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ORIGINAL ARTICLE

Diversity of aerobic and anaerobic ammonia-oxidizing bacteria in marine sponges

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Aerobic ammonia-oxidizing bacteria (AAOB) are known to have an important function in the marine nitrogen cycle. Anaerobic ammonium oxidation (anammox) carried out by some members of *Planctomycetales* is also an important process in marine ecosystems. Ammonia-monooxygenase gene (*amoA*) fragments were amplified to investigate the potential for nitrification and the diversity of the AAOB in two marine sponges *Ircinia strobilina* and *Mycale laxissima*. All of the AmoA sequences obtained from the two sponges clustered with the AmoA sequences of the *Betaproteobacteria Nitrosospira* spp. To investigate the anaerobic ammonia-oxidizing bacteria (AnAOB) in sponges, 16S rRNA gene fragments of *Planctomycetales* and anammox bacteria were also amplified with specific primers, and clone libraries were constructed. The *Planctomycetales* diversity detected in the two sponges was different. The *Planctomycetales* community in *M. laxissima* was affiliated with *Pirellula*, *Planctomyces* and anammox bacteria, while all of the *I. strobilina Planctomycetales* clones were solely affiliated with the candidate phylum '*Poribacteria*'. Interestingly, sequences related to anammox genera were recovered only from *M. laxissima*. This is the first report of anammox bacteria in marine sponges. It is intriguing to find AAOB and AnAOB in *M. laxissima*, but the nature of their interaction with the sponge host and with each other remains unclear. This work further supports the potential of sponge-associated microorganisms for nitrification and sheds light on anammox as a new aspect of the nitrogen cycle in marine sponges.
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Introduction

Sponges are among the most ancient animals, with a fossil record dating to ca. 580 million years (Li *et al.*, 1998; Müller *et al.*, 1999). They are sessile filter feeders that draw water through numerous tiny pores on their surface by the flagellar motion of their choanocyte cells. The water circulates through a series of channels in which suspended particulate organic matter is filtered out, dissolved nutrients are absorbed and metabolic wastes are released and flushed away (Reiswig, 1971; Reiswig, 1974; Pile, 1997). Sponges are hosts to diverse microorganisms that can constitute up to 60% of the total sponge biomass (Vacelet, 1975; Vacelet and Donadey, 1977; Wilkinson, 1978; Hentschel *et al.*, 2006). Sponge–microbe associations involve a diverse range of heterotrophic bacteria, cyanobacteria, facultative anaerobes, unicellular algae and archaea (Webster and Hill, 2001; Hentschel *et al.*, 2002; Montalvo *et al.*, 2005; Taylor *et al.*, 2007; Mohamed *et al.*, 2008b, c). Symbiotic relationships between sponges and microorganisms contribute to the sponges' health and nutrition. These relationships can involve more than one partner and can vary from mutualism to commensalism to parasitism. Sponges may offer nourishment and protection to their symbionts (Bultel-Poncé *et al.*, 1999), and the symbionts may benefit the nutrition of their host by translocation of metabolites through for example nitrogen fixation, nitrification and photosynthesis (Wilkinson and Fay, 1979; Wilkinson and Garrone, 1980; Bayer *et al.*, 2008; Mohamed *et al.*, 2008a).

There has been a growing effort in recent years to link the phylogenetic diversity and functional activity of sponge-associated microorganisms. 16S rRNA gene sequence-based community analysis of the microbial consortia of sponges has revealed a great deal about the diversity of bacteria and archaea associated with sponges. However, there is a paucity

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of knowledge regarding the metabolism, physiology and function of these microbes. Knowledge of the metabolic capabilities of the bacteria within sponges is critical for understanding of the symbiotic relationships between bacteria and sponges and the impact of this relationship on the biogeochemical nutrient cycling in sponge-dominated coral reef systems. Considerable effort has been expended on examining nitrogen transformations within sponges. A good example is biological nitrogen fixation. There have been many studies by us and others assaying the rates of nitrogen fixation in marine sponges (Wilkinson and Fay, 1979; Wilkinson *et al.*, 1999) and investigating the presence and expression of *nifH* genes in microbial communities associated with sponges (Mohamed *et al.*, 2008a). Nitrogen fixation by sponges' symbionts is possibly an important source of new nitrogen to the reef environment and may be a factor in the high productivity of coral reefs in oligotrophic tropical waters.

The biological nitrogen cycle is a complex interaction between many microorganisms catalyzing different pathways. Nitrification is an important process in the marine nitrogen cycle (Ward, 1986). It is an essential link between ammonification (decomposition of organic matter) and denitrification and subsequent removal of fixed nitrogen from the system. The first step in the nitrification process is the oxidation of ammonia to nitrite, performed by aerobic ammonia-oxidizing bacteria (AAOB) and archaea. The AAOB are phylogenetically affiliated with *Beta-* and *Gammaproteobacteria*. The nitrite-oxidizing bacteria (NOB) perform the second step of nitrification, which is the oxidation of nitrite to nitrate. The NOB are phylogenetically affiliated with genera *Nitrobacter* (*Alphaproteobacteria*), *Nitrococcus* (*Gammaproteobacteria*), *Nitrospina* (*Deltaproteobacteria*) and the phylum *Nitrospira*. Ammonia, which can be toxic to eukaryotes, is a metabolic waste product and could accumulate within sponge tissues. Therefore, sponges like many other marine invertebrates excrete ammonium as a waste product (Brusca and Brusca, 1990). Symbiotic nitrifying bacteria presumably metabolize nitrogen excreted by the sponges and other symbionts by oxidizing it to nitrate (Corredor *et al.*, 1988). Sponges have been found to assimilate and release dissolved inorganic nitrogen (ammonia, nitrite and nitrate) as well as dissolved and particulate organic nitrogen (DON and PON, respectively) (Corredor *et al.*, 1988; Diaz and Ward, 1997; Pile, 1997; Bayer *et al.*, 2008). In a study by Diaz and Ward (1997), nitrification capacity was investigated in tropical sponges and was related to the association between sponges and bacteria. More recently, Jiménez and Ribes (2007) evaluated nitrogen ingestion from particulate organic matter and DON excretion by Mediterranean sponges. They found differences in compounds that were excreted and in rates of excretion between sponge species, and most of the species exhibited significant nitrification rates.

