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Coral-associated ammonium oxidizing Crenarchaeota and their role in the coral holobiont nitrogen cycle

N. Siboni[1], E. Ben-Dov[1,2], A. Sivan[1], A. Kushmaro[1,3*]

1) The Environmental Biotechnology Laboratory, Department of Biotechnology Engineering, Ben-Gurion University of the Negev, P. O. Box 653, Be'er-Sheva 84105, Israel
2) Achva Academic College, MP Shikmim 79800, Israel
3) National Institute of Biotechnology in the Negev, Ben-Gurion University of the Negev, P. O. Box 653, Be'er-Sheva 84105, Israel

Abstract. Genetic comparison of Archaea associated with the surface mucus of corals from three genera, namely *Acanthastrea* sp., *Favia* sp. and *Fungia* sp. from the Gulf of Eilat, Israel and from Heron Island, Australia were studied. Sequencing of the 16S rRNA gene of the coral-associated microorganisms revealed dominance of Crenarchaeota (79%, on average). In this phylum, 87% of the sequences were similar (≥97%) to the Thermoprotei, with 76% of these being similar (≥97%) to the ammonium oxidizer, *Nitrosopumilus maritimus*. Analysis of archaeal *amoA* sequences obtained from the fungiid coral, *Fungia granulosa*, divided into three clades, all related to archaeal sequences previously obtained from the marine environment. These sequences were distantly related to *amoA* sequences previously found in association with other coral species. Preliminary experiments suggest that there is active oxidiation of ammonia to nitrite in the mucus of *F. granulosa*. Thus, coral-associated Archaea may contribute to nitrogen recycling in the holobiont, presumably by acting as a nutritional sink for excess ammonium trapped in the mucus layer, through nitrification processes.

Key words: Archaea, ammonia, *amoA*.

Introduction

Coral reefs develop in oligotrophic marine environments. The surface of scleractinian corals, the main reef builders, is covered by a layer of mucopolysaccharides. This mucus layer is made up of a gel consisting of an insoluble hydrated glycoprotein (Brown and Bythell 2005) that is constantly secreted by the coral tissue. At the point of contact of the mucus with the surrounding water, a boundary layer is formed (Segel and Ducklow 1982; Ritchie and Smith 2004). Recent research has demonstrated that corals contain large, diverse and specific populations of microorganisms, including Viruses, Bacteria, Archaea, fungi, algae and protozoa that apparently co-evolved with corals (reviewed by Rosenberg et al., 2007). The presence of archaeal groups in association with corals has been reported (Wegley et al. 2004; Kellogg 2004), although the roles assumed by the Archaea in the coral holobiont have not yet been studied.

Archaea comprise four phyla, namely the Crenarchaeota, Euryarchaeota, Korarchaeota, and Nanoarchaeota. In the ocean, planktonic Crenarchaeota are most abundant (Fuhrman 1992; Karner et al. 2001). Although Crenarchaeota constitute about 20% of the total marine picoplankton biomass world-wide (Karner et al. 2001), their roles in the marine biogeochemical cycle have not been thoroughly explored (Wuchter et al. 2006). Until recently, Archaea were considered to be uncommon partners for invertebrate symbioses. Archaea were found in association with sponge species (Preston et al. 1996; Webster et al. 2001; Pape et al. 2006), although the ecological functions assumed by these sponge-linked microorganisms remain unknown.

Wegley et al. (2004) showed by fluorescent in situ hybridization (FISH) that Archaea on the coral, *Porites astreoides*, accounted for nearly half of the prokaryotic community, implying that Archaea are abundant, diverse, and potentially important components of the coral holobiont.

Corals obtain and assimilate nitrogen through predation (Piniak et al. 2003). Additional nitrogen can be obtained through direct absorption from the surrounding water (Muscatine and D'Elia, 1978; Badgley et al., 2006), by integration via associated microorganisms, such as nitrogen-fixing cyanobacteria (Lesser et al. 2004), and from microorganisms trapped in the coral mucus (Wild et al. 2004). Nitrogen metabolized by the corals leads to the production of ammonia that can be excreted into the environment.

