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Evidence for a symbiosis between bacteria of the genus *Rhodobacter* and the marine sponge *Halichondria panicea*: harbor also for putatively toxic bacteria?

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Abstract *Halichondria panicea* (Pallas) is a marine sponge, abundantly occurring in the Adriatic Sea, North Sea, and Baltic Sea. It was the aim of the present study to investigate if this sponge species harbors bacteria. Cross sections through *H. panicea* were taken and inspected by electron microscopy. The micrographs showed that this sponge species is colonized by bacteria in its mesohyl compartment. To identify the bacteria, polymerase chain reaction (PCR) analysis of the 16S rRNA gene segment, typical for bacteria, was performed. DNA was isolated from sponge material that had been collected near Rovinj (Adriatic Sea), Helgoland (North Sea), and Kiel (Baltic Sea) and was amplified with bacterial primers by PCR. The data gathered indicate that in all samples bacteria belonging to the genus *Rhodobacter* (Proteobacteria, subdivision α) are dominant, suggesting that these bacteria live in symbiotic relationship with the sponge. In addition, the results show that the different samples taken contain further bacterial species, some of them belonging to the same genus even though found in sponges from different locations. The possibility of the presence of toxic bacteria was supported by the finding that organic extracts prepared from sponge samples displayed toxicity, when analyzed in vitro using leukemia cells.

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Introduction

It is a characteristic feature of most unusual and harmful marine algal blooms that they appear suddenly and show erratic fluctuations. Factors controlling, in some cases, the outbreak of such blooms might be climatic or seasonal. For example, the occurrence of the "brown-tide" caused by the algal chrysophyte *Aureococcus anophagefferens* shows no specific pattern except that the bloom appears in summer (Sieburth et al. 1988). Some views suggest that turbulence in the energy spectrum is the sole cause for the fluctuation in the appearance of harmful blooms (Platt and Denman 1975). The basis for the toxicity of blooms are one-celled organisms, e.g. bacteria, protozoa or algae as well as viruses. Viral infection of one-celled organisms, e.g. in algae, has been made responsible for harmful blooms (Van Etten et al. 1987) but it has also been suggested (i) that bacteria are the primary source of toxins usually attributed to toxic algae, e.g. in dinoflagellates (Kodama 1990), or (ii) that the eukaryotic organisms themselves, e.g. in diatoms (Todd 1993), are toxic; data were reviewed in Shimizu (1993).

At present neither sufficient data are available nor have investigations on the sources and harbors of toxic one-celled organisms been completed which might allow prediction of harmful blooms. It is known that sponges (Porifera) are one of the major animal phyla found in the marine hard-substrate benthos, both with respect to the number of species and to biomass (Sarà and Vacelet 1973). Sponges are sessile filter-feeders and ingest particles of sizes between 5 and 50 μm through the cells of the mesohyl and the pinacoderm, and microparticles (0.3 to 1 μm) via the cells of the choanocyte chambers. A sponge specimen of 1 kg may filter ≈24 000 liters d^{-1} (Vogel 1977). Plankton, e.g. dinoflagellates, contribute approximately 20% to the food of sponges; the remaining 80% are detrital organic particles (Margulis and Schwartz 1995). Consequently, sponges are constantly exposed to the environment to an extent which is

unique in the animal kingdom. Thus they are also suitable animals to study biomarkers for the monitoring of physical and chemical stress (reviewed in Müller and Müller 1997). For this purpose both biochemical (Bachinski et al. 1997) and molecular biological techniques have been applied (reviewed in Koziol et al. 1997).

Sponges are known as rich sources of bioactive secondary metabolites (Sarma et al. 1993). Some of these, like avarol, are synthesized by the sponge cells (Müller et al. 1986a), others, like okadaic acid, were initially isolated from a sponge (Tachibana et al. 1981) but later detected in the microalga *Prorocentrum lima* (Murakami et al. 1982). Sponges are thought to live in a symbiotic relationship (reviewed in Simpson 1984) with one-celled organisms such as (i) prokaryotes, bacteria (Vacelet 1970) and primarily cyanobacteria (Vacelet 1971) as well as (ii) eukaryotes, zooxanthellae (yellow symbiotic dinomastigotes) (Sarà and Liaci 1964) or (iii) zoochlorellae (green symbotic algae) (Gilbert and Allen 1973). These organisms occur both extracellularly and intracellularly (Wilkinson 1978). The molecular basis for the proposed symbiotic relationship is not sufficiently known. Evidence has been presented that growth of bacteria in *Halichondria panicea* is maintained by a lectin produced from the eukaryotic host (Müller et al. 1981).

