

## Reef-building coral *Goniastrea aspera* harbor a novel filamentous cyanobacterium in their skeleton

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**Abstract.** Reef-building corals harbor diverse communities of cyanobacteria, algae, bacteria and fungi within their skeleton. In spite of their potential significance, the interaction between these microbes and host corals is still obscure. Here we report a novel cyanobacterium within the skeleton of *Goniastrea aspera*, a massive reef-building coral that is predominantly found in shallow reef habitats. Characteristics of this cyanobacterium are: (1) non-branching filaments having a 1 µm diameter, (2) lack of heterocysts, (3) hormogonia formation, and (4) Chl *a* as the sole chlorophyll pigment. Consistent with these phenotypic traits, 16S rDNA sequence analysis showed a close association with this cyanobacterium to *Halomicronema*, a recently identified genus that includes species found in benthic microbial mats of hypersaline ponds. A possible interaction between *Halomicronema* sp. and the host coral is discussed in terms of stress tolerance.

**Key words:** Cyanobacteria, coral skeleton, endolithic algae, *Goniastrea aspera*, *Halomicronema* sp.

### Introduction

The obligate symbiotic relationship between reef-building corals and dinoflagellates (zooxanthellae) is well recognized (D'Elia and Wiebe 1990). In addition to this well-established relationship, coral tissue and coral skeleton are also known to harbor diverse microbial consortia (Rohwer et al. 2002; Le Campion-Alsumard et al. 1995a). Until recently, however, these coral-microbial interactions remain obscure (Le Campion-Alsumard et al. 1995b)

The presence of endolithic microorganisms in skeleton of corals was first discovered in 1902 by Durden (Durden 1902). Visible green bands usually observed in the skeletons of massive corals comprised of cyanobacteria, fungi, bacteria, red and green algae (Le Campion-Alsumard et al 1995a; Schlichter et al. 1997). Among endolithic microbes, *Ostreobium* spp. are frequently found in coral skeletons (Lukas 1974).

The function of bioerosion that contributes to the geochemical and sedimentological importance in the reef was documented (Kobluk and Risk 1977). Also, endolithic microorganisms have been considered to be one of the major primary producers in coral reef environments (Tribollet et al. 2006). Recent findings have shown that endolithic algae transfer photoassimilates to coral host (Fine and Loya 2002; Lesser et al. 2007). This serves an alternative nutrient source especially during bleaching events. More recently, endolithic algae was reported to possess a photoprotective role in the coral-algal photosynthesis during high-light stress (Yamazaki et al. 2008).

Coral skeleton covered by living coral tissue is a harsh environment for the growth of many organisms (Shashar and Stambler 1992). This environment partly shares a similarity with that for microbial mats that harbor diverse microbial communities (Fourçans et al. 2006). In the intertidal reefs in Okinawa, Japan, massive coral *Goniastrea aspera* is constantly being exposed to a strong sunlight and high salinity during low tides. In this study, we report an endolithic community found within the skeleton of *G. aspera*.

### Material and Methods

Colony of massive coral *Goniastrea aspera* (approximately 5-6 cm in diameter) was collected in June 2004 from a shallow intertidal pool of Bisezaki, Okinawa Japan. Coral tissue was removed using a WaterPik (Johannes and Wiebe 1970) and coral skeleton was crushed to small fragments (approximately 1 cm in diameter) with a chisel. Skeletal fragments were then trimmed off with anatomical scissors to small pieces < 2 mm in length). The crushed skeletal pieces of *G. aspera* containing green bands were investigated utilizing culture method, microscopic observations, pigments analysis and molecular techniques.

Small pieces of coral skeleton were incubated in a liquid A medium (Mitsui and Cao 1988). Nitrogenous compounds were omitted from this medium to prevent the growth of undesirable microorganisms. The culture medium was modified to include sterile coral tissue extraction. Repeated subcultures of

colonies were carried out on agar plates to obtain a pure culture of cyanobacterium. All cultures were incubated at 28°C under a 12h dark : 12h light (30  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) photoperiod. The cultured cyanobacterium was used for pigment analysis and DNA analysis.