Aerobic oxidation of ammonia by some microorganisms is catalyzed by ammonia

monooxygenase (AMO), an enzyme that converts ammonia to hydroxylamine, which is further converted to nitrite by hydroxylamine oxidoreductase (Nicol and Schleper 2006). A time series study in the North Sea revealed that the abundance of archaeal *amoA* was 1 to 2 orders of magnitude higher than those of *amoA* from nitrifying bacteria. Sea water incubated with ammonia was dominated by a single member of the crenarchaeotal phylogenetic cluster showing 99% sequence identity over the nearly complete 16S rRNA gene of the nitrifying Crenarchaeote, *Nitrosopumilus maritimus* (Wuchter et al. 2006). *N. maritimus* is a marine chemolithtotroph which aerobically oxidizes ammonia to nitrite with near stoichiometric conversion, using ammonia as its sole energy source (Könneke et al. 2005).

Wafar et al. (1990) suggested that nitrification occurs at significant rates in living coral colonies. The authors showed that nitrifying coral-associated microorganisms can vary from 4 to 260 x 10^3 cells per mg coral tissue. Recently putative archaeal *amoA* genes encoding ammonia monooxygenase subunit A were retrieved from several corals (Beman et al. 2007). Indeed, while archaeal *amoA* sequences were obtained from different species of coral-associated Archaea, no bacterial *amoA* sequence could be amplified from any of these samples.

In this report, we study coral-associated Archaea from three genera of corals (*Acanthastrea* sp., *Favia* sp. and *Fungia* sp.) from the Red Sea, Israel with those from Heron Island, Australia concentrating in Crenarchaeota. Based on our findings, the possible role of coral-associated Archaea in the coral holobiont is discussed.

Material and Methods

Samples of the hermatypic corals, *Fungia* sp., *Favia* sp. and *Acanthastrea* sp., were collected at depths of 5-10 m at Heron Island GBR Australia (23º26 'S, 151°54 'E), in July, 2006, and near the Inter-University Institute for Marine Science in the Gulf of Eilat (29°51 'N, 34°94 'E), Red-Sea, in June, 2007. Within one hour of collection, the corals were placed in aquaria filled with running sea water. On the same day, mucus was sampled from the corals using sterile bacteriological loops. Sea water from Eilat was collected by opening a sterile container on the same dives. Four additional *Fungia granulosa* individuals were collected in December, 2007 for mucus collection, for NO_2 production experiments. This was carried out by inverting the corals on a funnel for 2 min. Mucus for DNA extraction was sampled from two of the *F. granulosa* corals using bacteriological loops, as previously described (Barneah et al. 2007).

F. granulosa mucus (2 ml) was filtered through 0.1 μm filter. The filters were then placed in glass flasks with 10 ml sterile sea water and 1mM ammonium chloride at 22-25ºC and incubated with shaking for up to 28 days. Since streptomycin inhibits the growth of nitrifying bacteria (Könneke et al. 2005), to eliminate the activities of bacterial ammonia oxidation, two flasks were incubated with 500 mg L^{-1} of streptomycin. Eight milliliters of filtered mucus topped with 2 ml sterile sea water containing 1 mM ammonium chloride served as control. NO_2 was measured after 0, 2, 15 and 28 days, according to standard methods for the examination of water and wastewater (Clesceri et al. 1998).

Genomic DNA from the mucus samples was extracted by a PowerSoil purification kit (Mo Bio Laboratories Solana Beach, CA. USA), and stored at -20°C. To extract genomic DNA from the water column, 2 L of sea water collected from the same depths were passed through sterile 0.2 μm filters and the DNA was extracted as above. Total DNA was PCR-amplified using specific archaeal 16S rRNA gene modified primers; Arch-21F (5'-TTCCGGTTG ATCCYGCCG-3'), obtained from DeLong (1992) and Arch-915R (5'-GTGCTCCCCCGCCAATTC-3'), taken from Amann et al., (1995). An initial denaturing step of 4 min at 95°C was followed by 30 cycles of the following program pattern: 94°C for 30 sec, 54°C for 30 sec, and 72°C for 70 sec. The procedure was completed with a final elongation step at 72°C for 30 minutes. Archaeal *amoA* gene fragments were amplified using Arch-amoA-F (5'-STAATGGTCTGGCTTAGACG-3') and Arch-amoA-R (5'-GCGGCCATCCATCTGT ATGT-3') primers and conditions as previously described (Francis et al. 2007; Beman et al. 2007).

