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A New Species of Cyanobacterial Symbiont from the Marine Sponge *Chondrilla nucula*

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Abstract

Partial 16s rDNA sequencing results indicate that the unicellular cyanobacterial symbionts of the sponge species *Chondrilla nucula*, from the Mediterranean Sea, and *C. australiensis*, from Australia, are the same, and comprise a unique species. This cyanobacterium belongs to the genus *Synechococcus*, and occurs in the surface tissues of these sponges, as demonstrated with Fluorescent *In Situ* Hybridization (FISH) using a sequence-specific DNA probe, and with Transmission Electron Microscopy (TEM). We propose the name *Candidatus Synechococcus spongiarum* sp. nov. for this symbiont. The cyanobacterial symbiont "*Aphanocapsa feldmannii*" (Fremy), hosted by the marine sponge *Petrosia ficiformis* from the Mediterranean Sea, is a separate, but closely related, species also belonging to the genus *Synechococcus*.

Keywords: Cyanobacteria, sponge, symbiont, *Aphanocapsa*, *Synechococcus*

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1. Introduction

"*Aphanocapsa feldmannii*" was first described by Feldmann (1933) in the sponges *Ircinia variabilis* and *Petrosia ficiformis* (= *P. durae*), however this name has never been validated by the Botanical Code or the Bacteriological Code of Nomenclature. Since this time "*A. feldmannii*"-like symbionts have been reported in the surface tissues of more than 60 sponge species in 13 orders (Diaz, 1996), including *Chondrilla nucula*, from the Mediterranean Sea (Sarà and Liaci, 1964; Gaino et al., 1976). However, the placement of this symbiont in the genus *Aphanocapsa* has been questioned, as it does not resemble other members of this genus (Wilkinson, 1992). In addition, the conspecificity of specimens of this apparently widespread symbiont has not been established. The application of molecular techniques has revealed that morphologically similar cyanobacteria may be only distantly related (Ward et al., 1992), particularly those in the order Chroococcales which have very simple morphology.

The use of many molecular techniques to resolve questions related to the taxonomy of sponge cyanobacterial symbionts has been limited by the inability to culture these microbes. However, recent partial sequencing of 16S rDNA of "*Aphanocapsa feldmannii*"-like symbionts in the sponge species *Aplysina archeria* from the Caribbean (Diaz, 1996), *Aplysina aerophoba* from France and *Theonella swinhoei* from Palau (Hentschel et al., 2002), *Xestospongia muta* from the Caribbean (R. Gomez, University of Amsterdam, pers. com.), and *Theonella swinhoei* (GenBank accession number AF186426, not published) all indicate these symbionts belong to the genus *Synechococcus*.

The aim of this study was to identify "*Aphanocapsa feldmannii*"-like symbionts in *Chondrilla nucula* using partial 16S rDNA sequencing based on cyanobacterial-specific primers, and to confirm the occurrence of the sequenced organism in sponge tissues using Transmission Electron Microscopy observations and Fluorescent *In Situ* Hybridization. The partial 16S rDNA sequences of the cyanobacterial symbionts of *C. australiensis* and *Petrosia ficiformis* were compared to investigate possible conspecificity.

2. Materials and Methods

Specimens of *Petrosia ficiformis* and *Chondrilla nucula* were collected from the Mediterranean Sea (Golfe du Lion, Marseille, France, 43° 15'N, 5° 20'E), and *C. nucula* was also collected from the Ligurian Sea (Golfo di Genova, near Portofino, Italy, 44° 18'N, 9° 12'E), using SCUBA. *Chondrilla australiensis* was collected from Fremantle, Western Australia (32° 03'S, 115° 45'E) and Bateau Bay, New South Wales, Australia (33° 23'S, 151° 29'E). Partial 16S rDNA of

the symbionts of these sponges was amplified directly from tissue excised from fresh sponge or specimens preserved in 80% EtOH or 20% DMSO. The surface of each sample was scrubbed with a small toothbrush and rinsed with ddH_2O to remove surface contamination. Approximately 1 mm^3 was excised from the surface epithelium, and for specimens preserved in 80% EtOH, air dried for two hours. The excised piece was then placed directly into 25 μl of PCR mix. The cyanobacterial-specific primer cya359F (5'-GGGGAATYTTCCGCAATGGG-3') (Nübel et al., 1997) was used in combination with the reverse primer 1459R (5'-GGTAAYGACTTCGGGCRT-3') (Diaz, 1996) to amplify 16S rDNA.