The *amoA* gene encoding for the catalytic α-subunit of the ammonia-monooxygenase enzyme, the protein that catalyzes the rate-limiting step in ammonia oxidation, has been used as a phylogenetic marker to detect AAOB (Wood, 1986; Rotthauwe *et al.*, 1997). Recently, strong evidence was found that ammonia-oxidizing archaea (AOA) are potential nitrifiers in marine sponges. Hallam *et al.* (2006a, b) reported the presence of *amo* gene homologues within the sequenced genome of the uncultivated sponge symbiont *Cenarchaeum symbiosum.* Bayer *et al.* (2008) recovered *Nitrosospira* cluster 1 and Crenarchaeota group1 16S rRNA and *amoA* genes from the Mediterranean sponge *Aplysina aerophoba.* They linked the presence of bacterial and archaeal nitrifiers to the nitrification activities in living sponges. In a recent study by Steger *et al.* (2008), AOA were shown to be vertically transmitted through larvae in sponges, which is suggestive of their important symbiotic role in ammonia detoxification within sponges.

Planctomycetales are an unusual, gram negative, deep branching group of peptidoglycan-less budding bacteria with unique cell structure (Ward *et al.*, 2006). Members of *Planctomycetales* have important ecological functions in different marine environments, and they are found associated with mineral encrustation, crustations, algal blooms and sponges (Ward *et al.*, 2006). Recently, a novel group of *Planctomycetales* has been shown to perform anaerobic ammonia oxidation (anammox). Anammox was discovered in a laboratory scale denitrification process and was confirmed to be a microbially mediated process (van de Graaf *et al.*, 1995). The anammox process is important in biological nitrogen removal from water containing high nitrogen and carbon ratios. Anaerobic ammonia-oxidizing bacteria (AnAOB) have become a major focus of microbial ecology as they are estimated to contribute 30–50% to the nitrogen loss in the oceans (Codispoti *et al.*, 2001). Four candidate genera have been reported to perform anammox reactions, comprising *Candidatus* 'Brocadia' (Strous *et al.*, 1999; Kuenen and Jetten, 2001; Kartal *et al.*, 2008), *Candidatus* 'Kuenenia' (Schmid *et al.*, 2000; Egli *et al.*, 2003), *Candidatus* 'Scalindua' (Kuypers *et al.*, 2003) and *Candidatus* 'Anammoxoglobus' (Kartal *et al.*, 2007). Anammox bacteria grow very slowly and many have not yet been isolated in pure culture. Phylogenetic analysis of 16S rRNA gene, ^{15}N isotope incubation and lipid analysis showed the distribution of anammox bacteria in fresh water and marine sediments (Penton *et al.*, 2006), anoxic Black Sea (Kuypers *et al.*, 2003), oceanic upwelling systems (Kuypers *et al.*, 2005) and estuarine sediments (Tal *et al.*, 2005). However, no anammox bacteria were detected by PCR amplification targeting 16S rRNA genes from several sponges (Taylor *et al.*, 2007). In this study, we determined the diversity of the AAOB and AnAOB associated with two marine sponges *Ircinia strobilina* and *Mycale*

laxissima by analyzing *amoA* and anammox 16S rRNA gene markers.

Materials and methods

Sample collection

I. strobilina and *M. laxissima* were collected from Conch Reef, Key Largo, Florida (latitude 24°57.11′N, longitude 80°27.57′W) at a depth of ca. 20 m in summer 2005. Water salinity was 36 ppt and temperature was 26–27 °C. The sponges were collected by SCUBA, allowed to evacuate seawater then rinsed three times with sterile artificial seawater and frozen immediately. Sponge samples for RNA extraction were stored in RNA*later* RNA stabilization solution (Qiagen, Valencia, CA, USA). Two water samples were collected from the vicinity of the sponges in sterile 20-l containers and 15–20 l were filtered through 0.22-μm pore-size Sterivex filters (Millipore, Billerica, MA, USA). The Sterivex filters were frozen immediately and stored at −20 °C for isolation of nucleic acids.