The potential of sponges to harbor toxic one-celled organisms has not yet been studied and is one aim of this report. The sponge *Halichondria panicea* was selected because it is very abundant in the North and Baltic Seas, where it is found on red algae at a depth of 8 m (Barthel 1986). In the Kiel Bight, red algae communities cover an area of 500 km^2 (Breuer 1989; Witte and Barthel 1994); more than 15% of the sessile epifaunal biomass in this area can be attributed to *H. panicea*. In 1922, Babic described *H. panicea* also in the Adriatic Sea, where it is abundantly found in caves (Riedl 1966). It is assumed that the same species is present even in the Arctic, Antarctic, Atlantic, Pacific and Indian Oceans (Arndt 1935).

For the identification of potentially harmful one-celled organisms, several techniques can be applied, e.g. immunological techniques (Shapiro et al. 1989) or nucleic acid probe technology. Focusing on the latter methodology, which is characterized by its high sensitivity and specificity as well as its high fidelity, the following approaches are possible: (i) hybridization of distinct gene segments by probes using a solid phase system, like immobilization on nylon membranes (Macario and Macario 1990) or (ii) identification of variable domains, especially of the ribosomal genes (rDNA) (Stahl et al. 1988). A very powerful identification method is based on the polymerase chain reaction (PCR) which amplifies 5.8S rDNA from eukaryotes (Scholin and Anderson 1993) and 16S rDNA from bacteria, especially from those living in a marine ecosystem (Giovanni 1991).

In the present study the bacterial colonization of the sponge *Halichondria panicea* was determined by PCR technique with focus on whether the same bacterial species is dominant in specimens collected from the Adriatic and Baltic Seas as well as from different locations in the North Sea.

Materials and methods

Materials

Restriction endonucleases and other enzymes for recombinant DNA techniques and vectors were obtained from Stratagene (La Jolla, CA; USA), Qiagen (Hilden; Germany), Boehringer Mannheim (Mannheim; Germany), GibcoBRL (Grand Island, NY; USA), Amersham (Buckinghamshire; UK), USB (Cleveland, OH; USA), Dupont (Bad Homburg; Germany) and Promega (Madison, WI; USA).

Sponges

Live specimens of *Halichondria panicea* (Pallas) (Porifera: Demospongiae: Halichondriida: Halichondriidae) were collected at different locations. The first collection was from the Adriatic Sea, near Rovinj (Croatia), in the unpolluted, fjord-like bight Limski Kanal, from a cave at a depth of 5 m. A second collection was made in the North Sea, near Helgoland (Germany) from a depth of 8 to 10 m. Three sites, none of which is heavily polluted, were selected: (i) South Harbor (pre-harbor), internal site of the mole (low diesel load); (ii) North Harbor, internal site of the mole (lower diesel load); and (iii) Swimming Pool, external site of the mole (lowest diesel load). And the third was from the Baltic Sea near Kiel (Germany), from a depth around 2 to 12 m. The water in the Kiel Bight is brackish (salinity 13 to 17‰); the sampling was from red algae (*Coccotylus truncatus* and *Phycodrys rubens*) at "Boknis Eck – Hausgarten", a low-polluted area.

Soon after collection the material was frozen in liquid nitrogen until use.

Amplification, cloning and sequencing of partial 16S rRNA genes

DNA was extracted as follows. Tissue samples were ground on ice in a 20 m*M* Tris-HCl buffer (pH 7.2; containing 0.1 *M* NaCl, 1 m*M* EDTA, 1% Na-dodecyl sulfate). This material was incubated overnight with 50 μg ml^{-1} (final concentration) of proteinase K; after phenol-chloroform extraction the DNA was obtained by ethanol precipitation (Ausubel et al. 1995).

