SPONGE ASSOCIATED BACTERIA FROM BOREAL SPONGES

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ABSTRACT

This study represents the microbiological part of the BOSMAN project (Boreal Sponges; Sources of Marine Natural Products) and investigates the diversity of bacteria associated with sponges from boreal deep water regions. A variety of sponges were collected from the Sula Ridge and the shelf off the Norwegian coast with the aim to identify and characterize their associated microorganisms. Classical cultivation techniques as well as culture independent molecular methods were applied for this purpose. With both approaches a broad diversity of phylogenetic groups could be detected, but there was no correspondence between the results of the two methods. Each dataset provides a different part of the picture and thus contributes to a more comprehensive view of the sponge bacteria community.

KEY WORDS

Sponge, bacteria, boreal, 16S rDNA, microbial diversity.

INTRODUCTION

Research in the field of sponges and associated microorganisms have indicated a bacterial source of compounds with pharmacological properties, formerly attributed to the host organisms (KELECOM, 2002; PROKSCH *et al.,* 2002). Up to date most studies concentrated on sponges from tropical and Mediterranean regions. Investigations of cold water sponge-bacteria communities show that they also represent a reservoir for the production of specific bioactive substances. Corresponding data are mainly available from antarctic sponges (JAYATILAKE *et al.,* 1996; MCCLINTOCK & BAKER, 1997; AMSLER *et al.,* 2001) but also from sponges of the North and Baltic Sea (ALTHOFF *et al.,* 1998; PEROVIC *et al.,* 1998; WICKE *et al.,* 2000).

In the BMBF priority program "Marine Natural Products Research" the multidisciplinary project **BOSMAN** - **BO**real **S**ponges - Sources of **MA**rine **N**atural Products has undertaken the task of examining newly discovered compounds produced by marine Porifera and their associated bacteria in boreal habitats of the North Atlantic.

Our interest in the microbiological part of this project has been the analysis of the diversity of sponge associated bacteria. This was approached by culture dependent methods as well as by employing the culture independent method of

cloning and sequencing of the bacterial 16S rRNA genes and their phylogenetic affiliation. To our knowledge 16S rDNA libraries to examine microbial diversity in sponges have hitherto been constructed in two studies from warm water environments only (WEBSTER *et al.,* 2001a; HENTSCHEL *et al.,* 2002). Here we present first results from the analysis of a selected set of Demospongiae and their associated bacteria from a boreal habitat.

MATERIAL AND METHODS

Sample collection

The sponges were obtained from various sites at water depths of 233 - 330 m along the Sula Ridge off the Norwegian coast in 1999. Collection was done with a manned submersible providing an excellent means to obtain good quality specimen without producing unnecessary damage to the habitats. Microbiological analysis of 12 sponges belonging to the Demospongiae was performed in this study: *Geodia macandrewi* Bowerbank, 1858 (GM), *Isops phlegraei* Sollas, 1862 (IP), *Haliclona* sp. 1 (HAL), *Hemigellius pumiceus* (Fristedt, 1885) (HP), *Mycale lingua* (Bowerbank, 1858) (ML), *Oceanapia robusta* (Bowerbank, 1866) (OR), *Pachastrella* sp. (PA), *Petrosia crassa* Carter, 1876 (PC), *Phakellia ventilabrum* (Johnston, 1842) (PV), *Plakortis* sp. (P), *Polymastia sol* (Schmidt, 1870) (PS), *Thenea muricata* (Bowerbank, 1858) (TM). For molecular analysis *Geodia barretti* Bowerbank, 1858 from the Korsfjord (near Bergen, Norway) was collected.