DNA extraction and PCR amplification

A 1 cm^3 freeze-dried sponge tissue was ground using a sterile mortar and pestle. Total DNA was extracted from sponge samples using a TissueLyser system (Qiagen) and an AllPrep DNA/RNA mini kit (Qiagen). DNA was extracted from Sterivex filters using the phenol/chloroform protocol described by Somerville *et al.* (1989). The *Planctomycetales*-specific 16S rRNA genes were amplified from the extracted DNA using the planctomycete-specific primer PLA-46F and the universal bacterial primer 1392R (Chouari *et al.*, 2003). The anammox-specific PCR was carried out using AMX386 (Schmid *et al.*, 2005) and 1392R primers and either sponge DNA or planctomycete-PCR amplicon as a template. Both planctomycete- and anammox-specific PCR reactions were carried out with denaturation at 95 °C for 5 min, seven touchdown cycles (denaturation at 94 °C for 30 s, annealing at 62, 60, 59, 58, 57, 56, 55 °C for 30 s, and elongation at 72 °C for 1 min), 25 consistent cycles (at 94 °C for 30 s, at 54 °C for 30 s and 72 °C for 1 min), and final elongation at 72 °C for 10 min. The *amoA* genes were amplified from the extracted DNA using *amoA*-1F and *amoA*-2R primers (Rotthauwe *et al.*, 1997). PCR was carried out with 5 min at 94 °C, 42 cycles of 94 °C for 1 min, 60 °C for 1:30 min and 72 °C for 1:30 min, and a final 10 min extension cycle at 72 °C. Negative controls without DNA were included in all reactions to test for contamination. PCR products of the expected size and corresponding positions of the negative controls were gel-purified using a QIAquick gel extraction kit (Qiagen).

Cloning and sequencing

PCR products were ligated into PCR-XL-TOPO vector and transformed into OneShot TOP10 chemically competent *Escherichia coli* cells using the TOPO XL PCR Cloning Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). Plasmid DNA was isolated from individual clones and purified using Agencourt SprintPrep 384 HC kit (Agencourt Bioscience, Beverly, MA, USA). Sequencing was done using ABI PRISM genetic analyzer (Applied Biosystems, Foster City, CA, USA) and M13 forward and reverse primers.

RNA extraction and RT-PCR

Total RNA was extracted from sponge samples using the TissueLyser system (Qiagen) and the AllPrep DNA/RNA mini kit (Qiagen). RNA samples were purified using RNAeasy mini-spin column and DNA was digested by adding RNAase-free DNAase (Qiagen) to RNAeasy mini columns. Reverse transcription reactions were performed using *amoA*-2R primer and ThermoScript RT-PCR system (Invitrogen). The reverse transcription reaction was carried out at 50 °C for 30 min, followed by denaturation, RNase inactivation at 99 °C for 5 min, and final cooling at 4 °C for 5 min. After reverse transcription, *amoA* genes were amplified in cDNA samples using the PCR protocol described for the DNA samples. RNA samples without the RT step were used as negative controls to test for contaminating DNA.

Phylogenetic analysis

16S rRNA gene sequences were edited and assembled using VectorNTI software (Invitrogen Life Technologies, Carlsbad, CA, USA). They were checked for possible chimeras using CHECK_CHIMERA program of the Ribosomal Database Project (Maidak *et al.*, 1999). *amoA* gene sequences were edited and the vector sequences were clipped using PreGap4 and Gap4 from the Staden package (http://sourceforge.net/projects/staden/). The AmoA amino acid sequences were deduced from the *amoA* gene sequences. 16S rRNA and AmoA gene sequences were analyzed initially using the BLASTn tool to aid the selection of the closest reference sequences. All sequences were imported into ARB (Ludwig *et al.*, 2004), which was used to align homologous regions of our sequences and the nearest relative matches. This database was supplemented with relevant environmental sequences that had been submitted recently to GenBank. These sequences were selected based on the top BLAST hits of the clone sequences. Multiple alignments were checked manually and improved by the ARB editor tool, and the aligned sequences were analyzed by the neighbor-joining distance matrix method available in ARB. Neighbor-joining trees (Jukes-Cantor correction) (Saitou and Nei, 1987) were constructed and the robustness of the inferred tree topologies was evaluated after 100 bootstrap replicates of the neighbor-joining data. Bootstrap values were generated using Phylip (Felsenstein, 2004).

Rarefaction analysis and estimation of microbial diversity
DOTUR (distance-based OTU and richness) was used to assign operational taxonomic units (OTUs) to 16S rRNA and AmoA phylotypes, to generate collector's curves for observed unique OTUs and to determine the species richness and diversity indices (Schloss and Handelsman, 2005). OTUs of the 16S rRNA and *amoA* genes were defined as sequence groups in which sequences differed by 3 and 5%, respectively. The percentage of coverage of the clone libraries was calculated by dividing the observed number of OTUs by Chao1 estimate.

Nucleotide sequence accession numbers
The sequences determined in this study were submitted to GenBank under accession numbers FJ652464-548 (for the 16S rRNA gene clone sequences) and FJ652549-570 (for the *amoA* gene clone sequences).