Sequencing was performed on the symbionts from three specimens of *Chondrilla australiensis* from the two locations in Australia, and two specimens of C. *nucula* from the Mediterranean. BigDye Terminator Mix version 3 (Applied Biosystems, USA) was used in sequence reactions, following the manufacturer's instructions, and sequence runs were performed on an ABI automated sequencer. The symbionts of two specimens of *Petrosia ficiformis* were also sequenced. Forward and reverse strands were sequenced independently.

The 16S rDNA sequences of the most closely related organisms were obtained by a search using BLAST, and were included in the phylogenetic analysis. Sequences were aligned with ClustalX (Thompson et al., 1997) using unrestricted data, and corrected manually. Phylogenetic analysis of 16S rDNA sequences were performed with Mr Bayes, with a burn-in value of 50,000 generations out of 500,000 generations, and with Maximum Likelihood, using PAUP 4.0 beta 10 (Swofford, 2002), with 500 replicates. A general time reversible model was used, with gamma-distributed rate variation. Trees were drawn with TreeView (Page, 1996).

A DNA probe specific to the cyanobacteria from *Chondrilla nucula* was designed by analysis of the variable 16S rDNA regions of closely related cyanobacteria. BLAST searches confirmed the specificity of the probe. The probe was Cy5 fluorochrome 5' end-labeled (5'-(Cy5) GCAAGATCGCTACTCTTTGTCCGC-3') and synthesized by Genset, Singapore. The probe was diluted to a working concentration of 25 ng μl^{-1} with ddH_2O.

Chondrilla nucula from Marseille that had been preserved in 80% EtOH for one year was used for Fluorescent *In Situ* Hybridization (FISH), as the cyanobacterial symbionts in the tissues were no longer autofluorescent. The probe was not able to be tested against other species of cyanobacteria due to their strong autofluorescence when fresh or frozen. Approximately 1 cm^3 samples were cut from the sponge and fixed in 4% paraformaldehyde using standard procedures. Thin cross-sections, which included the surface layers of the sponge tissue, were cut by hand using a double-sided razor. Eight sections were cut and processed, five with the probe, and three as negative controls without the probe.

Sections were dehydrated in an EtOH series and air-dried. The cells were permeabilized by incubating in lysozyme (using standard procedures), rinsed with ddH_2O, and the EtOH series repeated. 18 μl of hybridization solution (0.9 M NaCl, 100 mM Tris, pH 7.2, 0.01% SDS) was applied to each section and 2 μl of fluorescently labelled probe added. The sections were incubated at 55°C for 3 hours. Sections were then washed in 500 ml of pre-warmed hybridization solution and washing solution (100 mM Tris, pH 7.2, 0.9 M NaCl) for 20 minutes each at 55°C, rinsed in ddH_2O, and air dried in the dark.

Probed sponge sections were examined with a confocal laser scanning microscope (BioRad MRC 1024), using a 647 nm excitation filter (far-red) and a 680 nm DF 32 emission filter. A series of 112 scans at 1 μm intervals, set to slow scanning speed and using a Kalman filter (three passes per scan), were taken using a 10× objective. A single section was also taken with a 60× objective to visualize individual cyanobacteria.

Fresh sponge biopsies were also examined with fluorescence microscopy and TEM, as described previously (Usher et al., 2001). The dimensions of cyanobacteria were measured using the scale bar on TEM images. To avoid misleading data, only non-dividing symbionts sectioned through the centre and showing a spiral view of the thylakoid were measured. 147 cyanobacterial symbionts were measured from four specimens of *Chondrilla australiensis* from Fremantle, and 13 were measured from one specimen of *C. nucula* from France. An analysis of variance (ANOVA) employing a general linear model with mixed effects was performed on the data using SAS software (USA). This enabled variables from multiple samples to be compared using unequal data sets.

