AFFINITIES OF THE *APHANOCAPSA FELDMANNI*-LIKE CYANOBACTERIA FROM THE MARINE SPONGE *XESTOSPONGIA MUTA* BASED ON GENETIC AND MORPHOLOGICAL ANALYSIS

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ABSTRACT

The marine sponge *Xestospongia muta* (Porifera: Demospongiae: Haplosclerida) harbours cyanobacteria in its peripheral tissue that have been described as having an *Aphanocapsa feldmanni*-type appearance. Through subsequent cell fractionation steps we obtained a virtually pure cell suspension of the cyanobacteria. Through amplification of a region of the 16S rRNA gene we found that these cyanobacteria seem closely related to *Prochlorococcus* (Cyanobacteria, *Prochlorophytes, Prochlorococcaceae*), and *Synechococcus* (Cyanobacteria, *Chroococcales, Synechococcus*). We furthermore developed a new method, to obtain a clear signal with FISH by bleaching the auto-fluorescence of cyanobacteria, with osmium tetroxide. The location and morphological characteristics of the cyanobacteria are described by both light and electron microscopy.

KEY WORDS

Xestospongia muta (Porifera), Cyanobacteria, *Aphanocapsa feldmanni*, FISH, 16S rDNA.

INTRODUCTION

Sponges (Porifera) are known to harbour various amounts of bacteria, that may occur either as a heterogeneous population of heterotrophic bacteria and cyanobacteria or frequently as a monospecific population (VACELET & DONADEY, 1977; WILKINSON, 1978). Bacteria have been found extracellularly (SARÀ, 1971), intracellularly (VACELET & DONADEY, 1977) and sometimes intranuclearly (FRIEDRICH *et al.,* 1999). Because the composition of the bacterial species found in the sponge is dissimilar to that in the surrounding seawater (SANTAVY & COLWELL, 1990) it has been suggested that there is a symbiotic relationship between some bacteria and their host (WILKINSON, 1980). Further evidence for this hypothesis is the observation that the same bacterium is found in different sponge species from separate geographic locations. WILKINSON (1983) has postulated that in the case of cyanobacterial symbionts nutrients are transferred to the host either directly as a food source or by nutrient translocation from bacteria to the host.

Xestospongia muta (Porifera: Demospongiae: Haplosclerida), a common sponge in Caribbean reefs, which can be found to a depth of at least 60 m, harbours a heterogeneous population of bacteria in its mesohyl and large quantities of an apparently monospecific population of cyanobacteria in its peripheral tissue. The cyanobacteria have been described as having an *Aphanocapsa feldmanni*-type appearance, *i.e.* (i) unicellular ovoid cells, which measure 1.1 - 3.0 μm by 0.6 - 2.0 μm (SARÀ, 1971; RÜTZLER, 1990), (ii) a spiral thylakoid (SARÀ, 1971; RÜTZLER, 1990; WILKINSON, 1992). Morphologically similar but as yet undefined cyanobacteria have been found in three species of *Xestospongia* (RÜTZLER, 1990) but also in other sponge species from different taxonomic groups and in different geographic regions such as in *Ircinia variabilis* and *Petrosia ficiformis* from the Mediterranean and *Theonella swinhoei* from the North Pacific Ocean and *Aplysina archeri* from the Caribbean (SARÀ, 1971; WILKINSON, 1978, 1983; BEWLEY *et al.,* 1996; DIAZ, 1997; REGOLI *et al.,* 2000).

We report here the genetical characterisation by 16S rRNA gene sequencing and subsequent *in situ* hybridisation of the *A. feldmanni*-like cyanobacteria in *X. muta* (*CyXm*). In general the hybridisation of cyanobacteria by FISH is hampered by their strong auto-fluorescence. Therefore we have developed a protocol in which the auto-fluorescence is repressed/quenched enabling us to visualise the mono labelled oligonucleotide without the necessity of amplification of the signal. To localise the cyanobacteria in the sponge tissue and for morphological characterisation sections for both light microscopy and transmission electron microscopy (TEM) were made.

MATERIAL AND METHODS

Sponge collection and sampling

Specimens of the Caribbean sponge *Xestospongia muta* were collected from the reefs of Curaçao (The Netherlands Antilles) by SCUBA diving from May 1998 until July 1998 and from November 1999 until March 2000. Specimens were carefully cut loose from the reef, placed in plastic sampling bags under water and transported to the laboratory in a cooler. Samples were processed within 2 h after collection for further use.