Results and discussion

Phylogenetic diversity of AAOB
The diversity of AAOB in sponges was investigated using the *amoA* gene. A PCR product of *ca.* 491 bp was recovered from DNA extracted from sponges *I. strobilina* and *M. laxissima*. No PCR amplification of *amoA* genes was detected in bacterial communities in two surrounding seawater samples. Two clone libraries of *amoA* genes were constructed for both sponges and 11 randomly selected clones from each library were sequenced. Amino acid sequences (AmoA) were deduced from the *amoA* gene sequences for phylogenetic analysis. The AmoA sequences fell into three clusters that were related to AmoA sequences of the *Betaproteobacteria Nitrosospira* spp. (90–92% identity) (Figure 1). Cluster 1 included three *M. laxissima* clones (designated ML-amoA) that were 99–100% identical to each other and 95% identical to an uncultured sediment clone (EU244515). Cluster 2 included five *I. strobilina* clones (designated IS-amoA) that were 97–99% identical to each other and 96–98% identical to an uncultured bacterium clone from an aquaculture wastewater (EU718514). Cluster 3 included six *I. strobilina* clones and eight *M. laxissima* clones that were 97–100% identical to each other. The closest AmoA sequence was from an uncultured bacterium clone from an estuarine sample (AY702580, 94–96% identity). None of the sequences were related to AmoA sequences of NOB. The rarefaction analysis of AmoA sequences showed that the number of clones sequenced represented a sufficient sample size (data not shown).

The absence of NOB and the affiliation of sponge-associated AmoA genes with *Nitrosospir*a AmoA sequences indicate that AAOB are the main nitrifiers in sponges. The presence of AmoA sequences that are solely related to AmoA sequences of *Nitrosospira* lineage is similar to the findings of Bayer *et al*. (2008) in the sponge *A. aerophoba*. *Nitrosomonas*-related AmoA sequences were not detected in the bacterial communities of our sponges. A study by Diaz *et al*. (2004) found *Nitrosomonas*-related 16S rRNA sequences in Caribbean sponges by an analysis based on denaturing gradient gel electrophoresis. The non-detection of *Nitrosomonas*-related AmoA genes in the two marine sponges may be due to low abundance of these genes or bias of the primers used to amplify the *amoA* genes toward those from the *Nitrosospira* lineage.

The expression of *amoA* genes of the AAOB was investigated by RT-PCR to detect whether the sponge-associated AAOB are metabolically active. No expression of *amoA* was detected in any of the sponges (data not shown). This could be because of low levels of *amoA* transcripts, below the detection limit of RT-PCR. It is also possible that the AAOB were not metabolically active at the time or the season of sampling (daytime, late summer). Bayer *et al*. (2008) found a seasonal variability of ammonium and nitrate excretion in *A. aerophoba*. They reported higher nitrification rates in spring rather than late summer, which can be correlated to a seasonal-dependent *amoA* mRNA expression and nitrification activity of the AAOB.

Phylogenetic diversity of AnAOB
16S rDNA gene fragments were amplified from extracted DNA using planctomycete-specific and anammox-specific primers. Non-*Planctomycetales* phylotypes were amplified with the planctomycete-specific primers due to non-specific primer binding, we excluded these sequences from the present study. Several phylotypes were found for *Verrucomicrobia* especially in the seawater clone library. Other non-*Planctomycetales* phylotypes were related to *Chlamydia*, *Chloroflexi, Cyanobacteria*, *Proteobacteria* and *Bacteroidetes*.

The PCR products obtained using planctomycete-specific primers indicated that members of *Planctomycetales* were present in the two sponges and surrounding seawater. Three clone libraries were constructed and 48, 42 and 36 randomly selected clones were sequenced from *I. strobilina*, *M. laxissima* and the seawater sample, respectively. Of the sequenced clones, 34 clones from *I. strobilina* (designated IS-Pla), 34 clones from *M. laxissima* (designated ML-Pla) and three clones from seawater (designated SWB-Pla) were affiliated with *Planctomycetales.* Three unique OTUs (<97% identity) were recovered from *I. strobilina*. They exclusively clustered with uncultured *Poribacteria* clones that were previously found in marine sponges (Fieseler *et al.*, 2004) (Figure 2). Fourteen unique OTUs were recovered from *M. laxissima*, one OTU clustered with *Planctomyces*, 11 OTUs with *Pirellula* and two

