DENATURING GRADIENT GEL ELECTROPHORESIS OF NITRIFYING MICROBES ASSOCIATED WITH TROPICAL SPONGES

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ABSTRACT

This study aims to explore the presence and phylogenetic diversity of ammonium oxidizing bacteria (AOB) among abundant sponges in mangrove communities. Using nested PCR amplifications, DGGE electrophoresis, and direct sequencing of DGGE bands, the presence of AOB sequences from five of the six common sponge species was revealed (*Haliclona implexiformis*, *Lissodendoryx isodictyalis*, *Tedania ignis*, *Geodia papyracea, Chondrilla nucula*). The phylotypes encountered belonged to the *Nitrosomonas eutropha/europa* lineage (cluster seven) of the β- Proteobacteria. This indicates an affinity of sponge-associated AOB with those that prevail on marine-derived particles. This study suggests a widespread occurrence of AOB among Caribbean sponges whose role in the nutrient cycling on mangrove ecosystems remains to be evaluated.

KEYWORDS

Sponges, nitrification, epibionts, AOB, mangroves.

INTRODUCTION

Sponges are one of the most diverse and abundant colonizers of red mangrove roots in various Caribbean mangrove systems (RÜTZLER *et al.,* 2000). Marine sponges harbour complex microbial communities within their tissues (De Vos *et al.,* 1991). These microbial associations provide the sponge with metabolic capabilities such as photosynthesis, nitrification, nitrogen fixation, and methane oxidation that are otherwise absent in eukaryotes (WILKINSON & FAY, 1979; WILKINSON, 1992; VACELET *et al.,* 1995; DIAZ & WARD, 1997). Various studies suggest a central role of sponges in the nitrogen cycling in Caribbean nutrient limited ecosystems (CORREDOR *et al.,* 1988; ELLISON & FARNSWORTH, 1996; DIAZ & WARD, 1997; PILE, 1997). Five species, three inhabiting coral reefs (*Svenzea zeai*, *Ircinia felix* and *Ircinia strobilina*), one growing on seagrass beds (*Oligoceras violacea*), and another one inhabiting both mangrove and coral reef systems (*Chondrilla nucula*) were shown to release large amounts of dissolved inorganic nitrogen (DIN) (CORREDOR *et al.,* 1988;

DIAZ & WARD, 1997; PILE, 1997). DIAZ & WARD (1997) demonstrated that three of those species are actively mediating microbial nitrification.

Nitrification is a two-step process, which involves two different phylogenetic groups of bacteria. Ammonium oxidizing bacteria (AOB) oxidize ammonium to nitrite, and nitrite oxidizing bacteria (NOB) oxidize nitrite to nitrate. Nitrifying microorganisms associated with sponges have not been characterized due to cultivation difficulties outside the host. However, the molecular detection of nitrifying bacteria using polymerase chain reaction (PCR) of 16S rDNA genes offers an alternative to study these microbial associates. AOB are especially suitable to these types of studies due to their monophyletic nature within the β-subclass of Proteobacteria (BOTHE *et al.,* 2000). A novel strain of AOB within this subclass, from the Caribbean reef sponge *Svenzea zeai,* was phylogenetically characterized previously (DIAZ, 1997). This sponge species shows the highest benthic potential nitrification rates (DIAZ & WARD, 1997).

Sponges from the mangroves might also be active sites of nitrification. The unusually high nitrate concentrations detected by VILLAREAL *et al.* (2000) from under the prop root fringe at the semi-enclosed mangrove pond in Manatee Cay, Belize, could be a direct consequence of "sponge-mediated" nitrification (DIAZ & WARD, 1997). This study explores the presence and identity of AOB among the most abundant sponges inhabiting mangrove ecosystem at Twin Cays, Belize. First we attempted the detection of AOB among the sponges using nested PCR that is biased towards AOB. Secondly we used Denaturing Gradient Gel Electrophoresis (DGGE) to study the identity and variability of AOB among different sponge species (MUYZER *et al.,* 1993; KOWALCHUCK *et al.,* 1998; MCCAIG *et al.,* 1999; CAMPBELL & CARY, 2001).