For microscopic observation, pieces of trimmed coral skeleton were ground in sterilized seawater using mortar and pestle. The samples were observed under a fluorescence microscope.

Pigments extracted from the green band and the cultured cyanobacterium were analyzed by thin layer chromatography analysis (TLC). Coral tissue was removed with a WaterPik as the described above. The tissue-removed skeletons were crushed in 90% acetone at 4°C, and were centrifuged at 10,000  $\times g$  for 2 min and the supernatant was used for TLC analysis. The cultured cyanobacterium was collected by centrifugation (15,000  $\times g$ , 3 min). The supernatant was then discarded and the cyanobacterial pellet was homogenized in 90% acetone at 4°C, and it was centrifuged at 15,000  $\times g$  for 3 min and the supernatant was used for TLC analysis. All procedures were carried out under a dim light. TLC analysis was carried out on a reverse phase  $C_{18}$  plate (MERCK) with 100% MeOH as the developer. The spots of chlorophyll pigments were visualized under a blacklight (UVP UVL-56).

Total genomic DNA of the cultured cyanobacterium was extracted using the UltraClean Soil DNA Kit (MoBio, Solana Beach, CA). PCR was performed using the primers of fD1 (5'-AGAGGATGATCAGCCACACTG-3') and rP2, which were designed for eubacterial 16S rDNA (Weisburg et al. 1991). The reaction mixture (50  $\mu\text{l}$ ) contained 0.15 mM deoxynucleotides (Takara, Tokyo, Japan), 0.2  $\mu\text{M}$  forward primer, 0.2  $\mu\text{M}$  reverse primer, 2  $\mu\text{l}$  of the PCR template and 0.05 U of recombinant *Taq* DNA polymerase (Takara) per  $\mu\text{l}$  in PCR buffer (Takara). The temperature program for 30 cycles of PCR was 94°C for 30 s, 55°C for 1 min, 72°C for 2 min, and 72°C for 5 min as the final extension after the last cycle. The amplified DNA fragment were purified by gel percolation. The sequence was determined in opposite orientations using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with a DNA Sequencer (ABI 3100 Avant; Applied Biosystems). The obtained nucleotide sequence has been deposited in DNA Data Bank of Japan (DDBJ) with an accession number AB257773. Related sequences were aligned with CLUSTAL X 1.83 software for multiple sequence alignment, and bases of ambiguous alignment were corrected or removed manually. Tree topology was constructed with the

neighbor joining method with MEGA 3.0 software (Kumar et al. 2004).

## Results

Figure 1A shows the localization of green bands observed in the transverse section of *G. aspera*. It exhibits two distinct layers of green band within the skeleton. Green bands were observed just beneath and in deep of the skeleton that were separated by a light green or whitish zone. The green bands were primarily composed of photosynthetic organisms having non-branching, narrow filament approximately 1  $\mu\text{m}$  in diameter as shown in Fig 1B and C.