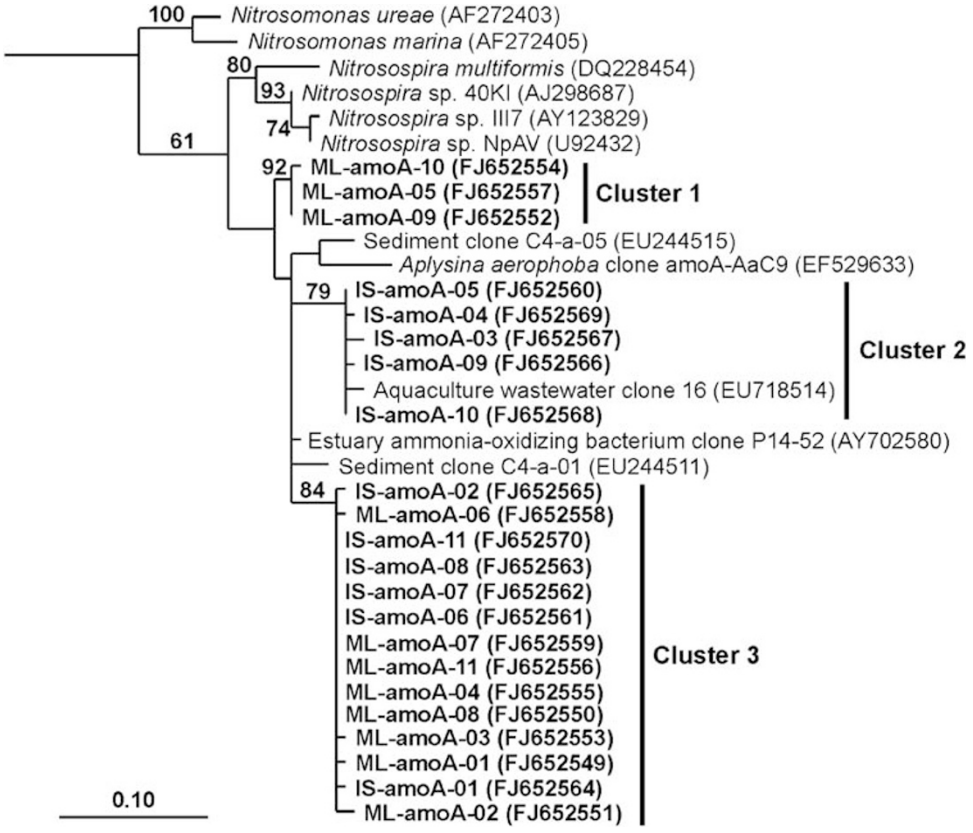

Figure 1 Phylogenetic neighbor-joining tree based on the 128-amino acid residue sequences of *amoA* gene retrieved from the bacterial communities of *I. strobilina* (IS-amoA) and *M. laxissima* (ML-amoA). Bootstrap values >50% are shown at each node. The root was determined using the AmoA sequence of *Nitrosomonas halophila* (GenBank accession no. AJ298704). The scale indicates the number of nucleotide substitutions per site.

OTUs exhibited a novel lineage. This novel lineage included six clones; one clone ML-Pla-01 was closely related to an uncultured sponge bacterium clone (EF092217, 93% identity) and the other five clones including ML-Pla-14 were closely related to an uncultured sediment clone (DQ833489, 93% identity) (Figure 2). The closest cultured relative to the six clones was the anammox bacterium *Candidatus* Brocadia fulgida (DQ459989) with a very low level of identity ranging from 77 to 81%. A comparison of three anammox signature oligonucleotides S-*Amx- 0368-a-A-16, S-*-Scabr-1114-a-A-22 and S-G-Sca-1309-a-A-21 (Schmid *et al.*, 2003) indicated that the *M. laxissima* novel anammox lineage was not identical (with 4–6 base mismatches) to the three signature nucleotides of *Candidatus* Kuenenia stuttgartiensis, *Ca.* B. fulgida and *Candidatus* Brocadia anammoxidans (Table 1). The *Planctomycetales* community of the seawater sample included two phylotypes. One phylotype had one clone SWB-Pla-31 that was closely related to an uncultured sponge *Poribacteria* clone (AY485286, 99% identity). It clustered with the *I. strobilina Poribacteria* group (88–98% identity). The other phylotype had four clones including SWB-Pla-37 and was closely related to an Arabian seawater clone (AY907752, 98–99% identity). It clustered with the novel anammox group detected in *M. laxissima* (80–91% identity) (Figure 2).

Although no 16S rRNA sequences corresponding to those from anammox bacteria were directly amplified from sponge DNA samples, such sequences were obtained only from *M. laxissima* by nested PCR using planctomycete 16S rRNA amplicons as templates, which indicated that AnAOB were present in this sponge. One clone library was constructed and 12 random clones were sequenced (designated ML-PlaAMX). They were 99% identical to each other, yielded one OTU and fell into one cluster with 16S rRNA gene sequences from known anammox bacteria. The closest relative to this OTU was an anammox *Planctomycetales* clone found in an anaerobic biofilter of a recirculating marine aquaculture system (AY769988, 97–98% identity) and *Ca.* B. fulgida found in an anammox wastewater treatment plant (DQ459989, 92–93% identical) (Figure 2). A comparison of three anammox signature oligonucleotides (Schmid *et al.*, 2003) indicated that ML-PlaAMX clones were identical to S-*Amx- 0368-a-A-16 sequences of *Ca.* K. stuttgartiensis, *Ca.* B. fulgida and *Ca.* B. anammoxidans, identical to S-*-Scabr-1114-a-A-22 sequence of *Ca.* B. anammoxidans and nearly identical (with two-base mismatches) to S-G-Sca-