MATERIAL AND METHODS

Sponge Collection

Sponge samples were collected at two sites in the mangrove islands of Twin Cays, in Central Belize: "Sponge Heaven" (16°49'42.4" N, 88°06'17.1" W min) and "The Lair" (16°49'46.0" N & 88°06'06.8" W, center of Lair channel).

Six sponges species were selected for this study (RÜTZLER *et al.,* 2000): *Haliclona implexiformis*, *Lissodendoryx isodictyalis*, *Tedania ignis*, *Spongia* sp., *Biemna caribea* and *Chondrilla nucula* were collected at the back reef in Carrie Bow Cay. A copper core was pushed through the sponge body. Then the surface tissues were removed by using a clean sterile razor blade. The sample was cut in smaller pieces (0.1 cc approximately). Approximately 0.5 cc of tissue was placed in a 1.5 ml Eppendorf tube containing up to 1ml of silica gel (chromatographic grade), by duplicate, for DNA extraction and PCR. Before placing the tissue in the tubes the excess liquid is blotted using a clean piece of tissue paper, which is immediately discarded. A water sample (2 l) from "The Lair" site was taken at the water surface in the forest fringe. The water was filtered through a 0.2 μm pre-combusted GF-F filter, which were then placed in 1 ml sucrose lyses buffer (40 mm EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris, pH 9.0). Samples are kept frozen (-20° C) until processed.

DNA extraction

DNA extraction from the sponges was performed using a CTAB extraction. Briefly, a tissue subsample (0.25 cc) was homogenized with two volumes of warm CTAB solution (500 μl of CTAB buffer and 2 μl of β-Mercaptoethanol at 65° C) and incubated 15 min at 65° C.

The mix is cooled briefly, mixed with one volume of 24:1 Chlorophorm:Isoamylalcohol solution, vortexed to mix the two layers, rocked slowly (20 min), and centrifuged at 12500 rpm (15 min). The resultant DNA pellet was washed twice with ethanol (70 %), dried overnight and resuspended in 50 µl of MilliQ water. Quantification of the DNA was performed on a DU 640B Spectrophotometer (Beckman Instruments). A concentration of 10 ng/ul solution was used for PCR amplification.

PCR amplification

A two-step "nested" PCR amplification was performed. Approximately 25 - 50 ng of isolated total genomic DNA was amplified (PCR I) using universal eubacterial primers of 16S rDNA 27F (5'AGAGTTTGATCCTGGCTCAG3'), and 804R (5'CTACCAGGGTATCTAA TCC3') (VOYTEK & WARD, 1995; CAMPBELL & CARY, 2001). Thermocycler conditions were: Hot start 94° C (1 min), followed by 35 cycles of 94° C (15 s), 58° C (30 s), 72° C (1 min). One µl of this PCR product (773 bp in length) was used for a second amplification (PCR II) using primers biased towards AOB bacteria. The forward primer NIT A (5' CTTAAGTGGGGAA TAACGCATCG 3', with positions 137 - 159, in *E. coli*) (VOYTEK & WARD, 1995) is well suited to amplify AOB of the β-subclass of Proteobacteria. The reverse primer NEU (5' CTTAAGTGGGGAATAACG CATCG 3' with positions 653 - 670, in *E. coli*) is specifically biased towards a sub-group of the *Nitrosomonas* cluster of AOB that contains *Nitrosoccocus mobilis* (MOBARRY *et al.,* 1996; UTÅKER & NES, 1998). *N. mobilis* 16S rDNA was determined as the closest sequence to an AOB sequence ever amplified from a sponge tissue (DIAZ, 1997).