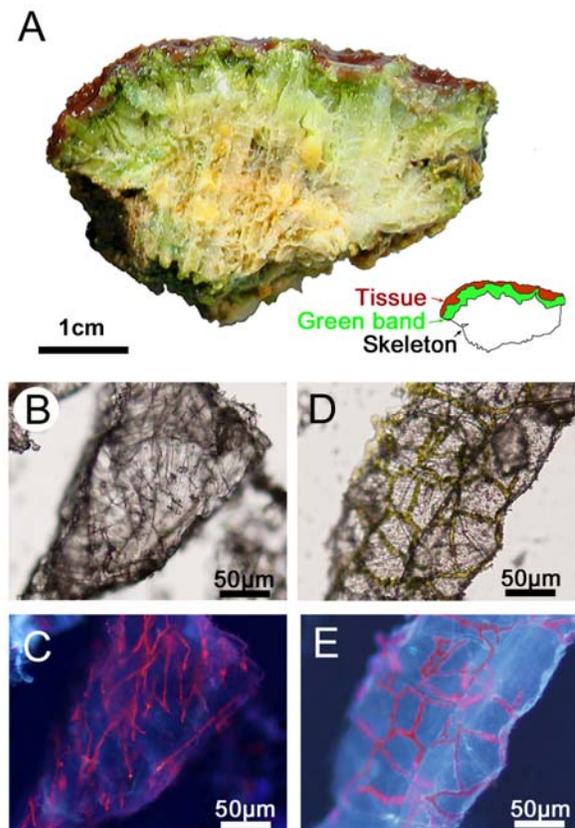


Figure 1: Layer of green band observed within the skeleton of *Goniastrea aspera*. (A) Dissected transverse section of *G. aspera* skeleton, (B and C) Photosynthetic organism found in skeletal fragments, (D and E) *Ostreobium* sp. (C) and (E) are epifluorescence images of (B) and (D), respectively.

*Ostreobium* sp. was observed on the fragments of the coral skeletons. The green alga showed repeatedly branching, non-septating 3  $\mu\text{m}$  diameter filaments having knobby surface irregularities (Fig 1D and E). In addition to *Ostreobium* sp., coccoid photosynthetic organisms with less than 1  $\mu\text{m}$  in diameter were also observed in the suspension of the green band and on the surface of skeletal fragments. These could be

attributed to the deposits during the process of tissue removal (data not shown).

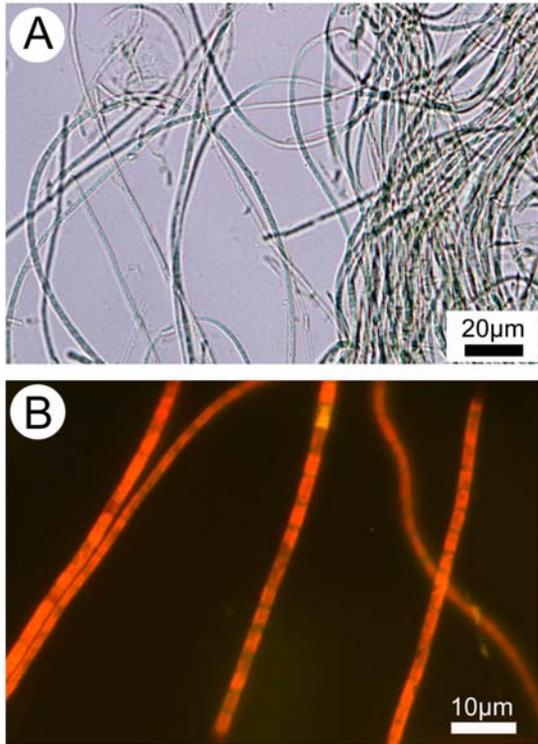


Figure 2: Morphology of the cultured filamentous cyanobacterium isolated from the green band in *G. aspera* skeleton. (A) Light micrograph of the filamentous cyanobacterium (B) Epifluorescence image of the filamentous cyanobacterium.

A cyanobacterium was successfully isolated and cultured with the modified A medium. This cultured cyanobacterium was non-branching, non-heterocyst forming, had narrow filaments around 1 µm in diameter and formed motile hormogonia (Fig 2). All these characteristics can be found in cyanobacteria belonging to the order Oscillatoriales.

Analysis of chlorophyll pigments using TLC revealed that both green band from coral skeleton and the isolated cyanobacterium contained chlorophyll *a* (Fig 3). However, chlorophyll *b* was only found in the extract obtained from the green band in the coral skeleton (Fig 3, lane A). Similar results were obtained with HPLC analysis (data not shown).

A phylogenetic tree constructed by the neighbor joining method is presented in Fig 4. A BLAST homology search of the determined sequence showed the closest identity (94%) to a 16S rDNA region of *Halomicronema excentricum*.