PCR and sequencing were performed as follows. The primers used were those of Rochelle et al. (1995), with modifications according to the following rationale. The forward primer, termed 8fn, was 5′-GGAGAGTTTGATCA/CTGGCT-3′ [located at the 5′-terminus of the 16S rRNA gene (Giovanni 1991)]; at a hybridization temperature T_{opt} of 58 °C (56 °C + 2 °C) it matched 100% with the bacteria: *Alcaligenes* 15–62 [Accession No. X86589], *Agromyces cerinus* [D45060], α-*Proteobacterium* (SAR 102) [L35460], *Acinetobacter* 1F-19 [X86591], *Agromyces fucosus* [D45061], *Acinetobacter* AC-40 [X86572], *Arthrobacter* AC-48 [X86594], *Agromyces mediolanus* [D45052], *Actinomadura globosa* [D50660], *Abiotrophia adjacens* [D50540]. This primer binds at nucleotide (nt) position 6–24 to the 16S rRNA gene from the *rrnB* cistron of *Escherichia coli* (Brosius et al. 1978). The reverse primer, termed 798rn, 5′-CCAGGGTATCTAATCCTGTT-3′ (T_{opt} of 58 °C) was designed to bind in the region approximately 700 to 900 nt downstream from the forward primer in the *E. coli* sequence; this size of rRNA is convenient to sequence. Highest identity was determined by FASTA (FASTA 1997) analysis from nt 780 to 799; this segment matches up to 100% with: *E. coli* [U70214], *Salmonella sofia* [X80677], *Paracoccus denitrificans* [X69159], *Paracoccus solventivorans* [Y07705], *Microscilla arenaria* [M60455], *Microscilla furvescens* [M58792], *Cytophaga diffluens* [M58765],

Antarcticum vesiculatum [M61002], *Flectobacillus glomera* [M58775] and *Cytophaga marina* [D12667].

The PCR reaction mixtures of 50 µl included 10 pmol of the primers, 200 µ*M* of each nucleotide, 1 ng of DNA and 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim). PCR amplifications were run on a GeneAmp 9600 thermal cycler (Perkin Elmer) under the following conditions: 30 cycles of 45 s at 95 °C, 45 s at 55 °C and 3 min at 74 °C. Negative controls with water instead of DNA resulted in no amplification. After electrophoresis the proper bands in the size range of approximately 800 bp were observed in the ethidium-bromide-stained gel. Amplification products were purified through a QIAquick Spin column (Qiagen), cloned in bulk into pGEM-T (Promega) and the clones were sequenced (Sequenase Version 2.0, USB). For a rapid screening to distinguish unique and duplicate clones, sequencing was performed with one single dideoxynucleotide (here ddG) as described by Schmidt et al. (1991). DNA sequencing was performed with an automatic DNA sequenator (Li-Cor 4000S).

Sequence comparisons

Sequences were analyzed using computer programs (BLAST 1997; FASTA 1997) and the data in the Ribosomal Database Project (RDP 1997). The phylogenetic tree was constructed on the basis of nucleotide sequence alignment, using the neighbour-joining method (Saitou and Nei 1987) and the program PHYLIP Ver. 3.5c (Felsenstein 1993). Multiple alignment was performed with CLUSTAL W Ver. 1.6 (Thompson et al. 1994), and the graphic presentation was prepared with GeneDoc (Nicholas and Nicholas 1997). Bootstrap analysis was performed with 1000 trials (Felsenstein 1985).

Electron microscopy

The tissue samples were dehydrated in an acetone series and the specimens were embedded in Spurr resin (Spurr 1969). Ultrathin sections were prepared and stained with uranyl acetate and lead citrate and examined with a Zeiss EM 9A transmission electron microscope.

Preparation of extracts and testing for bioactivity

Extraction of sponge samples was performed as described by Müller et al. (1985). First, 3 g of sponge tissue was homogenized in ethanol and subsequently extracted with ethyl acetate. The extracts were evaporated to dryness; 12 to 15 mg of tar-like residue per sample was obtained.

For the cytotoxicity studies L5178y mouse lymphoma cells were used. The cells were grown in Eagle's minimal essential medium, supplemented with 10% of horse serum in roller tube cultures as described by Müller and Zahn (1979). For the dose–response experiments, 5-ml cultures were initiated by inoculation of 5×10^3 cells ml^{-1} and were incubated at 37 °C for 72 h. Cell growth was determined with a Cytocomp counter (128-channel counter, system Michaelis) and the ED_{50} value (concentration which reduces cell number by 50%) was determined (Müller et al. 1986b).