The gel-purified PCR products were cloned into the PCRII-TOPO- A cloning vector, as specified by Invitrogen (Carlsbad, CA) and transformed into calcium chloride-competent *Escherichia coli* HD5a cells. More than 50 clones from each sample were amplified using M13-F and M13-R primers and sequenced, using the Arch-21F primer. All sequences were first compared with those in the GenBank database with the basic local alignment search tool (BLAST) network and check for chimeric sequences using Bellerophon (Huber et al. 2004).

Results

A total of 424 clones derived from 8 coral samples (>50 clones from each library) displayed a prevalence of crenarchaeotal sequences (79%), as compared to euryarchaeotal sequences (21%) (Fig. 1). Red-Sea water displayed opposite prevalence with dominants of Euryarchaeota (63%).

BLAST analysis revealed that of the coral-drived crenarchaeotal sequences, 87% were similar (≥97%) to the class Thermoprotei. Of this group, 76% were

similar (≥97%) to sequence of *Nitrosopumilus maritimus* (DQ085097) (Fig. 1), a marine Archaea that oxidizes ammonia Könneke et al., (2005).

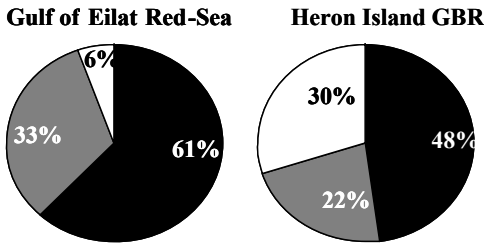

Fig. 1: Archaea distribution by sample location. Crenarchaeota similar to *Nitrosopumilus maritimus* (≥97%, FastGroup II) (black), other Crenarchaeota (gray) and Euryarchaeota (white).

Analysis of the archaeal *amoA* from two *F. granulosa* corals, Rs-Fg1 and Rs-Fg2 (24 sequences in each clone library), collected from the Red Sea, yielded three different clades, all related to sequences retrieved from the marine environment. Part of the nitrification cycle of the mucus-associated microorganisms was demonstrated by nitrite production.

Nitrification measurements of *F. granulosa* mucus-derived microbiota incubated with 1 mM ammonia revealed an increase of 50% after 15 days and a 9-fold increase after 28 days (Table 1). Production of nitrite after 28 days of incubation in a streptomycin-amended medium (designed to inhibit nitrifying bacteria) was slightly lower than in non streptomycin-amended samples.

Table1: NO_2 production by *F. granulosa* coral mucus-associated microorganisms during 28 days.

Days NO_2 (ppm)	0	2	15	28
Control	0	0	0.037	0.096
Coral mucus microorganisms	0	0	0.1186	1.076
Coral mucus microorganisms + streptomycin	0	0	0.118	0.856

Discussion

Phylogenetic analysis of archaeal 16S rRNA gene sequences retrieved from three corals species from the Red Sea (*Fungia* sp., *Favia* sp. and *Acanthastrea* sp.) displayed high similarity (up to 100%) to archaeal sequences retrieved from the same coral genera from the GBR. 67% of the 424 sequences from those locations were closely related (≥97%) to sequences derived from three scleractinian corals from the Virgin Islands (Kellogg 2004), implying the existence of a general coral-archaeal symbiotic association.

In general, the Crenarchaeota sequences were most abundant in coral samples from the Red Sea than in GBR (Fig. 1). A higher abundance of Crenarchaeota (73%) over Euryarcaeota (27%) was also observed by Wegley et al. (2004). By contrast, Kellogg (2004) reported a dominance of Euryarcaeota (average of 80%) in scleractinian corals from Carribian. This may a biogeographic distinction, or due to differences in sample collection methods.

The prevalence of Euryarchaeota over Crenarchaeota in the sea water sample was previously reported with water samples from oceanic photic zones (Delong et al. 2006; Delong 2007). This result strengthens the premise that conditions in the mucus differ from those in the surrounding medium.