Cultivation of sponge-associated bacteria

Sponges were homogenized, serially diluted in sterile seawater, and cultivated on different media using the spread plate technique: MBA (Bacto Marine Broth 2216 from Difco at pH 7.6 or pH 9), CSA (per liter: 1 g casein, 5 g $MgSO_4$ x $7H_2O$, 0.5 g K_2HPO_4 x $3H_2O$, 10 g soluble starch, 30 g sea salt), CDC (per liter: 10 g Bacto peptone, 5 g NaCl, appr. 6 ml 1N NaOH), BM_0 (BAUMANN & BAUMANN, 1981) and BM_0 supplemented with methanol (0.5 %). All media contained 15 g Bacto agar (Difco) per liter. Subcultivation was always performed on MB agar plates and at an incubation temperature of 8° C. Colony morphology was used as a selection criterion for further cultivation und subsequent molecular analysis.

DNA preparation and amplification of 16S rRNA genes

DNA was extracted from the bacterial colonies by using alkaline lysis buffer (0.25 % Na-dodecyl sulfate, 50 mM NaOH) and incubation at 95° C for 15 min. The PCR was performed with the universal bacterial primers: 616V, 5‘ AGA GTT TGA TYM TGG CTC AG and 1525R, 5‘ AAG GAG GTG WTC CAR CC. Cycling conditions were as follows: 1.5 min at 96° C, 35 cycles of 2 min at 96° C, 3 min at 57° C, 1.5 min at 72° C, and a last extension step at 72° C for 10 min. The PCR products were purified on QIAquick spin columns (Qiagen) and their quantity and quality was visualized on agarose gels.

Cloning of bacterial 16S rDNA

Sponge tissue was homogenized on ice with sterile artificial seawater, suspended in alkaline lysis buffer (see above), and incubated at 95° C for 15 min. After a 1:10 dilution in AE buffer (Qiagen) PCR was performed with two different universal bacterial primer pairs: 616V / 1525R (see above) and 63f / 1387R (63f, 5‘ CAG GCC TAA CAC ATG CAA GTC and 1387R, 5‘ GGG CGG WGT GTA CAA GGC). Cloning of the PCR products was performed with the TOPO TA cloning kit (Invitrogen) according to the supplier‘s protocol. To determine the size of the plasmid inserts individual *Escherichia coli* clones were lysed and inserts were amplified using the M13 primers: forward, 5‘ CTG GCC GTC GTT TTA and reverse, 5‘ CAG GAA ACA GCT ATG A with the following cycling conditions: 10 min at

94° C, 25 cycles of 94° C for 1 min, 54° C for 1 min, 72° C for 2 min, and the final extension step at 72° C for 10 min. The resulting products were checked by agarose gel electrophoresis and candidates with the correct size were chosen for a nested PCR with universal bacterial primers (shown above).

Cycle sequencing and phylogenetic analysis

The PCR products resulting from amplification of the 16S rDNA of the cultured bacteria as well as of the cloned fragments in *E. coli* were partially sequenced (approx. 500 bp) with the primer 610R (5‘ ACC GCG GCT GCT GGC AC) using the PE Gene Amp PCR System 9700 (Perkin Elmer) and ABI Prism 310 (PE Biosystems). Sequence data were edited with Chromas version 1.45 (http://www.technelysium. com.au/), phylogenetically analysed by the ARB software program (http://www.arb-home.de/), and compared to the GenBank database (http://www.ncbi.nlm.nih.gov/). The bacterial isolates were initially grouped using MALDI-TOF mass spectrometry. On the basis of 280 16S rDNA sequences of the bacterial isolates and their phylogenetic affiliation with the GenBank this method provided a rapid means to detect duplicates and allowed the classification of the bacteria into distinct phylogenetic groups by comparing their characteristic mass spectral patterns. This method will be described elsewhere (R. Dieckmann *et al.,* unpubl. data).

RESULTS

Culture-dependent diversity

In this study more than 700 bacterial strains were isolated on a variety of media from 12 boreal sponges collected at the Sula Ridge.