DGGE analysis

Denaturing gradient gel electrophoresis (DGGE) was performed as described by CAMPBELL & CARY (2001). Briefly, 1 µl of the amplification product from PCRII was used for a third amplification (PCR III), which targeted the V3 region of 16S ribosomal genes that is located within the sequence amplified in PCR II. The primers used were 338F/GC clamp and 519R (MUYZER *et al.,* 1993). PCR was performed in 50 µl PCR mixtures which contained 0.1 mM, 1.25U of Taq polymerase (Promega), 1x Promega Taq buffer, 2 mM $MgCl_2$, and each deoxyribonucleoside triphosphate at a concentration of 200 µM. Thermocycler conditions for PCR III were: a) Hot start, 94° C for 5 min; b) 21 cycles of 80° C (5 s), 94° C (1 min), 65° C (1 min, decreasing 0.5° C per cycle), and 72° C (1 min); c) 8 cycles of 94° C (1 min), 55° C (1 min), and 72° C (1 min); d) 72° C (5 min) and 4° C Hold (Campbell, pers. comm.). Amplification products from PCR III approximately 1000 - 1500 ng (15 - 25 µl of product) were run in a 25 - 55 % denaturing gradient, 6 - 8 % acrylamide gel (100 % denaturant was 7 M urea plus 40 % deionized formamide) for 5 h a constant voltage of 130 V at a temperature of 60° C by using a Dcode universal mutation detection system (Bio-Rad). DNA bands were visualized with a UV transilluminator and were photographed by using Alpha-Imager system (Alpha Innotech).

Individual DGGE fragments were stabbed with an aerosol-free pipette tip, and resuspended in 20 µl of sterile milliQ water. One half of the sample was used in a 50 µl PCR mixture as described above. Amplification products were checked for purity by DGGE of a portion of the sample. Excess primer was removed from PCR products by passage through a Quiagen PCR purification column (Quiagen) according to the manufacturer's instructions and were quantified by UV spectophotometry. Approximately 40 ng of purified products were used in 10 µl Big Dye terminator sequencing reaction mixtures (ABI) with 519R primer. Sequences read ranged from 88 - 146 bp in length.

The sequences from the DGGE bands were deposited on Genbank using Bankit, and were given Genbank accession numbers (Tab. I).

Tab. I. Phylogenetic affinities of "DGGE band" sequences from this study (see Fig. 1) after comparison using "Sequence Match", and "Similarity matrix" analyses from the Ribosomal Database Project (RDP), and "Blast" analysis from the National Center for Biotechnology Institute (NCBI). S = percentage similarity to its closest sequence on the Database.

DGGE band number	Sample source	Phylotype	Genebank#	Sequence (bp)	Closest sequence (BLAST)	Phylogenetic identity (Sequence match-RDP)
1	Water	Tdv (1)	AY337330	91	*Thermodesulfovibrio islandicus* str R1ha3 (gb # 0.6171314)	TDV *yellowstoni* group (*Leptospirillum-Nitrospira*)
2	Water	Act	AY337331	93	*Actinobacubum schaalii* (gb # 0.639137)	*Actynomyces* subgroup (Gram Positive)
3	Water	Tdv (2)	AY337332	99	*Thermodesulfovibrio* TGLLS1 (gb # ABO21303)	= DGGE band 1
4	*H. implexiformis*	Nmn (3)	AY337325	116	*Nitrosomonas* sp. str. GH 22 (gb # ABO000701)	*Nitrosomonas europae* subgroup (β-Proteobacteria)
5	*G. palpyrawa*	Nmn (2)	AY337326	102	*Nitrosomonas eutropha* C-91, Nm 57 (gb # AJ298739)	= DGGE band 4
6	*G. palpyrawa*	Nmn (1)	AY337323	100	*Nitrosomonas* sp. str. Koll 21 (gb # AJ224941)	= DGGE band 4
7	*Spongia* sp.	Tdv (1)	AY337333	142	*Thermodesulfovibrio islandicus* str R1ha3 (gb # 0.6171314)	= DGGE band 1
8	*Tedania ignis*	Nmn (1)	AY337327	139	= DGGE band 6	= DGGE band 4
9	*L. isodyctialis*	Nmn (4)	AY337328	92	*Nitrosomonas* sp. str. HPC 10 (gb # ABO000702)	= DGGE band 4
10	*L. isodyctialis*	Nmn (1)	AY337329	116	= DGGE band 6	= DGGE band 4
11	*C. mucula*	Nmn (1)	AY337324	127	= DGGE band 6	= DGGE band 4