Results

Identification of bacteria in *Halichondria panicea* in situ

Ultrathin sections were prepared from a specimen of *H. panicea* collected near Rovinj and inspected by transmission electron microscopy. The results revealed that the sponge cells are surrounded by numerous bacteria, present in the mesohyl compartment of the sponge (Fig. 1a). At higher magnification rodshaped bacteria of ≈0.5 µm width and ≈1.1 µm length appeared (Fig. 1b).

Identification of bacteria by PCR amplification

PCR amplification, cloning and subsequent sequencing were performed as described in "Materials and methods". For the sequence analyses the 5′-terminus of the 16S rRNA gene, covering Domain I and most of Domain II, was selected. For rapid screening, the clones were analyzed using a single dideoxynucleotide as described by Schmidt et al. (1991). As an example, the sequencing pattern for a sample from the North Sea (North Harbor) is shown; among the 20 clones analyzed, two clones displayed unique patterns (Fig. 2). In total, 244 clones were screened by the one-lane analysis (data not shown).

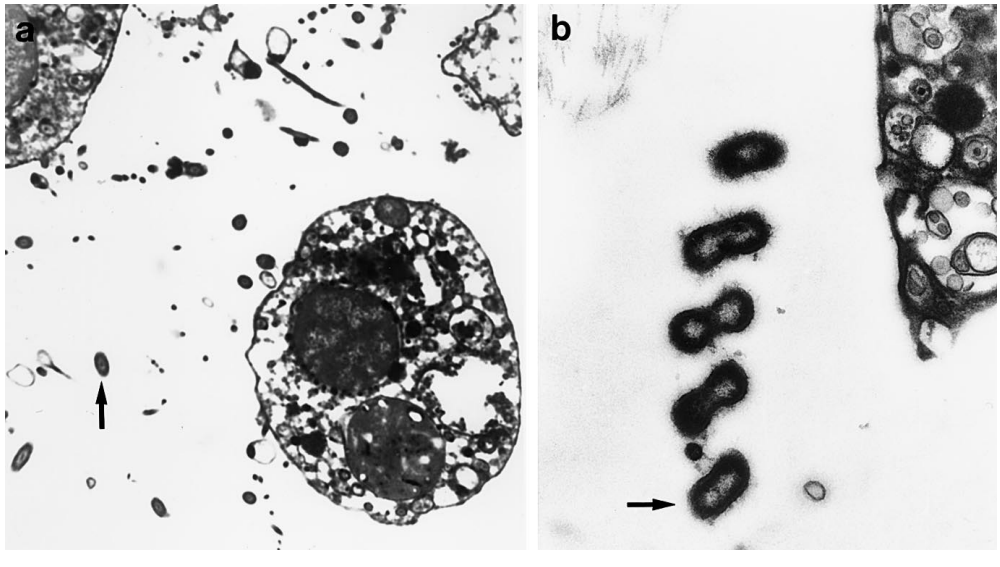

Fig. 1 *Halichondria panicea*. Transmission electron micrographs from a thin section through the mesohyl of *H. panicea*. The specimen was collected at Rovinj (Croatia). Bacteria indicated by arrows. Magnification: 2400× (**a**); 12 000× (**b**)