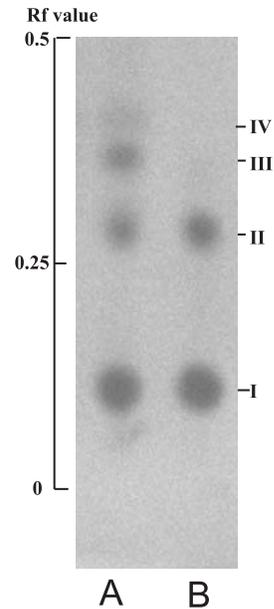


Figure 3: TLC chromatogram of chlorophyll pigments. Lane A, pigments extracted from the green band found in *G. aspera* skeleton. Lane B, pigments extracted from the isolated filamentous cyanobacterium. (I) Pheophytin *a* (II) Chlorophyll *a* (III) Pheophorbide *a* (IV) Chlorophyll *b*.

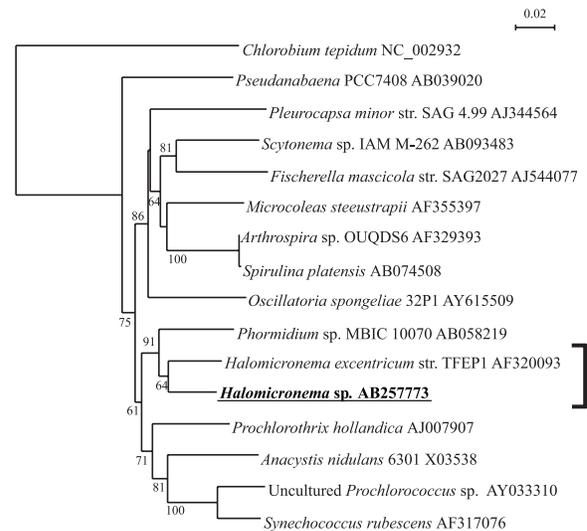


Figure 4: A phylogenetic tree constructed by the neighbor-joining method on the basis of 1029 bases 16S rDNA partial sequence. The sequence of *Chlorobium tepidum* was used as an outgroup. The scale bar represents a 0.05 substitution per nucleotide position. Numbers indicate the percentage of bootstrap support out of 1000 resampling data from the neighbor-joining method.

## Discussion

To the best of our knowledge, this is the first report of a *Halomicronema* sp. inhabiting in the skeleton of a live coral. Species of the genus *Halomicronema* are one of the dominant cyanobacteria found in hypersaline microbial mat. They are characterized moderately halophilic and thermophilic (Abed et al. 2002; Fourçans et al. 2006). The present study

suggests that the internal environments of coral skeletons could be similar to the habitats for hypersaline microbial mats.

Shallow intertidal pools in coral reefs are harsh environments that accompany daily and seasonal changes in salinity and water temperature with large extents. The massive coral *G. aspera* can be dominantly found in such harsh habitats. It is interesting to note that even after the mass bleaching event in 1998, many *G. aspera* survived and increased in abundance around Okinawa Island (Loya et al. 2001). It appears that *G. aspera* is relatively stress tolerant.

Although further studies are needed to clarify the biological functions of the endolithic microbes in *G. aspera*, we consider it plausible that the colonization brings a positive effect on the coral host. In fact, under high-light stress conditions, the *Acropora digitifera* colonies infected by endolithic microbes exhibited a photosynthetic activity higher than that of uninfected ones, a result suggesting a positive contribution of the microbes to the host physiology (Yamazaki et al. 2008). Endolithic microbes within coral skeleton may act as “secondary or facultative symbionts” (Yamazaki et al. 2008).

Endolithic algae found in coral skeletons have been reported to facilitate the host recovery from a bleached condition (Fine et al. 2002). *Halomicronema* was reported to have halophilic and thermophilic characteristics. High temperature and high salinity conditions in a shallow intertidal pool may allow the dominance of *Halomicronema* sp. within *G. aspera* skeleton. The presence of such stress-resistant microbes would contribute to the overall stress tolerance of *G. aspera*.