Morphological analysis

Sponge samples (5 by 5 mm) were prefixed and stored in 1 % formaldehyde and 0.04 % glutaraldehyde buffered with 0.1 M cacodylate buffer (pH 7.4) adjusted to 1070 mosM with NaCl. These low fixative concentrations appeared sufficient for initial preservation of the specimen allowing visualisation of the (cyano) bacteria both for fluorescence and electron microscopy. For thin sectioning the samples were post-fixed in 4 % formaldehyde and 1 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 - 6 h, rinsed five times in PBS (phosphate buffered saline) and placed in 0.1 % or 1 % osmium tetroxide buffered with 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hour, rinsed three times in distilled water and dehydrated by passing the samples through an ethanol series (50, 70, 80, 90, 96 and 100 %), then passed through a 1:1 solution of propylene oxide/ 100 % ethanol and twice in 100 % propylene oxide, 30 min each step. Finally the samples were infiltrated with a 1:1 solution of propylene oxide and Epon overnight, and embedded in Epon which was left to polymerise at a temperature of 60° C for three days. Sections of 2.5 μm (for fluorescence microscopy) or ultra-thin (silver) sections (for transmission electron microscopy) were cut with an ultramicrotome using a diamond knife. The sections were either stained with a solution of 1 % toluidine blue for 1 min, rinsed with distilled water and alcohol and mounted with Entellan (Merck) for examination under the light microscope, or stained with 1 % aqueous uranyl acetate for 30 min and with Reynold's lead citrate for 1 min and placed on grids for

examination under the TEM. Sections were examined either with an Olympus BX-60 fluorescence microscope equipped with a HBO 100W mercury lamp or with a TEM.

Molecular phylogenetic analysis of the cyanobacteria from *Xestospongia muta* (*CyXm*)

DNA extraction, amplification, cloning and sequencing

Pure cyanobacterial cell suspensions were obtained by dissociation of the peripheral tissue (ectosome) of the sponge samples, followed by differential and Percoll gradient centrifugation as described elsewhere (RICHELLE-MAURER *et al.,* 2001, 2002). The peripheral tissue was carefully separated from the inner part of the sponge, cut in small cubes and squeezed through a 100 µm nylon mesh in cold calcium/magnesium-free artificial seawater (CMF, pH 7.4; CZIHAK, 1975) containing 10 mM EDTA. The suspensions were subsequently filtered through a 50 µm nylon mesh to remove cell aggregates and debris. Differential centrifugation of the resulting cell suspensions (1000 g and 5000 g, 20 min), yielded two fractions which were used for Percoll discontinuous gradient (30 % / 75 % in CMF) centrifugation. Almost pure cyanobacterial fractions were recovered from the 30 % / 75 % interface. The cells were preserved in 100 % ethanol. Total DNA was extracted from these fractions using a QIAamp DNA isolation kit (QIAgen Corp.). DNA was amplified with touchdown PCR using the cyanobacterial 16S rRNA gene primers CYA 359f and CYA 781r (NÜBEL *et al.*, 1997). For the PCR mixture a total volume of 50 µl was used containing 1 µl DNA template, 5 U/l super*Taq* polymerase, 5 mol/µl primer and buffer (Boehringer Mannheim). The PCR was run as follows: an initial denaturation step of 3 min at 94° C; 37 cycles with a denaturation step at 94° C for 40 sec, the annealing temperature decreasing by 1° C after each cycle; starting at 60° C and ending at 50° C and then continuing at 50° C, 30 sec each step, extension was at 72° C for 1 min and a final extension step at 72° C for 15 min. The resulting PCR products were checked by gel electrophoresis and purified using a BIO 101 Geneclean II kit (Biogene). The products were then cloned into a pGEM-T vector (Promega pGEM-T Vector System Kit) according to the manufacturer's recommendations and inserted in *Escherichia coli*. DNA was extracted from 16 different clones with a plasmid miniprep according to SAMBROOK *et al.* (1989). These DNA extracts were bidirectionally sequenced by using infrared dye-labelled M13-primers and a Li-Cor automated DNA sequencer. The *CyXm* 16S sequence is deposited in GenBank under accession number AY320038.

Phylogenetic reconstruction

We narrowed down the taxonomic position of the sequenced cyanobacyteria (*CyXm*) with phylogenetic reconstructions of cyanobacterial 16S rDNA gene tree.