Sequence Analyses

These sequences were aligned to a database of previously determined 16s ribosomal gene sequences by using Blast (National Center for Biotechnology Institute, www.ncbi.nlm.nih.gov/blast) and the "Similarity matrix" and "Sequence Match" analyses (Ribosomal Database Project, rdp.cme.msu.edu/cgis). Distances between the aligned sequences were calculated using the Kimura two-parameter test, in the DNAdist program from Phylip 3.5 (FELSENSTEIN, 1993, University of Washington). Only positions that could be unambiguously aligned were considered for the analyses. A consensus tree was constructed using the Neighbor Joining method (SAITOU & NEI, 1987) and the program CONSENSE (Phylip 3.5). in order to evaluate the robustness of the inferred trees, a BOOTSTRAP analysis consisting of 5000 re-samplings of the data was performed using SEQBOOT (Phylip 3.5).

RESULTS

To investigate if AOB bacteria were present among common mangrove sponge epibionts we carried a nested PCR approach of 16S ribosomal gene sequences. Both eubacterial and AOB amplicons were obtained from genomic DNA samples derived from the six species studied and the water sample suggesting that eubacteria and AOB are present among the microbial communities associated to those samples. The third amplification of sponge-derived products (V3 region, PCR III) when run in a DGGE gel produced one or two major bands. The water-derived amplification products produced four major bands, and the positive control sample (*Nitrosomonas europae*) produced one band (Fig. 1).

Sequences were obtained from 11 of the major bands observed, and compared against known sequences using analyses provided by the Ribosomal Database Project (sequence match and similarity matrix) and NCBI (Blast). The phylogenetic affinities of the DGGE fragments sequences and their Genbank accession numbers are indicated on Tab. I. All the bands from the sponges species (except DGGE band 7 of *Spongia* sp.) are ammonium oxidizers within the *Nitrosomonas europa* subgroup of the *Nitrosomonas* group of from the β-subdivision of the Proteobacteria division of Bacteria.

Four phylotypes were distinguished. Nmn (1) was the most common phylotype represented by DGGE bands number 6, 8, 10, and 11, corresponding to *Tedania ignis, G. papyracea*, *L. isodictyalis* and *C. nucula,* respectively. Nmn (1) corresponded to a group with 97 % similarity to *Nitrosomonas* sp. stroll 21. Three other phylotypes, which shared similar migration pattern (III, Fig. 1), corresponded to: Nmn (2), DGGE band 5 from *G. papyracea,* closest to *Nitrosomonas eutropha* C-91, Nmn (3), DGGE band 4 from H. *implexiformis*, closest to *Nitrosomonas* sp. str. GH 22, and Nmn (4), DGGE band 9 from *L. isodictyalis,* closest to *Nitrosomonas* sp. str. HPC 10. Most of the AOB sequences found present a 97 % similarity with the closest sequences on the database. *H. implexiformis*'s DGGE band 4 presents a lower similarity of 93 % to its closest sequence.

Two of the bands from the water sample (DGGE bands 1, and 3) showed an affinity to *Thermodesulfovibrio* sp., from the TDV *yellowstoni* group of the *Leptospirillium-Nitrospira* division of Bacteria. Tdv (1) corresponded to Tdv island. 0.6171314, *T. islandicus* str R1ha3, and Tdv (2) corresponded to *Thermodesulfovibrio*, TGLLS1 0.8861400. Tdv (1) was revealed as the closest sequence from the *Spongia* sp. DGGE band 7. A third DGGE band sequenced from the water sample (DGGE band 2)

showed a sequence similar to *Actinobaculuum schaalii,* from *Actynomyces* subgroup of the Gram Positive division of Bacteria (Tab. I).