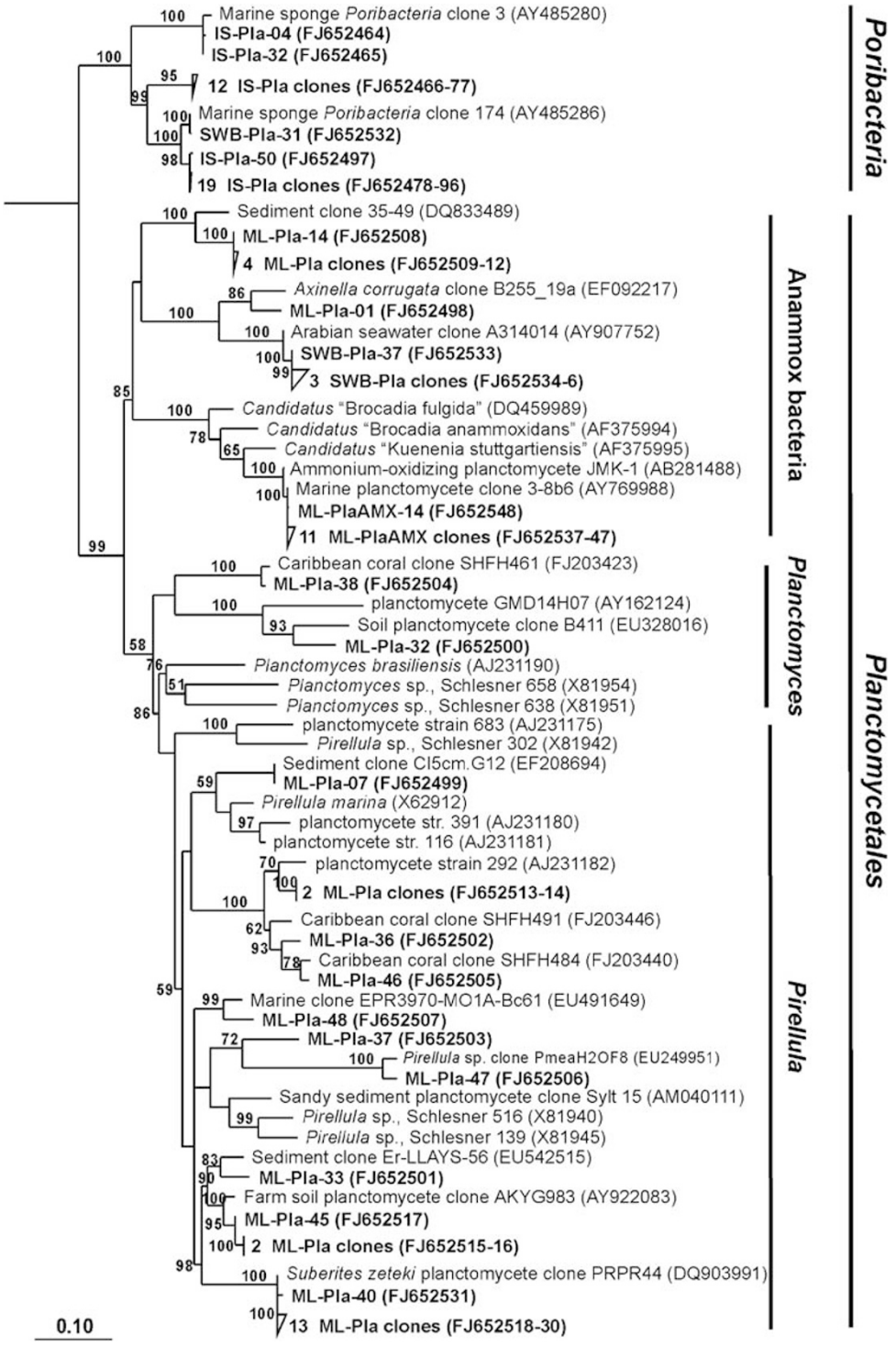

Figure 2 Phylogenetic neighbor-joining tree showing the affiliation of 16S rRNA sequences recovered from *I. strobilina* (IS-Pla), *M. laxissima* (ML-Pla) and the surrounding seawater (SWB-Pla) using planctomycete-specific primers. It also shows the affiliation of 16S rRNA sequences recovered from *M. laxissima* (ML-PlaAMX) using the anammox-specific primers to representative anammox bacteria within the *Planctomycetales*. The number and origin of clones within each polygon is shown in bold. Bootstrap values >50% are shown at each node. The root was determined using the 16S rRNA gene sequence of *Parachlamydia acanthamoebae* (GenBank accession no. Y07556). The scale indicates the number of nucleotide substitutions per site.

1309-a-A-21 sequence of *Ca.* K. stuttgartiensis (Table 1).

The use of 16S rRNA gene primers specific to *Planctomycetales* revealed higher *Planctomycetales* diversity in *I. strobilina* and *M. laxissima* than previously found with universal bacterial 16S rRNA primers. In our previous studies of the microbial communities associated with these sponges using

Table 1 Comparison of *M. laxissima* (ML-PlaAMX and ML-Pla) and seawater (SWB-Pla) anammox-related sequences with anammox-signature nucleotides

	Anammox-related signature nucleotides		
16S rRNA gene sequences	S-*-Amx-0368-a-A-16	S-*-Scabr-1114-a-A-22	S-G-Sca-1309-a-A-21
Kuenenia stuttgartiensis	CGCAATGCCCGAAAGG	CTTGTCTTTAGTTGCTAACGGG	GGAGGCTGAAACTCGCCTCCA
Brocadia anammoxidans	----------	----------C-T-A--	----------C---------
Brocadia fulgida	----------	----------C---A--	----------C---------
ML-PlaAMX-01	----------	----------C-T-A--	--------C---------G--
ML-PlaAMX-02	----------	----------C-T-A--	--------C---------G--
ML-PlaAMX-03	----------	----------C-T-A--	--------C---------G--
ML-PlaAMX-05	----------	----------C-T-A--	--------C---------G--
ML-PlaAMX-06	----------	----------C-T-A--	--------C---------G--
ML-PlaAMX-07	----------	----------C-T-A--	--------C---------G--
ML-PlaAMX-09	----------	----------C-T-A--	--------C---------G--
ML-PlaAMX-10	----------	----------C-T-A--	--------C---------G--
ML-PlaAMX-11	----------	----------C-T-A--	--------C---------G--
ML-PlaAMX-14	----------	----------C-T-A--	--------C---------G--
ML-PlaAMX-15	----------	----------C-T-A--	--------C---------G--
ML-PlaAMX-16	----------	----------C-T-A--	--------C---------G--
ML-Pla-01	-------GG------C	---A--G--------C-G-G--	--------C---------G--
ML-Pla-09	-------GGG-C--CC	------C--------C-G-G-A	--------C---------G--
ML-Pla-15	-------GGG-C--CC	------C--------C-G-G-A	-C------C--T------G--
ML-Pla-24	-------GGG-C--CC	------C--------C-G-G-A	-AG-----C--------CT--
ML-Pla-41	-------GGG-C--CC	------C--------C-G-G-A	-AG-----C--------CT--
SWB-Pla-21	-A-----GA------T	---A--G--------C-G-G--	-AG-----C--------CT--
SWB-Pla-32	-A-----GA------T	---A--G--------C-G-G--	-C------C--T------G--
SWB-Pla-37	-A-----GA------T	---A--G--------C-G-G--	-C------C--T------G--
SWB-Pla-47	-A-----GA------T	---A--G--------C-G-G--	-C------C--T------G--