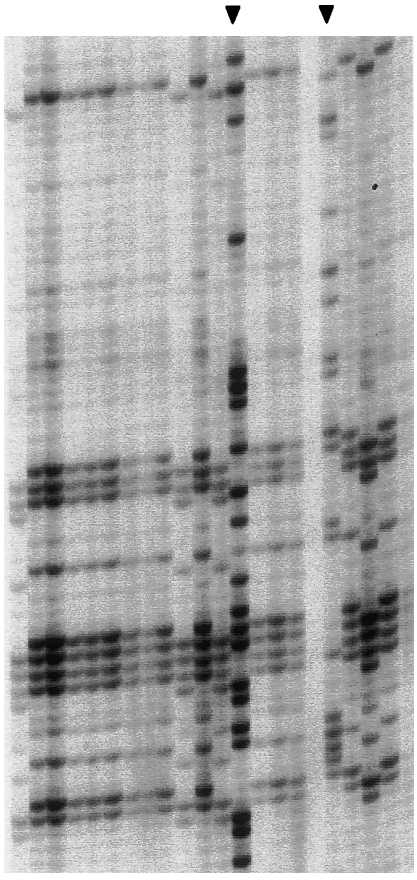

Fig. 2 Single-nucleotide sequence pattern for 20 bacterial clones, by using one dideoxynucleotide in the PCR reaction. Details under "Materials and methods". A sample from the North Sea (HpaNS1) is shown. Lanes of the two unique clones marked by arrowheads; in the remaining lanes some shifts of the sequencing patterns are present

Multiple as well as unique clones were subsequently sequenced to completion. Sequences were analyzed using the computer programs BLAST, FASTA and RDP; the highest scores of similarity (percentage of nucleotide identity) obtained from one of the three programs are listed in Tables 1 to 3.

Classification of the bacteria

From the sponge sample collected in the Adriatic Sea, DNA was extracted and then amplified by PCR using the primer sets (see under "Materials and methods") which are characteristic for 16S rRNA. To distinguish between unique and duplicate clones, sequencing reactions were carried out with a single dideoxynucleotide. Among the 121 clones analyzed, 14 clones contained different patterns. Representatives of these clones were selected and subjected to detailed sequencing. All inserts were found to code for 16S rDNA segments; they are abbreviated as HpaAS. Four identical clones displayed highest similarity to *Rhodobacter veldkampii* with 90% (termed HpaAS1), while five other identical clones showed, with 89%, again the highest similarity to *R. veldkampii* (HpaAS2). All other clones, containing different inserts were present only once or twice. The relationships, with respect to nucleotide identity between the bacterial clones, obtained from the sponge and the bacterial sequences compiled in the data banks are summarized in Table 1.

In a similar approach the samples from the other locations were investigated. From the North Sea three samples obtained from *Halichondria panicea*, which had been collected from different locations off Helgoland, were analyzed: (i) North Harbor, abbreviated HpaNS1; (ii) South Harbor, HpaNS2; and (iii) Swimming Pool, HpaNS3. The results revealed that the sample obtained from North Harbor contained three different bacterial specimens, from South Harbor six and from Swimming Pool three distinguishable sequences (Table 2). It is striking that the "*Rhodobacter*"-like sequences isolated from *H. panicea*, which had been collected from different sites off Helgoland, were found to have identical nucleotide sequences; they were therefore termed HpaNS1–3.

Table 1 *Halichondria panicea*. Sequence similarity between bacterial 16S rRNA sequences in *H. panicea* from the Adriatic Sea (HpaAS) with sequences present in the data banks

Clones, Accession No.	Highest similarity (%) [Accession No.]	Division
HpaAS1		
Z88581	*Rhodobacter veldkampii*; 90% [D16421]	Proteobacteria-α
HpaAS2		
Z88582	*Rhodobacter veldkampii*; 89% [D16421]	Proteobacteria-α
Z88580	*Rhodospirillum molischianum*; 85% [M59067]	Proteobacteria-α
Z88583	*Azospirillum brasilense*; 85% [Z29617]	Proteobacteria-α
Z88584	*Azospirillum brasilense*; 86% [Z29617]	Proteobacteria-α
Z88585	*Bdellovibrio stolpii*; 81% [M34125]	Proteobacteria-δ
Z88587	*Cytophaga latercula*; 84% [M58769]	Cytophagales
Z88588	*Psychroserpens burtonensis*; 85% [U62913]	Cytophagales
Z88579	*Codakia orbiculata* symbiont; 84% [X84979]	Proteobacteria-γ
Z88589	*Codakia orbiculata* symbiont; 85% [X84979]	Proteobacteria-γ
Z88586	PBN1; 84% [X83532]	Proteobacteria-β
Z88592	PBN1; 84% [X83532]	Proteobacteria-β
Z88590	*Rhizobium loti*; 88% [D14514]	Proteobacteria-α
Z88591	*Azospirillium brasilense*; 85% [Z29617]	Proteobacteria-α