Interestingly, almost all (87%) coral Crenarchaeota sequences analysed by BLAST were most closely related (≥97%) to the Thermoprotei class and 76% of these sequences were highly similar (≥97%) to that of *Nitrosopumilus maritimus* (DQ085097), whereas only 27% of the sea water-associated archaeal clone library sequences were similar to this strain. *N. maritimus*, isolated from the rocky substratum of a tropical marine tank, is an autotrophic crenarchaeote that is able to obtain its energy by oxidizing ammonium, producing nitrite as the end product (Könneke et al. 2005; Delong 2007). Positive correlations between the abundance of Crenarchaeota and ambient nitrite concentrations were observed in the Arabian Sea (Sinninghe et al. 2002) and in Santa Barbara (Murray et al. 1999). It is possible that the coral mucus layer is rich in ammonium and other metabolic by-products of corals, providing a rich nutritive source for these microorganisms.

Though ammonium can be utilized by coral zooxanthellae, very high levels may be detrimental to the coral holobiont. This may be due to the fact that high ammonium levels cause an inhibition of carbohydrate assimilation by algae (Azov and Goldman 1982) or affect other metabolic parameters, such as an inhibition of nitrate uptake (Badgley et al., 2006). It is feasible that mucus-associated Archaea act in the coral holobiont as a sink for excess ammonia, recycling it through nitrification during the day, when oxygen is in excess due to photosynthesis of the zooxanthella symbionts.

I this study we, screen the archaeal *amoA* gene in the Red Sea *Fungia* sp. Results showed that coral-associated archaeal *amoA* genes clustered into three clades, all related to sequences obtained from the marine environment. Surprisingly, *amoA* genes of coral-associated Archaea from Bermuda (Beman et al., 2007) and the *amoA* gene of *N. maritimus* created a separate cluster with very low similarity (79%-85%) to the *amoA* sequences from our coral-associated

Archaea .This may be due to the fact that Archaea from the Bermuda were from corals that were not in as close association to the substrate as the Red Sea fungiids. On the other hand, most of our Crenarchaeota 16S rRNA gene sequences were closely related (≥97%) to *N. maritimus*. These differences may be explained by the choice of genes for comparison, namely the highly conserved 16S rRNA gene (Gutell et al., 1994) and the functional and variable *amoA* gene.

The 16S rRNA gene library of coral-associated Archaea showed that at least 50% of the clones were similar (≥97%) to ammonium oxidizer, *N. maritimus*. To further confirm the presence of ammonia oxidizing Archaea, NO_2 production by coral mucus microorganisms was investigated (Table 1) using streptomycin, an antibiotic that affects Bacteria but not Archaea (Könneke et al., 2005). Results showed an increase in nitrification in the mucus of *F. granulosa* throughout the experiment. Moreover, there was only a slightly lower production of nitrite after 28 days of incubation in a streptomycin-amended medium, as opposed to non-amended samples. These results provide evidence for the possible widespread presence of ammonia oxidizers in coral reefs. Similarly, Wuchter et al., (2006) showed that the addition of nutrients to North Sea water was followed by an enrichment of Crenarchaeota and was correlated with the concomitant decrease of ammonia and increase of nitrite concentrations.

Nutrients from the environment have to move across a physical boundary layer, as well as through the mucus layer, to reach the coral tissue. Nutrients from the coral tissue must pass an opposite route to reach the environment. Such movement of nutrients is governed by a very slow diffusion rate (Segel and Ducklow, 1982). It is likely that a microbe growing on the mucus surface has access to dissolved nutrients before the coral does and, conversely, has access to coral-borne nutrients before the planktonic microorganism population does. Therefore, the coral mucus may be a better living substrate for ammonia-oxidizing Archaea than the water column because these Archaea would encounter ammonia before planktonic organisms and at higher concentrations.

We, therefore, suggest the following role for mucus-associated Archaea in the coral holobiont nitrogen cycle. During the day, the mucus conditions are oxic, and Archaea similar to *N. maritimus* oxidize ammonia to nitrite, which may be assimilated into the coral. At night, the mucus conditions are anoxic and microorganisms, like the archaeal H. salifodinae, may convert nitrite into nitrogen via denitrification pathways. This implies that Archaea play an essential role in coral reef and coral holobiont physiology.

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