3. Results

The cyanobacterial symbionts of *Chondrilla nucula* and *C. australiensis* were an average size of 0.95 μm by 0.68 μm (standard deviations 0.2 and 0.15, respectively). The differences in the mean dimensions of symbionts from these two sponge species was not significant at the 90% level. The symbionts have a spiral thylakoid around the perimeter of the cell (Fig. 1), and the cell centre was finely granulated, with ribosomes of approximately 22 nm often observed. The width of the lumen space of the thylakoid varied, and some cells were observed with an undulating outermost membrane (Fig. 1), perhaps of host origin. Cells were observed dividing by pinching in the centre. The symbionts of *C. nucula* from the Mediterranean Sea (isolate KU43Fr) are the designated type strain for this species. EtOH preserved samples *C. nucula* and *C. australiensis* have been deposited at the Western Australian Museum (WAM).

The GenBank accession numbers for the cyanobacterial symbionts are as follows: *C. nucula* from France (WAM Z13268T) is AY190186; *C. nucula* from

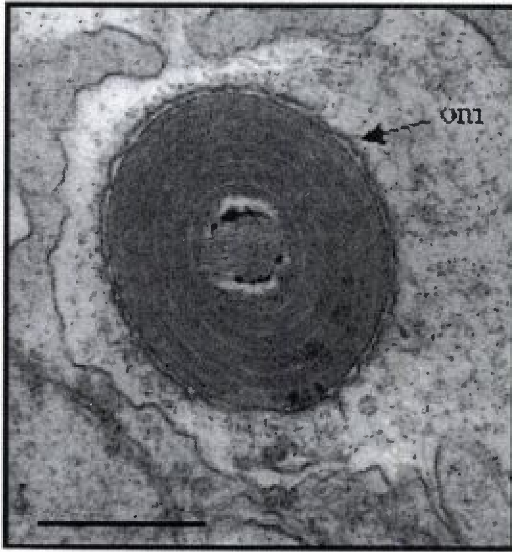

Figure 1. Transmission Electron Micrograph of KU43Fr in *Chondrilla nucula* from Marseille, France. om = outer membrane. Scale bar = 500 nm.

Italy (WAM Z13261) is AY190180; *C. australiensis* (WAM Z13257 from Fremantle, Australia, ochre; WAM Z13255 Fremantle, Australia, maroon; WAM Z13254, Bateau Bay, NSW) are AY190176, AY190177, and AY190178, respectively. The GenBank accession number for the cyanobacterial symbionts of *Petrosia ficiformis* (WAM Z12499) is AY190187.

Partial 16S rDNA sequences 950 and 949 nucleotides long were obtained from the symbionts of *Chondrilla nucula* from the two locations in the Mediterranean. These sequences had 100% sequence similarity to each other, and were treated as the same sequence in phylogenetic analysis. Sequences of 950 and 915 base pairs were obtained from the symbionts of *C. australiensis* at Fremantle and Bateau Bay, respectively. The sequence from Bateau Bay had 100% sequence similarity to the sequences from *C. nucula*, and 99.6% sequence similarity to the symbionts of *C. australiensis* at Fremantle. The two most closely related organisms derived from the BLAST search were the planktonic *Synechococcus* WH 8103 and *Prochlorococcus* NATL 2, which had 94.3% and 95.4% sequence similarity, respectively, to the sequences from *C. nucula*. A 16S rDNA sequence 943 nucleotides long was also obtained from the cyanobacterial symbiont of *Petrosia ficiformis*, which had 95.1% sequence similarity to the *C. nucula* sequences, and 94.6% sequence similarity to the *C. australiensis* symbionts at Fremantle. Phylogenetic trees produced by both MrBayes and Maximum Likelihood revealed four distinct groups of closely related