Table 2 *Halichondria panicea*. Sequence similarity between bacterial 16S rRNA sequences in *H. panicea* from Helgoland, North Sea: North Harbor (HpaNS1), South Harbor (HpaNS2) and Swimming Pool (HpaNS3) with sequences present in the data banks

Clone, Accession No.	Highest similarity (%) [Accession No.]	Division
HpaNS1 (North Harbor)		
Z88567	*Rhodobacter veldkampii*; 89% [D16421]	Proteobacteria-α
Z88566	*Vibrio logei*; 89% [X74708]	Proteobacteria-γ
Z88566	*Vibrio logei*; 87% [X74708]	Proteobacteria-γ
Z88566	*Vibrio logei*; 87% [X74708]	Proteobacteria-γ
HpaNS2 (South Harbor)		
Z88567	*Rhodobacter veldkampii*; 89% [D16421]	Proteobacteria-α
Z88566	*Vibrio logei*; 89% [X74708]	Proteobacteria-γ
Z88570	*Sphingomonas adhaesiva*; 87% [X74708]	Proteobacteria-α
Z88572	*Rizobium* sp.; 96% [U50168]	Proteobacteria-α
Z88571	*Burkholderia cepacia*; 96% [X87275]	Proteobacteria-β
Z88573	*Lamellibrachia columella* endosymbiont; 89% [U77481]	Proteobacteria-γ
HpaNS3 (Swimming Pool)		
Z88567	*Rhodobacter veldkampii*; 89% [D16421]	Proteobacteria-α
Z88568	*Burkholderia cepacia*; 96% [X87275]	Proteobacteria-β
Z88569	*Erythrobacter* sp.; 90% [M59063]	Proteobacteria-α

Table 3 *Halichondria panicea*. Sequence similarity between bacterial 16S rRNA sequences in *H. panicea* from the Baltic Sea (HpaBS) with sequences present in the data banks

Clone, Accession No.	Highest similarity (%) [Accession No.]	Division
HpaBS		
Z88578	*Rhodobacter veldkampii*; 89% [D16421]	Proteobacteria-α
Z88574	*Cytophaga uliginosa*; 88% [M62799]	Cytophagales
Z88575	*Rhodospirillum salinarum*; 84% [M59069]	Proteobacteria-α
Z88576	*Ehrlichia risticii*; 83% [M21290]	Proteobacteria-α
Z88577	*Microscilla aggregans*; 86% [M58791]	Cytophagales

In parallel the bacteria were analyzed in *Halichondria panicea*, obtained from the Baltic Sea. Again the "*Rhodobacter*"-like sequences were identified (HpaBS), displaying an identity of 89% to *R. veldkampii* (Table 3). In addition, four more bacterial sequences of lower identities were detected and analyzed.

Phylogenetic analysis of the potential *Rhodobacter* sp. symbiont

The sequence data of the 16S rDNA gene segment show that the samples obtained from the Adriatic Sea (HpaAS1 and HpaAS2), the North Sea (HpaNS1–3) and the Baltic Sea (HpaBS) have high similarity, with 90 to 89%, to the Proteobacterium (α subdivision) *Rhodobacter veldkampii*, a purple, nonsulfur bacterium [Accession No. D16421 – Gracilicutes: Anoxyphotobacteria (Hiraishi and Ueda 1994)].

The phylogenetic tree inferred from the neighbour-joining method shows that the 16S rRNA "*Rhodobacter*"-like sequences, isolated from *Halichondria panicea*, are related to members of the Proteobacteria, subdivision α (Fig. 3). In the trichotomous tree the "*Rhodobacter*"-like sequences are together with other members of the α-subdivision, *Roseobacter algocolus*, the *Prionitis lanceolata* gall symbiont and unidentified *Alpha proteobacterium 307* in one branch. Slightly distantly related are the sequences from *Rhodobacter veldkampii*, *Rhodobacter capsulatus*, *Rhodovulum sulfidophilum* and *Rhodovulum adriaticum*. For the establishment of the tree the sequence from *Escherichia coli* (Proteobacteria, subdivision γ) was used as outgroup.

