3; Table 2). Interestingly, the respective host sponges were collected from different geographic regions and are phylogenetically only distantly related. While *A. aerophoba* (subclass Ceractinomorpha) was collected from the Mediterranean (global positioning system, GPS: 42°29′N; 03°08′E), *T. swinhoei* (subclass Tetractinomorpha) was collected off the W. Caroline Islands, Palau (GPS: 07°23′N; 134°38′E). Nevertheless, the 16S rDNA sequences of this cluster are more closely related to each other than to any acidobacterial sequence in the database. The phylum Acidobacteria typically contains terrestrial bacteria, for which only few culturable representatives exist. Accordingly, the genetic and metabolic diversity is still largely undescribed (Hugenholtz et al. 1998; Barns et al. 1999).

The 16S rDNA sequences represented by permanent DGGE bands 5 (mesohyl core tissue) and 12 (surface tissue) also belong to a monophyletic sequence cluster that contains only sponge-derived sequences. Due to the remarkably low sequence similarity to other 16S rDNA sequences deposited in public databases, the precise phylogenetic position of this cluster remains unknown. Here, the sponge host *T. swinhoei* was collected off the coast of Hachijo-jima Island, Japan (GPS: 33°38′N; 139°48′E) (Hentschel et al. 2002) and *R. odorabile* (subclass Ceractinomorpha) was collected off Davies Reef, Australia (18°49′S; 147°38′E) (Webster et al. 2001). Both monophyletic 16S rDNA sequence clusters appear to be specific to marine sponges, as their members have not been recovered from other marine habitats so far. The identification of the acidobacterial sequence cluster and the sequence cluster of uncertain phylogenetic affiliation in the sponge *A. cavernicola* extends the abundance of the respective microorganisms to yet another sponge species.

Verongida sponges are characterized by an abundance of bromotyrosine-derived compounds of which >100 analogues have been identified to date (Carney and Rinehart 1995). Due to structurally different pigments, the color of *A. cavernicola* is sulfidic-yellow, while *A. aerophoba* is more ochre-yellow colored. The major *A. cavernicola*–specific metabolite, aerothionin, is matched by the *A. aerophoba*–specific compound isofistularin-3, which contains the same spiro-cyclohexadieneisoxazoline moieties, albeit connected via different diamino compounds. These compounds are mostly contained within host cells, termed spherulous cells, but are also found in the sponge fibers (Thompson et al. 1983; Turon et al. 2000). As shown previously these precursors are converted into the highly bioactive compounds aeroplysinin-1 and dienone following breakdown of the cellular compartmentation (Weiss et al. 1996; Ebel et al. 1997; Goldenstein et al. 2000; Thoms 2000). Despite the different precursor metabolites in the two sponges, the products of this conversion (aeroplysinin-1 and the dienone) are the same.

In order to determine whether the natural product profile of *A. cavernicola* changes following transplantation of the sponge, HPLC analysis was performed. Since no changes in natural product profiles were observed in the transplanted sponges when compared to specimens from the original location, only one representative HPLC diagram is shown (Fig. 4). Moreover, a shift of the compound pattern towards the metabolite profile characteristic for *A. aerophoba*, as one might have expected by increased illumination, was not observed.

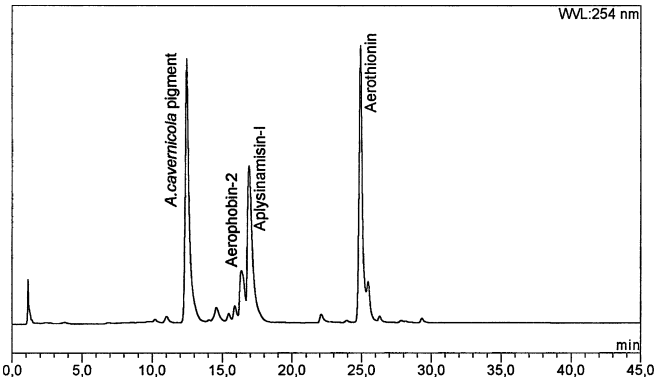

Fig. 4 *Aplysina cavernicola*. High performance liquid chromatography (HPLC) chart of a methanolic extract from *A. cavernicola* collected from the original location after 3 months. The HPLC profile is representative of the other sponges analyzed (data not shown)

These results are consistent with previous observations by Ebel et al. (1999), who have shown that *A. aerophoba* metabolite patterns are stable, even if the examined individuals were sampled at locations >2000 km apart. Betancourt-Lozano et al. (1998) also came to the conclusion that the antimicrobially active compounds of *A. fistularis* were produced continuously throughout the annual cycle. Friedrich et al. (2001) have shown that microbial composition and metabolite patterns in *A. aerophoba* did not change upon exposure to antibiotics or under starvation conditions. Because the cumulative experimental (starvation, antibiotics) and environmental parameters (geographic location, sunlight, annual cycle) had no apparent effect on secondary metabolite profiles or concentrations, it is concluded that the expression of these compounds is constitutive rather than inducible in *Aplysina* sponges. However, for the lack of change in microbial community composition, conclusions regarding a possible involvement of bacteria in the biosynthesis of the brominated compounds cannot be drawn.

In summary, the transplantation of *A. cavernicola* into shallower habitats resulted in tissue degradation, particularly at the shallower, light-exposed locations, which was likely caused by stress due to increased illumination. In spite of these morphological changes of the host, the bacterial community was largely unaffected. The application of DGGE allowed, to our knowledge for the first time, the differentiation between the variable and permanent fraction of a sponge-associated microbial community. The variable bacterial fraction is phylogenetically related to common seawater bacteria, while seven (out of 12) permanent DGGE bands represented sequences that group with a cluster that have recently been identified as members of an ocean-spanning, previously elusive and sponge-specific microbial community. Four permanent DGGE bands represented a 16S rDNA sequence that was most closely related to *B. cepacia*, which is not typically known from a marine environment. The lack of transition from *A. cavernicola* to *A. aerophoba* following transplantation, both with regard to appearance and alkaloid profiles, lends support to the hypothesis that these taxa should be regarded as two separate sponge species rather than two different phenotypes, merely shaped by their different environments. Finally, the cumulative lack of variability of secondary metabolites points towards the constitutive nature of these secondary metabolites.

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