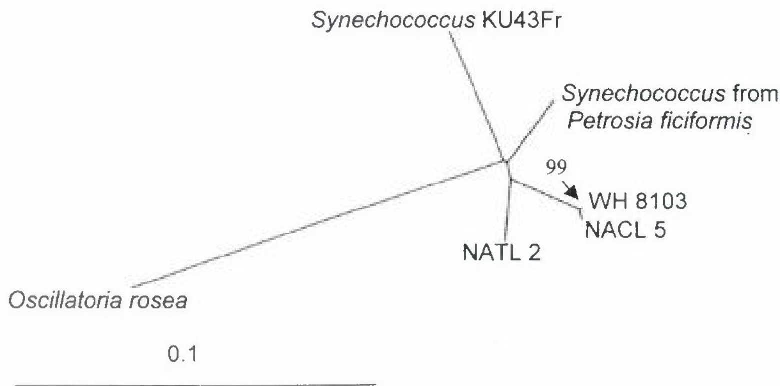

Figure 2. Consensus tree of partial 16S rDNA sequences of symbiotic cyanobacteria, *Synechococcus* KU43Fr and *Synechococcus* from *Petrosia ficiformis*, and sequences from free-living cyanobacterial obtained from GenBank (*Synechococcus* WH 8103, and NACL 5, *Prochlorococcus* NATL 2 and *Oscillatoria rosea* M220), constructed by the MrBayes. Outgroup is *Oscillatoria rosea*. The probability value of the node of the branching point of WH 8103 and NACL 5 is 99%. Other probability values are below 50%. Bar = 0.1 substitutions per site.

cyanobacteria (Fig. 2), with a close relationship between *Synechococcus* KU43Fr and the symbionts of *P. ficiformis*. The bootstrap value for the branching of WH 8103 and NACL 5 was 99%. The bootstrap values of other branching points were below 50%, indicating that the evolutionary relationships between *Synechococcus* KU43FR, the symbionts of *P. ficiformis* and NATL 2 are not well defined. The sequence alignment and phylogenetic tree have been deposited on TreeBASE, submission SN1611.

Autofluorescence microscopy showed that cyanobacterial symbionts in both *Chondrilla* species were present only in the surface tissues, to a depth of about 1 mm. This was confirmed with TEM, which also revealed heterotrophic bacterial symbionts distributed throughout the matrix and surface tissues.

In EtOH preserved *Chondrilla nucula* hybridized with the *Synechococcus* KU43Fr-specific DNA probe, fluorescence with the far-red excitation filter was confined to the surface tissues where fluorescent spots approximately 1 μm in size were observed (Fig. 3). This corresponded to the location and size of cyanobacterial symbionts observed using autofluorescence microscopy and TEM. Heterotrophic bacteria in the sponge matrix did not bind the probe, and canals could be seen where cyanobacterial symbionts did not occur (Fig. 3A). Non-probed negative controls did not fluoresce at these wavelengths.