Different bacterial morphotypes from each sponge were selected and the phylogenetic relationship to known bacterial classes was determined for 378 isolates. The species were found to be distributed across 15 genera of the Eubacteria at widely different frequencies. The majority of them grouped within the Gammaproteobacteria (85 %), the Alphaproteobacteria comprised approximately 12 % of the total number whereas representatives of Firmicutes and Bacteroidetes were rare (3 %, 0.5 %) (Tab. I). The most abundant bacterial isolates in the Gammaproteobacteria group belonged to the genus *Pseudoalteromonas*. Altogether more than 50 % of all isolated strains of known phylogeny from different sponges were affiliated to this genus and clustered closely together. The most abundant species within that genus were affiliated to *P. carrageenovora*, *P. nigrifaciens*, *P. haloplanktis*, *P. atlantica*, and *P. antarctica*. Other isolates from the Gammaproteobacteria were represented by members of the genera *Pseudomonas*, *Vibrio, Alteromonas, Marinobacter*, *Colwellia*, and *Shewanella* (in decreasing numbers). Isolates from the class Alphaproteobacteria were phylogenetically affiliated to *Erythrobacter*, *Roseobacter*, *Sphingomonas*, and one to *Agrobacterium*. Isolates from the phylum Firmicutes were represented by the genera *Bacillus* and *Marinococcus* whereas two isolates from the phylum Bacteroidetes were affiliated to *Cytophaga* and *Flavobacterium*. The diversity of culturable bacteria was dependent on the media used. The highest diversity was achieved by MBA medium, in contrast to CSA where the Alphaproteobacteria, in particular the genus *Erythrobacter* was enriched (data not shown).

Tab. I. Phylogenetic distribution of cultivated bacteria, isolated from 12 boreal sponges (for abbreviations see Materials and Methods).

phylum	class	genus	number of isolates per sponge	total
Proteobacteria	γ-proteobacteria		HAL: 3, IP: 1	4
		Pseudoalteromonas	GM: 9, HAL: 56, IP: 72, OR: 3, P: 17, PC: 1, PV: 50, PS: 12, TM: 2	222
		Alteromonas	HAL: 11, IP: 2, PV: 1, PS: 1	15
		Colwellia	HAL: 4	4
		Marinobacter	HAL: 4, IP: 1, P: 3	8
		Shewanella	TM: 1	1
		Pseudomonas	HAL: 14 , HP: 2, ML: 2, OR: 6, P: 8, PA: 1, PV: 3, PS: 11	47
		Vibrio	IP: 8, PA: 1 , PS: 6, TM: 4	19
	α-proteobacteria		PS: 5	5
		Erythrobacter	HAL: 17, OR: 2, PS: 10, TM: 1	30
		Sphingomonas	HAL: 1, OR: 1	2
		Roseobacter	HAL: 5, IP: 1, PS: 2	8
		Agrobacterium	PS: 1	1
Firmicutes	Bacilli	*Bacillus*	P: 5	5
		Marinococcus	HAL: 5	5
Bacteroidetes		*Cytophaga*	PS: 1	1
		Flavobacterium	PS: 1	1
				378

Tab. II. Phylogenetic affiliation of 69 clones from *Geodia barretti* based on partial sequencing of 16S rDNA.

phylum	class	number of clones
Proteobacteria	γ-proteobacteria	3
	α-proteobacteria	2
Unclassified group of bacteria located between Cyanobacteria and Spirochaetes		4
Unclassified group of bacteria located between Spirochaetes and Deferribacteres		24
Nitrospirae		1
Acidobacteria		26
Unclassified group of bacteria located between Acidobacteria and Actinobacteria		2
Actinobacteria		4
Firmicutes		1
Aquificae		2

Culture–independent diversity

The bacterial profile of the marine sponge *Geodia barretti*, sampled off the coast of Norway, was analysed by cloning and sequencing of the 16S rDNA. The distribution of 69 partially sequenced clones in the phylogenetic tree is shown in Tab. II. All clones were representatives of the superkingdom Bacteria. The majority of the clones were either phylogenetically affiliated to the Acidobacteria (26 clones; 38 %)

or represented an unclassified group (24 clones; 35 %) placed between the phyla Deferribacteres and Spirochaetes. Clones which appeared in lower frequency (below 6 % of the total number) fit either into the taxonomic units of the Gammaproteobacteria, Alphaproteobacteria, Nitrospira, Actinobacteria, Firmicutes, Aquificae, or they clustered in unclassified groups placed between the Acidobacteria and Actinobacteria as well as between the Spirochaetes and Cyanobacteria.

