Microbial nitrification in Mediterranean sponges: possible involvement of ammonia-oxidizing Betaproteobacteria

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Abstract: The aim of this study was to assess the potential for nitrification in *Aplysina aerophoba* Schmidt 1862 using a combined physiological and molecular approach. Whole animals were incubated in experimental aquaria and the concentrations of ammonia, nitrate and nitrite were determined in the incubation water using colorimetric assays. Nitrate excretion rates reached values of up to 3.6 µmol g⁻¹ fresh weight day⁻¹ (equivalent to 830 nmol g⁻¹ dry weight h⁻¹) and were matched by ammonia excretion rates of up to 0.56 ± 0.09 µmol g⁻¹ fresh weight day⁻¹. An accumulation of nitrite was not detected in any of the experiments. Control experiments without sponges showed no variation in nitrogen species in the incubation water. A slight increase in ammonia excretion was observed over 11 days of maintenance in holding tanks that were constantly supplied with fresh, untreated Mediterranean seawater. Other sponges from the same habitat (*Dysidea avara* Schmidt 1862, *Tethya* sp., *Chondrosia reniformis* Nardo 1847) showed high rates of ammonia excretion but nitrate excretion was significantly reduced or absent. Using specific PCR primers, 16S rRNA genes of the betaproteobacterial clade of the Nitrosospira cluster 1 were recovered from *A. aerophoba*, *D. avara* and *Tethya* sp. tissues. In conclusion, this study provides physiological and molecular evidence for the presence of nitrifying bacteria in *A. aerophoba* while the potential for nitrification in the other sponges remains to be investigated.

Keywords: Ammonia-oxidizing Betaproteobacteria, microbial consortia, nitrification, 16S rRNA gene, sponge

**Introduction**

Sponges (Porifera) are evolutionarily ancient metazoans with a fossil record dating back nearly 600 million years in time (Li *et al.* 1998). Today, an estimated 13,000 species, classified in three classes (Demospongiae, Calcarea, Hexactinellida) populate virtually all benthic marine and freshwater habitats (Hooper and van Soest 2002). Sponges have a primitive morphology lacking true organs or tissues. Most metabolic functions are carried out by totipotent, amoeboid cells, termed archaeocytes that move freely through the mesohyl matrix. Inhalant and exhalant canals build an aquiferous system through which water is actively pumped by flagellated choanocytes (Brusca and Brusca 1990). As filter-feeders, sponges efficiently take up nutrients like organic particles and microorganisms from the seawater, leaving the expelled water essentially sterile (Reiswig 1974, Pile 1997, Wehrl *et al.* 2007).

Despite the fact that sponges feed on microorganisms, large amounts of extracellular microorganisms populate the mesohyl matrix of many demosponges (for recent reviews, see Hentschel *et al.* 2003, 2006, Imhoff and Stöhr 2003, Hill 2004). Bacterial numbers may constitute as much as 40 - 60 % of the total biomass exceeding concentrations of seawater by two to four orders of magnitude. Molecular diversity analyses showed that the sponge microbiota is phylogenetically complex, yet highly sponge-specific. Members of eight eubacterial phyla [Proteobacteria (Alpha-, Gamma-, Deltaproteobacteria), Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Gemmatimonadetes and Nitrospira], members of the recently discovered candidate phylum ‘Poribacteria’ (Fieseler *et al.* 2004), and of one archaeal phylum (Crenarchaeota) are numerically abundant and metabolically active in sponges. As none of these sponge-specific microorganisms have been obtained in pure culture, their function, metabolism, and possibly nutritional interactions with the host sponge are virtually unknown.

In this study, we aimed to investigate the process of microbial nitrification in Mediterranean demosponges. Nitrification describes the oxidation of ammonia (NH₃) to nitrite (NO₂⁻) by ammonia-oxidizing bacteria (AOB) and archaea (AOA) and subsequently to nitrate (NO₃⁻) by nitrite-oxidizing bacteria (NOB) for energy purposes (Kowalchuk and Stephen 2001). Several lines of evidence suggest that marine sponges are indeed a reservoir for...
nitrifying microorganisms. Firstly, sponges and many other marine invertebrates release ammonia as a metabolic waste product (Wang and Douglas 1998, Davy et al. 2002) and nitrate excretion has already been documented in Caribbean (Corredor et al. 1988, Pile 1996, Diaz and Ward 1997) and Mediterranean (Jimenez and Ribes 2007) sponges. Secondly, 16S rRNA gene sequences of several clades of ammonia-oxidizing Beta- and Gammaproteobacteria and nitrite-oxidizing Nitrospina were recovered from sponge tissues, making microbial nitrification a likely scenario (Hentschel et al. 2002, Diaz et al. 2004). In the present study, a combination of physiological and phylogenetic approaches was employed to explore the potential of microbial nitrification in Aplysina aerophoba Schmidt 1862 and several other Mediterranean demosponges.