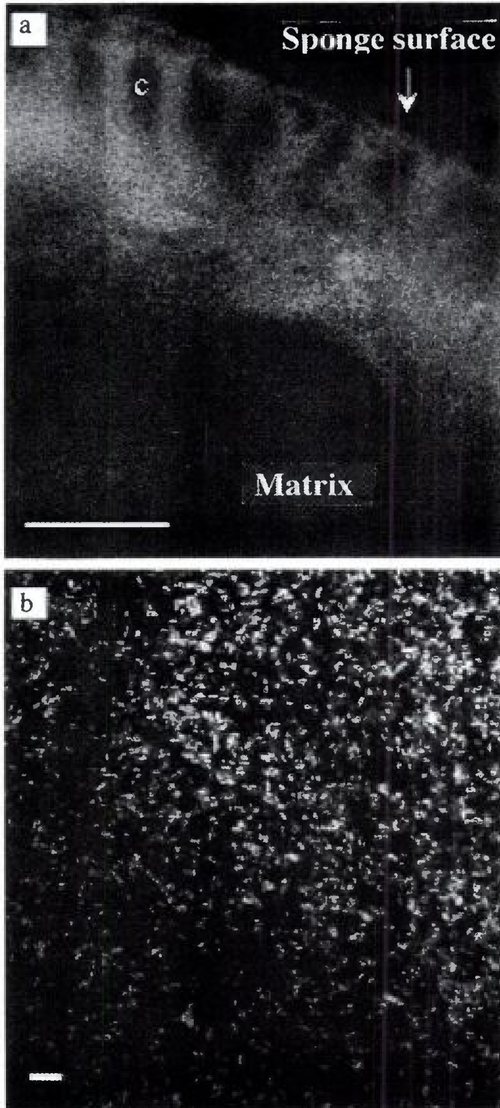

Figure 3. Cross sections of *Chondrilla nucula* after probing with a KU43Fr-specific DNA probe (Cy5 fluorochrome 5' end-labeled). Florescence was visualized with confocal microscopy using far red excitation and a 680 nm DF 32 emission filter. a) An overall view showing the sponge surface, and the matrix of the sponge where cyanobacterial symbionts do not occur. Surface tissues show strong florescence indicating the presence of KU43Fr, but heterotrophic bacteria that occur in the matrix are not fluorescent. Canals (c) leading to the sponge surface can be seen as dark areas where KU43Fr does not occur. Scale bar = 300 μm. b) Individual KU43Fr can be seen in the surface tissues of *Chondrilla nucula* due to probe fluorescence. Scale bar = 10 μm.

4. Discussion

Partial 16S rDNA sequence results indicate that the symbionts of *Chondrilla nucula*, *C. australiensis* and *Petrosia ficiformis*, previously identified as "*Aphanocapsa feldmannii*", belong to *Synechococcus* group 6 (marine cluster A: Honda et al., 1999; Robertson et al., 2001). It therefore appears that "*A. feldmannii*", originally described in *P. ficiformis* (Feldmann, 1933), would be more correctly referred to as "*Synechococcus feldmannii*". The symbionts of *Chondrilla nucula* and *C. australiensis* are the same species. While closely related to the symbionts in *Petrosia ficiformis*, they are considered a separate species as they have less than 97% sequence similarity to the *P. ficiformis* cyanobacteria, and are therefore unlikely to meet the 70% DNA-DNA hybridization criteria of conspecificity (Stackenbrandt and Goebel, 1994; Lyra et al., 2001). In addition, these two symbionts can be distinguished by size and subtle ultrastructural differences (pers. obs.). However, caution should be used in applying morphological characteristics alone to distinguish between different species of *Synechococcus*.

On the basis of a unique 16S rDNA sequence and FISH using a sequence-specific DNA probe, KU43Fr is a new species in the genus *Synechococcus*. We propose the *Candidatus* name *Synechococcus spongiarum* sp. nov., with the symbionts of *C. nucula* from the Mediterranean Sea the designated type strain for this species.

The 16S rDNA sequences obtained for these symbionts have a slightly higher percent similarity with *Prochlorococcus* NATL 2 (95.4%) than with *Synechococcus* WH 8103 (94.3%). However, their strong yellow/orange autofluorescence at blue wavelengths points to high levels of phycoerythrin, a phycobiliprotein that is lacking in *Prochlorococcus* NATL 2 (Urbach et al., 1998), further indicating that this cyanobacterium belongs in the genus *Synechococcus*. In addition, "*Aphanocapsa feldmannii*" have been reported to contain chlorophyll *a*, but not chlorophyll *b* (Larkum et al., 1988; Bavestrello et al., 1992), phycoerythrin and phycocyanin (Larkum et al., 1988). This is consistent with the genus *Synechococcus*, rather than *Prochlorococcus*, which has divinyl chlorophyll *a* and *b* (Chisholm et al., 1992).

Molecular techniques now provide the opportunity to understand the genetic diversity of cyanobacterial symbionts of marine sponges, and their relationships to sponge families and biogeography. This study indicates that more than one species has been referred to as "*Aphanocapsa feldmannii*", that these symbionts co-exist in the same regions, and are more correctly assigned to the genus *Synechococcus*. However, further studies examining cyanobacterial symbionts from different regions of the world are required to determine the extent of the biodiversity represented by these important photosynthetic symbionts.

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