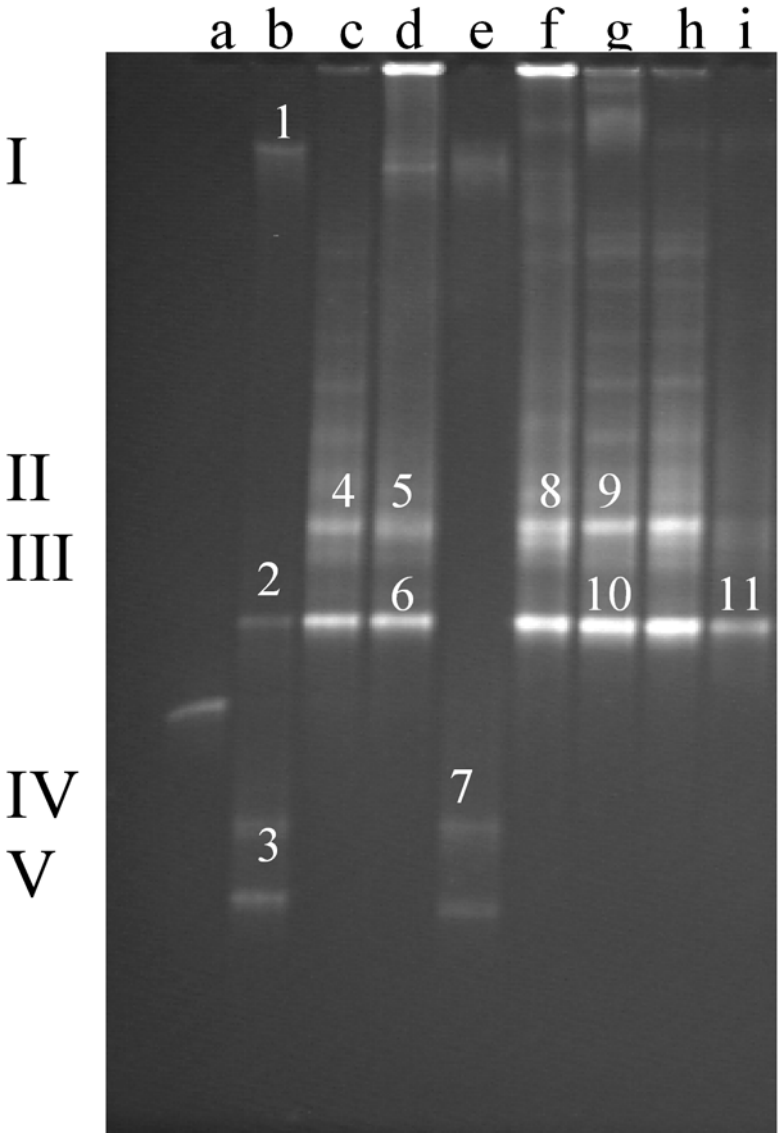

Fig. 1. DGGE of 16S ribosomal gene fragments (V3 region) from bacteria associated to sponge tissues. The V3 PCR products were amplified from 16S bacterial PCR products derived from: **a**. *N. europae* isolate, **b**. Surface water sample from Twin Cays, **c**. *H. implexiformis,* **d**. *G. papyracea,* **e**. *Spongia* sp., **f**. *T. ignis,* **g**. *L. isodictyalis,* **h**. *B. caribea,* **i**. *C. nucula.* Bands below each number were subsequently sequenced and analyzed.