**Materials and methods**

**Animal collection**

Whole, intact colonies of the sponges A. aerophoba, Dysidea avara Schmidt 1862, Tethya sp. and Chondrosia reniformis Nardo 1847 were collected by SCUBA diving offshore Banyuls-sur-Mer (France) (42°43’N, 10°08’E) and from Rovinj (Croatia) (45°05’N, 13°38’E) at depths from 2-20 m. The animals were in the range of 30-50 g wet weight (about 15 g for D. avara). Small tissue pieces were removed from freshly collected animals, immediately frozen in liquid nitrogen and stored at -80°C until use. Whole, intact animals were maintained in > 1000 L volume, flow-through holding tanks that were constantly supplied with fresh, untreated Mediterranean seawater prior to the experiments.

**Sponge incubations**

Individual specimens were placed into aquaria containing three liters of fresh, untreated Mediterranean seawater. A constant water current was generated by small aquarium pumps (Vita Tech 300, Vitakraft, Germany). Only sponges that were in good physiological condition as judged by their regular filtration activity were chosen for the experiments. The experiments were performed in triplicate while a fourth aquarium without a sponge served as a control. In time intervals, 10 ml aliquots were removed from each aquarium, placed on ice and frozen at -20°C until use.

**Determination of ammonia, nitrate and nitrite concentrations**

The ammonia concentration was determined with the Indol-phenol-blue reaction (Parsons et al. 1984). The concentration of nitrite (NO$_2^-$) was determined by the Griess reaction (Parsons et al. 1984). Nitrate (NO$_3^-$) concentrations were measured indirectly after conversion to nitrite by the nir mutant E. coli strain JBC 606 as described in Pospesel et al. (1998). Ammonia and nitrate standard curves ranging from 0-100 µM were performed for each measurement series and fresh standards were prepared on a weekly basis.

**DNA extraction and PCR**

For the amplification of 16S rRNA genes from ammonia-oxidizing Betaproteobacteria, the primers AOB189f (5’-GGG GAA AAG CAG GGG ATC G-3’) and AOB1224r (5’-CGC CAT TGT ATT ACG TGT GA-3’) were used, that originally had been designed as the FISH probes NSO190 and NSO1224, respectively (Loy et al. 2003). The PCR reaction mix contained 1 x PCR reaction buffer (Qiagen), 2 mM of each primer, 0.2 mM dNTPs (Sigma) and 1.25 U Taq Polymerase (Qiagen) in a final volume of 50 µl. The PCR protocol was as follows: 1 min initial denaturation at 94°C followed by 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 56°C for 30 sec and elongation at 72°C for 5 sec. The PCR was terminated with a final elongation step at 72°C for 5 min.

**Cloning, RFLP-Analysis and Sequencing**

Purified PCR products (PCR purification kit, Qiagen) were ligated into the pGEMT-easy vector (Promega) and transformed by electroporation into competent E. coli XL 1-Blue cells. The enzymes Msp I and Hae III were used for restriction fragment length polymorphism (RFLP) analysis. Plasmid DNA was isolated from selected clones by standard miniprep procedures (Sambrook et al. 1989) and sequencing was performed on an ABI 377XL automated sequencer (Applied Biosystems).

**Phylogenetic analysis**

Sequences obtained in this study were checked for chimeras with the program Pintail. Sponge sequences together with reference sequences [received from GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST)] were aligned automatically with ClustalX and the alignment was subsequently corrected manually in Align. Neighbor Joining (with Jukes-Cantor correction) and Maximum Parsimony trees were constructed using the ARB software package (Ludwig et al. 2004).

**Results and discussion**

**In vivo sponge incubations**

For A. aerophoba, nitrate excretion rates of 3.6 ± 0.27 µmol g$^{-1}$ fresh weight day$^{-1}$ (equivalent to 830 nmol g$^{-1}$ dry weight h$^{-1}$) were determined which corresponded to an ammonia excretion of 0.56 ± 0.09 µmol g$^{-1}$ fresh weight day$^{-1}$ (n = 4 ± S.E.) (Fig. 1A). The nitrate excretion rate was about six fold higher than the ammonia excretion rate. Ammonia and nitrate did not appear in the incubation water in sponge-free control aquaria. Nitrite was not detected in any of the incubations. In order to measure ammonia uptake rates, 100 or 200 µM NH$_4$Cl final concentrations were added to the incubation water. Aplysina aerophoba was capable of ammonia uptake which corresponded to a nitrate excretion rate of 9.2 and 5.0 µmol g$^{-1}$ fresh weight day$^{-1}$ (Fig. 1B). Aplysina aerophoba was not capable of taking up nitrate which was tested at a concentration of 100 µM (data not shown).
Ammonia and nitrate excretion rates of *A. aerophoba* were determined in correlation to the maintenance time in holding tanks (Fig. 2). After one day of maintenance, the ammonia excretion rate was $0.54 \pm 0.07 \mu$mol g$^{-1}$ fresh weight day$^{-1}$. After six and 11 days of maintenance, the ammonia excretion rates were slightly increased ($1.04 \pm 0.05$ and $1.11 \pm 0.05 \mu$mol g$^{-1}$ fresh weight day$^{-1}$, respectively, Krustal-Wallis Test $p=0.051$). Nitrate excretion rates were similar over time of maintenance, ranging from $0.74 \pm 0.15$ (Fig. 2A), to $0.94 \pm 0.36$ (Fig. 2B) and $0.87 \pm 0.43 \mu$mol g$^{-1}$ fresh weight day$^{-1}$ (Fig. 2C). Interestingly, a correlation between ammonia and nitrate excretion and sponge pumping activity was evident. While ammonia was excreted at almost double rates in non-pumping sponges, nitrate was not excreted in sponges whose osculi were closed as judged by visual inspection (data not shown). The observation that the mesohyl of non-pumping sponges becomes anaerobic within 15 min (Hoffmann *et al.*. unpublished) would be consistent with an inhibition of the aerobic process of nitrification. However, possible differences in the diffusion process of ammonia and nitrate in non-pumping sponges cannot be excluded.