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#### References

- Abed RMM, Garcia-Pichel F, Hernández-Mariné M (2002) Polyphasic characterization of benthic, moderately halophilic, moderately thermophilic cyanobacteria with very thin trichomes and the proposal of *Halomicronema excentricum* gen. nov., sp. nov. Arch Microbiol 177:361-370
- D'Elia CF, Wiebe WJ (1990) Biogeochemical nutrient cycles in coral reef systems. In Dubinsky Z (ed) Coral reefs: Ecosystems of the world vol 25. Elsevier, Netherlands, pp 49-74
- Durden JE (1902) Endolithic algae as agents in the disintegration of corals. Bull Am Mus Nat. Hist 16:323-332
- Fine M, Loya Y (2002) Endolithic algae: an alternative source of photoassimilates during coral bleaching. Proc R Soc Lond B 269:1205-1210
- Fourçans A, Solé A, Diestra E, Ranchou-Peyruse A, Esteve I, Caumette P, Duran R (2006) Vertical migration of phototrophic bacterial populations in a hypersaline microbial mat from Salins-de-Giraud (Camargue, France). FEMS Microbiol Ecol 57:367-377
- Johannes RE, Wiebe WJ (1970) A method for determination of coral tissue biomass and composition. Limnol Oceanogr 21:540-647
- Kobluk DR, Risk MJ (1977) Rate and nature of infestation of a carbonate substratum by a boring alga. J Exp Mar Biol Ecol 27:107-115
- Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150-163
- Le Campion-Alsumard T, Golubic S, Hutchings P (1995a) Microbial endoliths in skeletons of live and dead corals: *Porites lobata* (Moorea, French Polynesia) Mar Ecol Prog Ser 117:149-157
- Le Campion-Alsumard T, Golubic S, Priess K (1995b) Fungi in corals: symbiosis or disease? Interaction between polyps and fungi causes pearl-like skeleton biomineralization. Mar Ecol Prog Ser 117:137-147
- Lesser MP, Falcón LI, Rodríguez-Román A, Enríquez S, Hoegh-Guldberg O, Iglesias-Prieto R (2007) Nitrogen fixation by symbiotic cyanobacteria provides a source of nitrogen for the scleractinian coral *Montastraea cavernosa*. Mar Ecol Prog Ser 346:143-152
- Loya Y, Sakai K, Yamazato K, Nakano Y, Sambali H, van Woesik R (2001) Coral bleaching: the winners and the losers. Ecol Lett 4:122-131
- Lukas KJ (1974) Two species of the chlorophyte genus *Ostreobium* from skeletons of Atlantic and Caribbean reef corals. J Phycol 10:331-335
- Mitsui A, Cao S (1988) Isolation and culture of marine nitrogen fixing unicellular cyanobacterium, *Synechococcus*. Methods Enzymol 167:105-113
- Rohwer F, Breitbart M, Jara J, Azam F, Knowlton N (2001) Diversity of bacteria associated with the caribbean coral *Montastraea franksi*. Coral Reefs 20:85-91
- Schlichter D, Kampmann H, Conrady S (1997) Trophic potential and photoecology of endolithic algae living within coral skeletons. Mar Ecol 18:299-317
- Shashar N, Stambler N (1992) Endolithic algae within corals – life in an extreme environment. J Exp Mar Biol Ecol 163:277-286
- Tribollet A, Langdon C, Golubic S, Atkinson M (2006) Endolithic microflora are major primary producers in dead carbonate substrates of Hawaiian coral reefs. J Phycol 42:292-303
- Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697-703
- Yamazaki SS, Nakamura T, Yamasaki H (2008) Photoprotective role of endolithic algae colonized in coral skeleton for the host photosynthesis. In Allen JF, Gantt E, Golbeck JH, Osmond B (eds) Photosynthesis energy from the sun: 14<sup>th</sup> International Congress on Photosynthesis. Springer, Heidelberg, pp 1391-1395