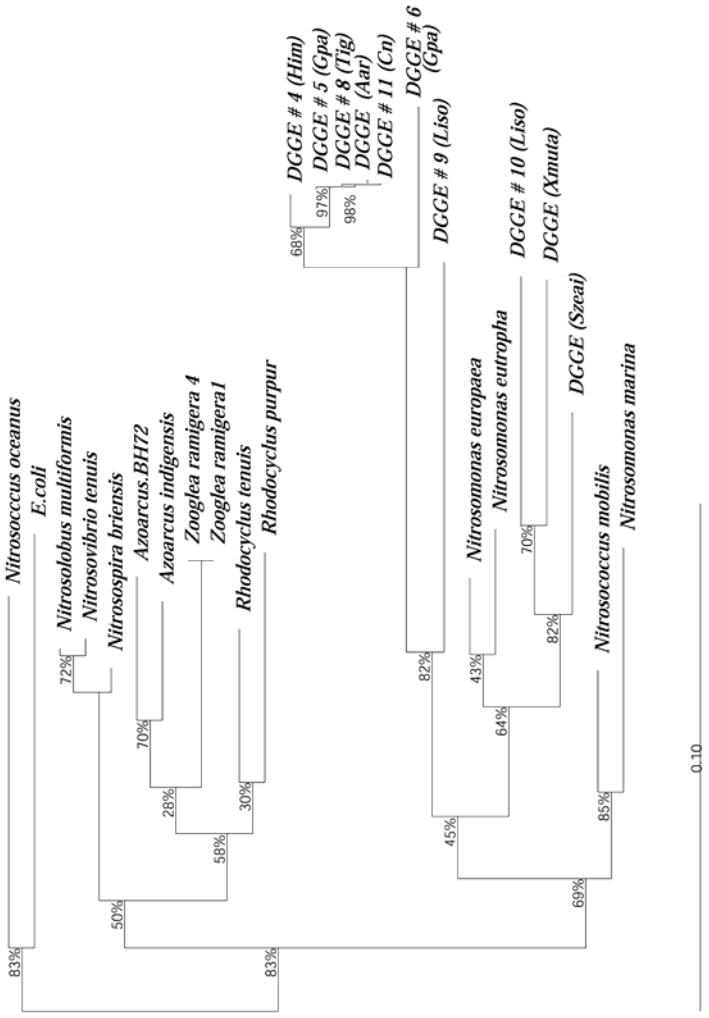

Fig. 2. Distance matrix phylogenetic tree illustrating the relationships of sponge associated AOB 16S ribosomal sequences in a phylogenetic tree of ammonium oxidizers, and other bacteria within the β subdivision of Proteobacteria. One consensus tree was obtained using the Neighbor Joining method and the program CONSENSE (Phylip 3.5). *E. coli*, and *Nitrosococcus oceanus* (an AOB of the delta subdivision) were used as outgroups. Sponge-derived sequences are labelled as follows: DGGE # 4 (Himp)= upper band from *H. implexiformi)*, DGGE # 5 (Gpap)= upper band from *G. papyracea*, DGGE # 8 (Tign) = band from *T. ignis*, DGGE (Aar)= band from *Aplysina archeri* (genbank # AY337334), DGGE #11 (Cn) = band from *C. nucula*, DGGE # 6 (Gpap)=*Geodia papyracea*, DGGE # 9 (Liso)= upper band from *L. isodictyalis*, DGGE # 10 (Liso)= lower band from *L. isodictyalis*, DGGE (Xmu)= band from *Xestospongia muta* (genbank # AY337336), and DGGE (Szeai) = band from *Svenzea zeai* (genbank # AY337335). Genbank accession numbers for all the numbered DGGE bands are shown on Tab. I.

A distance matrix analysis illustrating the relationships of sponge associated AOB 16S ribosomal sequences in a phylogenetic tree of ammonium oxidizers, and other bacteria within the β-subdivision of Proteobacteria, is shown in Fig. 2. One consensus tree was obtained using the Neighbor Joining method and the program CONSENSE (Phylip 3.5). *E. coli*, and *Nitrosococcus oceanus* (an AOB of the δ subdivision of Proteobacteria) were used as outgroups. The sponge-derived sequences used to construct this tree are all AOB sequences obtained from sponges inhabiting mangrove systems: *H. implexiformis* DGGE band 4, *G. papyracea* DGGE bands 5 and 6, *T. ignis* DGGE band 8, *C. nucula* DGGE band 11, *L. isodictyalis* DGGE bands 9, and 10, and from sponges inhabiting coral reefs: DGGE fragments from *Aplysina archeri*, *Xestospongia muta*, and *Svenzea zeai* (DIAZ, unpubl. data). Genbank accession numbers are listed on Tab. I, except for: DGGE band from *A. archeri* (genbank #AY337334), DGGE band from *X. muta* (genbank #AY337336), and DGGE band from *S. zeai* (genbank #337335). All the sponge-derived AOB sequences group within the branch of the *Nitrosomonas europa* subgroup of β-Proteobacteria, and separate in two sub-groups (Fig. 2). The sequences are not grouped by habitat (mangrove or coral reef) or by sponge species. The affinities between sponge-derived AOB fragments suggested by this NJ analyses, differs slightly from the ones suggested by the RDP, and NCBI analyses for two of the DGGE fragments (DGGE band 6, *G. papyracea* and DGGE band 10 *L. isodictyalis*). This is probably due to the fact that the sequence length used for the NJ analyses was shorter (91 bp) than the one used for the other analyses (91 - 165 bp).