Table 2 Richness and diversity estimates for *Planctomycetales* 16S rRNA gene clone libraries from *I. strobilina* and *M. laxissima* using planctomycete-specific primers and from *M. laxissima* using anammox-specific primers

Source[a]	*Distance*[b]	*Richness*[c]	*ACE*[d]	*Chao1*[e]	*Shannon*[f]	*1/Simpson*[g]	*Coverage*[h] (%)
IS-*Planctomycetales* ($n=34$)	0.2	1	0	1	0	1	100
	0.03	3	3	3	0.9	2.2	100
ML-*Planctomycetales* ($n=34$)	0.2	4	8	5	0.7	1.7	80
	0.03	14	45	37	2.1	5.3	38
ML-anammox ($n=12$)	0.2	1	0	1	0	1	100
	0.03	1	0	1	0	1	100

[a]*n*, number of gene sequences analyzed.
[b]80% identity was estimated as the phylum-level distance ($D=0.20$), and 97% identity was estimated as the species-level distance ($D=0.03$).
[c]Richness is based on observed unique OTUs.
[d]Nonparametric statistical prediction of total richness of different OTUs based on distribution of abundant (>10) and rare ($\leqslant 10$) OTUs.
[e]Nonparametric statistical predictions of total richness of OTUs based on distribution of singletons and doubletons.
[f]Shannon diversity index. A higher number represents more diversity.
[g]Reciprocal of Simpson's diversity index. A higher number represents more diversity.
[h]Percentage of coverage: percentage of observed number of OTUs divided by Chao1 estimate.

universal bacterial primers, *Planctomycetales* were not detected in *I. strobilina* and only one clone was detected in *M. laxissima* (Mohamed *et al.*, 2008b, c). Although the universal primers detected a wide range of bacterial phylotypes, PCR with group specific primers confirmed major limitations of universal primers, which are the inability to anneal equally well to all bacterial 16S rDNA and to amplify rRNA gene fragments from all the bacteria present in the sponges.

To determine whether the number of clones sequenced from *I. strobilina* and *M. laxissima* libraries using planctomycete-specific primers and from the *M. laxissima* library using anammox-specific primers were representative, rarefaction analyses were performed (Table 2; Figure 3). The rarefaction curves for *I. strobilina Planctomycetales* clones reached saturation at the phylum (distance = 0.20) and species (distance = 0.03) levels, indicating sufficient sampling of this clone library (Figure 3). The rarefaction curves for the *M. laxissima Planctomycetales* clones reached saturation at the phylum level, where richness reached an asymptotic maximum, but not at the

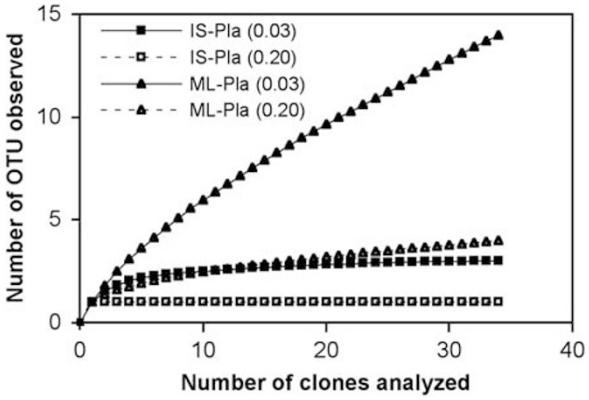

Figure 3 Rarefaction curves for the 16S rRNA gene sequences retrieved by using the planctomycete-specific primers from *I. strobilina* (IS-Pla) and *M. laxissima* (ML-Pla). Operational taxonomic units were defined at estimated distances at both phylum ($D=0.20$) and species ($D=0.03$) levels.

species level, indicating that further sampling of the clone library would have revealed additional diversity. The rarefaction curves for the *M. laxissima* clones that were recovered using anammox-specific primers reached saturation at the phylum and species levels, indicating sufficient sampling of this clone library (data not shown). The greatest number of OTUs was found in the *M. laxissima Planctomycetales* library. Higher Shannon and Simpson indices were observed for the same library indicating higher diversity of the *Planctomycetales* community in *M. laxissima* than *I. strobilina* (Table 2). The coverage estimate (number of OTUs observed/Chao1 estimate) indicated that we sampled 100% of the diversity in the *I. strobilina Planctomycetales* library while we sampled only 38% of the diversity in the *M. laxissima Planctomycetales* library, by sequencing 34 clones (Table 2). Given the high bacterial diversity and the low sequence coverage we achieved for the *M. laxissima* library, we expect that many more *Planctomycetales* species may be found in this sponge.