Cytotoxicity

To determine, if the sponge specimens display differential toxicity with respect to the locations where the samples were collected, organic extracts were prepared and toxicity tests were performed using leukemic cells; results are given in Table 4. It was found that the extract, isolated from the sponge sample from the Adriatic Sea displayed the lowest toxicity – expressed as an ED_{50} value – with 13.8 μg ml^{-1}, while extracts from the samples Swimming Pool, Helgoland (9.8 μg ml^{-1}) → Baltic Sea (6.5 μg ml^{-1}) → North Harbor, Helgoland (4.3 μg ml^{-1}) → South Harbor, Helgoland (2.1 μg ml^{-1}) displayed, in this order, significantly higher toxicitiy.

Discussion

The fact that sponges are associated with bacteria has been known since the contributions of Vacelet in the early 1970s (see "Introduction"). However, the function of sponge-associated bacteria remains unclear. Evidence has been presented suggesting a symbiotic relationship between bacteria and sponges (Müller et al. 1981); it can

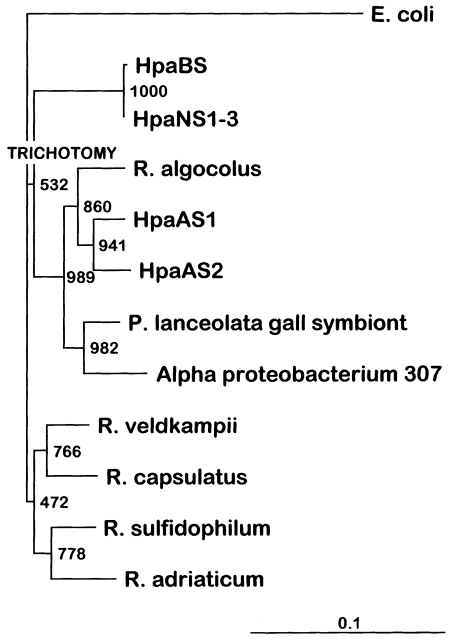

Fig. 3 Phylogenetic tree showing relationships between clones from samples from the Adriatic Sea (HpaAS1 and HpaAS2), North Sea (HpaNS1–3), as well as from the Baltic Sea (HpaBS) and the partial, corresponding sequence of 16S rRNA of *Roseobacter algocolus* (R. algocolus; Accession Number X78314), *Prionitis lanceolata* gall symbiont (P. lanceolata gall symbiont; U37762), unidentified *Alpha proteobacterium 307* (Alpha proteobacterium 307; U14583), *Rhodobacter veldkampii* (R. veldkampii; D16421), *Rhodobacter capsulatus* (R. capsulatus; D16428), *Rhodovulum sulfidophilum* (R. sulfidophilum; D16423) and *Rhodovulum adriaticum* (R. adriaticum; D16418). These sequences of the Proteobacteria, subdivision α, were compared with one of the members of the Proteobacteria, subdivision γ, and with *Escherichia coli* (E. coli; J01695) as an outgroup. The tree was constructed by the neighbour-joining method. Scale bar indicates a distance of 0.1 fixed mutation per nucleotide position. Numbers refer to bootstrap values for each node out of 1000 replicate resamplings

Table 4 *Halichondria panicea*. Cytotoxic activity of samples collected from different locations

Location	Cytotoxicity, ED_{50} (μg ml^{-1})
North Sea, Helgoland:	
South Harbor	2.1 ± 0.2
North Harbor	4.3 ± 0.3
Swimming pool	9.8 ± 1.3
Baltic Sea, Kiel Bight	6.5 ± 0.8
Adriatic Sea	13.8 ± 1.6

furthermore be assumed that bacteria are ingested with food and remain in the sponge body, establishing a commensalistic and/or an antagonistic interaction. To answer these questions one segment of the 16S rRNA gene was selected to characterize the bacterial microflora in the sponge *Halichondria panicea*.

Whole DNA was extracted from specimens of *Halichondria panicea* collected from three very distant, up to 7500 km apart, locations: Rovinj (Adriatic Sea), Helgoland (North Sea) and Kiel (Baltic Sea). 16S rRNA gene analysis revealed that the specimens from all locations contained bacteria; a sequence similarity of >90% was found with respect to species belonging to the genus *Rhodobacter*, and within this group most related to *R. veldkampii*. Members belonging to this genus are phototrophic, purple, nonsulfur bacteria (Imhoff et al. 1984). *Rhodobacter* species are gram-negative and facultatively photoheterotrophic bacteria. They grow either anaerobically or phototrophically in the presence of sulfide as an electron donor (Imhoff 1989). *Rhodobacter* species are found both in freshwater and terrestrial environments, but are also common in marine and hypersaline habitats. Based on nucleotide sequence data some marine *Rhodobacter* species have been transferred to the genus *Rhodovulum* (Hiraishi and Ueda 1994).