DISCUSSION AND CONCLUSIONS

The aim of this study was to test the application of a relatively rapid method of microbial molecular detection to explore the presence and diversity of ammonium oxidizing bacteria among common sponges in mangrove communities. The detection of AOB from five of the six common sponges inhabiting studied suggests that the association of AOB is widespread among Caribbean sponges. All the AOB phylotypes encountered among the sponges species here studied belong to the *Nitrosomonas eutropha/europa* lineage (cluster seven) of the β-Proteobacteria (HEAD *et al.,* 1993; KOWALCHUCK *et al.,* 1998). This seems to indicate an affinity of sponge associated AOB with the AOB that prevail on marine-derived particles (PHILLIPS *et al.,* 1999; BOTHE *et al.,* 2000).

DGGE electrophoresis was useful to distinguish the diversity of AOB bands within the sponges studied. However, similar migration patterns did not necessarily implied phylogenetic similarities (for example, see the diversity among level II in the DGGE gel, Fig. 1).

In this study we used one primer that targets all subgroups of AOB within the β Proteobacteria (Nit A) and one primer that targets the *Nitrosomonas eutropha/europa* lineage (NEU). The primer NEU was selected due to its affinity to *N. mobilis*, within the *Nitrosomonas* subgroup (MOBARRY *et al.,* 1996) which was the closest relative to the only known AOB sequence ever amplified from a sponge (DIAZ, 1997). This approach, revealed only members of two AOB branches within the *Nitrosomonas europa* subgroup of β-Proteobacteria and might not be portraying all the AOB members. For example, the relative high specificity of NEU towards the *Nitrosomonas*

subgroup of AOB within the β-Proteobacteria might have precluded the detection of other AOB from *Spongia* or from the water sample. Therefore, we can't assure that a complete representation of the AOB community in these sponges was obtained. The use of a much more general set of primers for AOB amplifications such as Nit A (136 F), and Nit B (1213 R) would probably allow a more complete characterization of AOB populations from the sponge tissues (see UTÅKER & NES, 1998). Furthermore, due to the complexity and heterogeneity of AOB distribution encountered in the sea it is probably important that various set of primers with affinities for distinct AOB subgroups are used (UTÅKER & NES, 1998; BOTHE *et al.,* 2000).

The amplification of non-AOB fragments from the water, and the *Spongia* sp. sample should be discussed. The use of universal eubacterial primers in the last amplification before the DGGE run (V3 amplification) opened the risk of amplifying from eubacterial products that could've been carried from the first PCR amplification (PCR I). The most probable explanation is the non-specific nature of the products for these two samples. To solve this problem we suggest to avoid the re-amplification of smeary PCR products, and eliminated the third and last eubacterial-biased PCR amplification. A better nested amplification approach might be: a first eubacterial amplification, followed by a general AOB one (Nit A and Nit B) which produces a piece approximately 1100 bp in length. Then a third AOB-specific amplification that takes into account the size limit to of DNA fragments that can be discerned by the DGGE technique (<500 bp). The appropriate combination of AOB primers may be developed from the approximately 30 different AOB primers reported in the literature (MOBARRY *et al.,* 1996; UTÅKER & NES, 1998).

A distance matrix phylogenetic analysis, of the AOB fragments here studied, suggested the existence of at least two types of AOB within the *Nitrosomonas europa* subgroup of ß-Proteobacteria. However, a clearer and more solid picture of the relationships between these AOB associates of sponges requires the study of longer sequences from the 16S ribosomal gene. We consider that the construction of a 16S rDNA library might help to better characterize the phylogenetic nature of this AOB associates. Furthermore, the physical localization of these bacteria seems crucial to understand the nature and biology of this important organismal consortia. The importance of sponges as DIN providers has been proved for the Indopacific symbiotic association between the sponge *Haliclona cymiformis* and the red alga *Ceratodictyon spongiosum* (DAVY *et al.,* 2002), where the ammonia excreted by the sponge directly supplies the N requirement of the algae. The actual role of sponges and their associated nitrifying microbes in the nitrogen budget of tropical marine ecosystems remains to be evaluated.

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