DISCUSSION AND CONCLUSIONS

In this study we used conventional cultivation strategies in combination with the molecular approach of 16S rDNA cloning to investigate the microbial communities in selected sponges.

The culturable microbial community of the boreal sponges shows a composition not unusual for a marine habitat with representatives of the Gammaproteobacteria, Alphaproteobacteria, and of the Firmicutes as well as the Bacteroidetes (HENTSCHEL *et al.,* 2001). It is however heavily dominated by Gammaproteobacteria, in particular by *Pseudoalteromonas*. This genus is often found in a wide range of marine locations including marine eukaryotic organisms (FRIEDRICH *et al.,* 1999; HOLMSTRÖM & KJELLEBERG, 1999). The most abundant isolates within that genus were affiliated to the closely related *P. carrageenovora*, *P. nigrifaciens*, *P. atlantica*, and *P. antarctica*. *P. haloplanktis* was also observed, this species has been mentioned in the context of water around sampling sites (HENTSCHEL *et al.,* 2001). In all the examined sponges where the MBA medium was used for cultivation (*Isops phlegraei*, *Haliclona* sp. 1, *Phakellia ventilabrum*, and *Plakortis* sp.) this dominance of *Pseudoalteromonas* was observed. In contrast reports on different culturable bacterial communities have identified members of the Alphaproteobacteria as dominant sponge associates (WEBSTER & HILL, 2001; OLSON *et al.,* 2002). Although the number of strains isolated was highest on MBA, the bacterial genera other than *Pseudoalteromonas* were represented in relatively low numbers as compared to other media which supported the growth of only few colonies but on which genera from the Alphaproteobacteria (in particular on CSA) appeared enriched. Due to the big difference in cultivation efficiency and the fact, that altogether in a given habitat only a minor proportion of the bacteria can be cultured (JANNASCH & JONES, 1959; WEBSTER *et al.,* 2001a) no conclusion can be drawn as to the actual composition of the bacterial community based on these culture data only.

A great contribution to the detection of sponge-bacteria relationships has been achieved with the use of molecular cloning techniques (PRESTON *et al.,* 1996; ALTHOFF *et al.,* 1998; WEBSTER *et al.,* 2001a,b; WEBB & MAAS, 2002; HENTSCHEL *et al.,* 2002). We used this molecular approach first for the analysis of the diversity of sponge associated bacteria from the boreal sponge *Geodia barretti*. The phylogenetic analysis of the 69 cloned DNA fragments shows that some groups of clones correlated with published cloning data including the Actinobacteria, Acidobacteria, Nitrospira, Alpha- and Gammaproteobacteria, and we also found a cluster of uncertain affiliation located between the Acidobacteria and Actinobacteria as shown by HENTSCHEL *et al.* (2002). But we also detected members of phyla which, to our knowledge, have so far not been described in molecular cloning approaches from sponges: two sequences were phylogenetically affiliated into the vicinity of Aquificae,

some clustered in the region between Deferribacteres and Spirochaetes and between Spirochaetes and Cyanobacteria. Final phylogenetic affiliations of the complete sequences of the cloned 16S rDNA fragments are in progress as well as the expansion of the clone library. The phylogenetic analysis of the 69 cloned DNA fragments indicated the presence of bacteria across a wide range of phyla but essentially no correspondence with our cultivated bacteria was found.

Our results show the necessity for different cultivation methods. Presently we concentrate on anaerobic cultivation approaches and use continuous culture systems operated under oligotrophic conditions mimicking the nutritional surroundings of the sponge in order to enrich for bacteria which are normally overgrown by fast growing species. In addition, as molecular data about boreal sponge-bacteria communities are rare, we propose to compare more sponges from the same habitat. Ongoing work includes FISH (fluorescence in situ hybridisation) to determine if the bacteria detected by the above described methods are present in the investigated sponges.

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