It is striking that in all *Halichondria panicea* specimens analyzed, the *Rhodobacter*/*Rhodovulum* bacteria dominate. The phylogenetic tree, based on the partial 16S rDNA gene sequences, shows that "*Rhodobacter*"-like sequences obtained from bacteria of the sponge *H. panicea* should be grouped with the Proteobacteria of the subdivision α.

These nucleotide sequence data may imply that *Rhodobacter* species are symbionts of *Halichondria panicea*; the potential symbiotic function must still be studied. In addition, it becomes obvious that the *Rhodobacter* species/strains occurring in this sponge are closely related, regardless of whether they were collected from the North, Baltic, or Adriatic Seas.

This finding may also be of interest for the taxonomical grouping of *Halichondria panicea*, as it is still under debate whether *H. panicea* should actually be separated into different species (Diaz et al. 1993). Even though the spicules appear to be identical in the specimens collected in different areas, the shapes of the individual sponges often clearly differ (Diaz et al. 1993). It has been reported that wave forces affect the size of spicules of *H. panicea* (Palumbi 1986). Furthermore, *H. panicea* has been shown to display a high level of genetic polymorphism (Solé-Cava and Thorpe 1990); the value for the mean heterozygosity per locus in this sponge species was determined to be 0.23, while this value commonly ranges between 0.02 and 0.15 in plant and animal species.

The existence of bacteria in *Halichondria panicea* was further substantiated by electron microscopical analysis. The bacteria were found to be abundantly present in the mesohyl of this sponge species. Future in situ hybridization studies with specific nucleotide probes, based on the sequence analyses presented here, will be performed to identify which of the bacterial species live within the organism, i.e. in the mesohyl, or are present on the sponge surface and/or colonize the channel system.

Our 16S rRNA gene analysis also showed that a series of other bacterial species are present in *Halichondria panicea*. In spite of the fact that species belonging to the same genus, e.g. *Cytophaga* or *Rhodospirillum*, are found in samples collected from distant locations, we cannot presently rule out the possibility that some of the

bacteria identified are epibionts. It is also premature to discuss which of the bacteria present in *H. panicea* might be toxic, as it is a known fact that different species of the same genus show different patterns of toxicity.

It has only been possible in a few cases to correlate a given toxicity found in an animal, with a given bacterial species. One example is the toxic compound okadaic acid present in the sponge *Halichondria okadai* (Tachibana et al. 1981), which is produced by the microalga *Prorocentrum lima* (Murakami et al. 1982). However, because sponges are filter-feeders, like bivalves (Gallacher and Birkbeck 1995), the possibility that they accumulate toxin-producing protists from the surrounding water appears realistic and will be tested in future exposure experiments.

The possibility that sponges, such as *Halichondria panicea*, may indeed harbor toxic microorganisms can be deduced from our finding that extracts isolated from *H. panicea*, which had been collected at different sites, displayed a significantly varying toxicity on human leukemia cells in vitro. The toxicity of the samples collected near Helgoland was sixfold higher than that associated with the same species from the Adriatic Sea.

Most known toxins occurring during blooms are neurotoxins that act on the sodium channels of both vertebrates and invertebrates, e.g. tetrodotoxin, saxitoxin (Catterall 1985) or brevetoxin (Shimizu 1988). In contrast to voltage-gated ion channels, which are characterized by fast responses, other neuronal receptors, like the metabotropic glutamate receptors (mGluRs) react relatively slowly (Nakanishi 1994).

In conclusion, these data show that the marine sponge *Halichondria panicea* harbors a dominant – very likely symbiotic – bacterial species belonging to the genus *Rhodobacter*. In addition we detected further bacterial species in *H. panicea*, depending upon the location from which the sponge samples were taken. Due to the different toxicities present in organic extracts prepared from these samples, we assume that toxic bacteria might be present in this sponge species.

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