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With respect to microbial loads in the mesohyl tissue, sponges are characterized as high and low microbial abundance sponges (Hentschel *et al.* 2006). Accordingly, ammonia and nitrate excretion rates were determined in the Mediterranean...
Phylogenetic analysis

In total, 200 clones were compared by restriction fragment length polymorphism analysis and four major restriction patterns were detected. After removal of five chimeras, nine, one, and two sequences from A. aerophoba, D. avara and Tethya sp. libraries, respectively, were used for phylogenetic tree construction (Fig. 4). All twelve sequences fell into the marine Nitrosospira cluster 1 of the Betaproteobacteria together with marine seawater and sediment sequences. Except Aplysina aerophoba (F) clone 5, the sequences obtained in this study build a subcluster with a high in-cluster similarity (98.5-99.9%). It is noteworthy that sequences were also recovered from the bacteria-free sponge D. avara as well as Tethya sp. whose mesohyl shows moderate amounts of microorganisms (Thiel et al. 2007). While it cannot be excluded that the cloned AOB sequences represent seawater bacteria rather than true sponge symbionts, the high nitrate excretion rates, at least for A. aerophoba, would argue for a specific and probably symbiotic association. Although the primers used in this study also match Nitrosomonas species, no such bacteria could be detected in the clone libraries. Our findings expand those by Diaz (1997) and Diaz et al. (2004) who had reported on the identification of members of the Nitrosomonas eutropha/europa lineage (Betaproteobacteria) in five tropical sponges.

Modelling nitrogen fluxes in the sponge-microbe association

The following scenario, depicted in Fig. 5, is proposed based on this and other studies. The sponge host excretes ammonia as a metabolic waste product, which in turn, is oxidized to nitrite by ammonia-oxidizing bacteria (AOB), such as Nitrosospira (this study), Nitrosococcus (Hentschel et al. 2002) or members of the Nitrosomonas eutropha/ europa lineage (Diaz et al. 2004). Nitrite is further oxidized to nitrate by nitrite-oxidizing bacteria (NOB), such as Nitrospina or members of the phylum Nitrospira (Hentschel et al. 2002). The coordinated action of members of these two groups might then be responsible for the conversion of ammonia to nitrate in A. aerophoba and possibly also in other sponges. In addition to eubacteria, the involvement of archaea should be considered in future studies. Recent literature illustrates

Fig. 3: Ammonia and nitrate excretion by D. avara (A), Tethya sp. (B), and C. reniformis (C), (each species, n = 3 ± S.E.). Symbols represent ammonia (♦) and nitrate (▲) concentrations.

Fig. 4: Distance 16S rRNA (1053 bp) gene phylogeny showing the relationships between ammonia-oxidizing Betaproteobacteria using the ARB program package. Sponge derived sequences are shown in bold. Neighbor-joining and maximum parsimony (100 pseudoreplicates) bootstrap values are indicated. Arrow to outgroup (Nitrosococcus oceani Nc1 (A298727)). Designation of clusters is adapted from Freitag and Prosser (2003). Scale bar indicates 1% sequence divergence.
that archaea, rather than bacteria, might in fact be involved in nitrification in marine and terrestrial ecosystems (Leininger et al. 2006, Wuchter et al. 2006). In fact, close relatives of the Cenarchaeum symbiosum lineage that are also present in sponges have recently been isolated and were shown to be capable of nitrification (Könneke et al. 2005). Additionally, it needs to be investigated whether nitrate serves as an energy substrate for denitrifying microorganisms under anaerobic conditions. In conclusion, this study contributes to an ongoing effort to link microbial diversity with function in these phylogenetically highly diverse, elusive and so far uncultivated marine sponge-associated microbial communities.

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