All of the clones recovered from *I. strobilina* using planctomycete-specific primers were affiliated with candidate phylum '*Poribacteria*'. This sponge-specific candidate phylum was described by Fieseler *et al*. (2004). The '*Poribacteria*' lineage is slightly related to the *Planctomycetes*, *Chlamydia* and *Verrucomicrobia* phyla. It has cell compartmentalization in the form of an unusual nucleoid-like structure, a unique feature only found in members of the phylum *Planctomycetes*.

The recovery of one anammox phylotype from the *M. laxissima* library using anammox-specific primers and two other putative anammox phylotypes using planctomycete-specific primers indicate the presence of three major types of AnAOB in *M. laxissima*. This is the first study in which 16S rRNA genes of anammox bacteria have been detected in sponges. These sequences were distantly (<92% identity) related to known candidate anammox genera. We therefore propose that these sponge-associated AnAOB represent a novel type of AnAOB. Future work aims to taxonomically classify the novel AnAOB in *M. laxissima* as new candidate genera. Fluorescent *in situ* hybridization probes will be used to target and quantify the dominant AnAOB in the total bacterial 16S rRNA genes in *M. laxissima*.

Microbial interactions and nitrogen cycling in marine sponges

The presence of AAOB in marine sponges is consistent with the aerobic conditions in the mesohyl of sponges (Hoffmann *et al*., 2005). The presence of AnAOB in *M. laxissima* is consistent with anaerobic environments or microniches in deeper and interior sponge tissues, resulting from respiratory depletion of oxygen in the water pumped through the sponge. Anaerobic bacterial metabolic activity has been demonstrated previously in sponges (Hoffmann *et al*., 2005; Mohamed *et al*., 2008a). An interesting question that is raised by our findings is whether AAOB and AnAOB in marine sponges are competitors or natural partners? One possibility is that each population has a different niche within the sponge tissues so that the AAOB are present and active under aerobic conditions in areas like the mesohyl, while the AnAOB are present and active under anaerobic conditions. Another possibility is that the AAOB and the AnAOB are natural partners that constitute a stable community in sponges such as *M. laxissima*. It is conceivable that under oxygen-limited conditions at oxic/anoxic interfaces, the AAOB cooperate with the AnAOB for efficient nitrogen removal from sponges. The AAOB would oxidize part of the ammonium to nitrite keeping the oxygen concentration low, while the AnAOB would then convert the produced nitrite and the remaining ammonium to dinitrogen gas (Schmid *et al*., 2002). This oxygen-limited combined process of nitrogen removal was patented as the CANON process (completely autotrophic nitrogen removal over nitrite) and was used for wastewater treatment (Sliekers *et al*., 2003; Third *et al*., 2005). The CANON process relies on the stable interaction between only two bacterial populations, *Nitrosomonas*-like aerobic and *Planctomycete*-like AnAOB. There is recent information about the flexibility of the metabolism of AAOB and their abilities to survive without oxygen as well as strong indications that AAOB might be capable of anaerobic ammonia oxidation (anoxic denitrification) (Schmid *et al*., 2002). They can oxidize ammonia in the absence of oxygen using nitrite as the terminal electron leading to the formation of dinitrogen gas. This possibility does not rule out competition between the AAOB and AnAOB in sponges when ammonia concentrations are low and the affinities of both groups toward ammonia will determine the outcome of the

competition. In this scenario, the reason that NOB were not detected could be due to their inability to compete with the AAOB for oxygen and with the AnAOB for nitrite.

Conclusions

In this study, the presence of AmoA sequences in marine sponges indicates that nitrification is a metabolic capability of sponge symbionts. All of the AAOB in *I. strobilina* and *M. laxissima* clustered with *Nitrosospira* spp. The 16S rRNA screening approach revealed the presence of AnAOB in *M. laxissima*. This is the first molecular evidence for the presence of anammox bacteria in sponges suggesting that sponges are source from which to enrich and cultivate novel anammox bacteria. These AnAOB are distantly related to known candidate anammox genera, and they likely represent novel genera. It is not clear whether the AAOB and AnAOB in sponges are natural partners, competitors, or two separate populations that have different niches within sponge tissues. We are still far from understanding the complexity of the nitrogen cycle in marine sponges. Sponges have significant coverage and constitute a major portion of the biomass of many coral reefs. Therefore, microbially mediated nitrogen metabolism in sponges is expected to have a major impact on the nitrogen budget of coral reefs. Clearly, there are still many open questions and knowledge gaps in understanding the sponge–microorganism associations and nutrient cycling in marine sponges. However, our research adds significantly to the ongoing effort to unravel metabolic capabilities of sponge-associated microbiota.

Note added in proof

A very recent publication [Hoffmann F, Radax R, Woebken D, Holtappels M, Lavik G, Rapp HT, Schläppy M-L, Schleper C, Kuypers MMM. (2009). Complex nitrogen cycling in the sponge *Geodia baretti*. *Environ Microbiol* Published online 18 May 2009; doi:10.1111/j.1462-2920.2009.01944.x] reports anammox rates and 16S rRNA gene sequences closely related to *Candidatus* Scalindua anammox bacteria in the sponge *Geodia baretti*.

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