We aligned the sequence of *CyXm* to the homologous fragments of 88 representative other cyanobacteria published in GenBank (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/) including multiple sequences of *Synechocystis*, *Aphanocapsa feldmanni* (Pacific and Caribbean isolates from *Aplysina* spp., DIAZ, 1997), *Synechococcus* and *Prochlorococcus*. We performed an alignment with clustalX (JEANNMOUGIN *et al.,* 1998) under default settings and improved it by eye. The *CyXm* 16S sequence alignment to the EMBL database is available upon request. The resulting matrix contained 388 characters of which 80 were excluded because of ambiguous alignment. The relative best fitting substitution model was estimated by MODELTEST 3.06 (POSADA & CRANDALL, 1998) based on the likelihood-ratio-test. Bayesian phylogenetic analyses were performed using MrBayes 2.01 (HUELSENBECK & RONQUIST, 2001) using one cold Metropolis-coupled Markov Chain and three incrementally heated with T=0.2, random starting trees and default starting values for sequence evolution model parameters and prior distributions. We ran each Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) for 500,000 generations with trees sampled every 100th generation and discarded the first 100,000 generations (1000 trees, burn-in). In addition, we performed a minimum evolution (ME) analysis on the estimated maximum-

likelihood model using Paup *4.08b (SWOFFORD, 2000). Minimum evolution trees were bootstrapped with 1000 replicates.

Fluorescence *In Situ* Hybridisation (FISH)

Test organisms

Samples of *Prochlorococcus marinus* PCC9511 and *Synechococcus* sp. PCC7001 were obtained from the Pasteur Culture Collection of Cyanobacteria (PCC, Institut Pasteur, Paris, France)

Oligonucleotide design

CyXm specific oligonucleotides for *in situ* hybridisation were designed from the variable regions of the SSU rRNA gene. Three target sites were selected which are unique for the isolated cyanobacteria according to the CHECK PROBE (RDP) and BLAST (GenBank) database. The cyanobacterial probes CYA 762 and CYA 664 developed by SCHÖNHUBER *et al.* (1999) were altered to match the *CyXm* sequence. These probes were selected because they have shown to hybridise *in situ* and could thus serve as a positive control. Oligonucleotide probes were synthesised by MWG (Biotech, Ebersberg, Germany) and Metabion (Martinsried, Germany) (Tab. I). Oligonucleotide probes were 5' end labelled with Cy3. As positive control the universal bacterial probe EUB338 (AMANN *et al.,* 1990) was used. Bn1253r (HAYGOOD & DAVIDSON, 1997) was used as negative control (Tab. I).

Quenching and permeabilisation

The sponge fractions obtained by differential centrifugation and from Percoll gradients were fixed overnight in 3.7 % formaldehyde/PBS (pH 7.4). After fixation the samples were rinsed 3 times in PBS for 10 min and stored in a 1:1 solution of PBS and 96 % ethanol at –20° C. Before hybridisation the cells were spotted on 2 % AAS (3-aminopropyltriethoxysilane, Sigma) coated object slides and air dried. To permeabilise the cells the object slide was passed through a flame twice. The cyanobacteria in *X. muta* showed strong autofluorescence due to photosynthetic pigments. This hampered the observation of positively labelled cells by FISH. Therefore the auto-fluorescence was quenched by applying osmium tetroxide (osmium tetroxide, Agar Scientific LTD.). A drop of 20 µl 0.5 % osmium tetroxide was placed on the cells and covered with a cover slip. After an incubation period of 60 min the slides were rinsed three times with demineralised water. As cyanobacteria have an outer membrane it is advisable to permeabilise the cells prior to hybridisation. Different proteinase-K concentrations and incubation times were tested. The best results were obtained when the object slides were placed in Coplin jars containing 1 µg of proteinase-K (Boehringer Mannheim) per ml pre-warmed (37° C) 0.1 M Tris-HCl, 50 mM EDTA (pH 8.0) and incubated for 1min at 37° C.

Hybridisation

Before hybridisation the cyanobacteria were dehydrated by passage through an ethanol series (50, 80, and 96 %). Hybridisation buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 8, 0.01 % SDS) and variable amounts of formamide (20, 30, 35, 40 50 and 60 %) containing the probe (5 ng/µl hybridisation buffer) were applied to the slides and incubated at 46° C in a humidity chamber for 4 to 16 h. The slides were removed from the humidity chamber and immersed in 100 ml washing solution (20 mM Tris-HCl, pH 8, 0.01 % SDS) with variable concentrations of NaCl (0.25, 0.125, 0.088, 0.062, 0.031 and 0.016 M NaCl respectively) depending on the formamide concentration used, at 46° C for 20 min. The slides were gently rinsed off with sterile water, air dried and mounted in Citifluor (Citifluor Ltd, London, UK) containing DAPI (4', 6-diamidino-2-phenylindole; 1 µg/ml) and sealed with a cover slip. The slides were viewed with an Olympus BX-60 fluorescence microscope equipped with a HBO 100W mercury lamp

using a 100x Uplan F1 objective (Olympus) and the filter sets: U-MWU (excitation at 330 to 385 nm for DAPI) and the U-MNG (excitation at 530-550 nm for the Cy3 label).

To distinguish between positive label, non-specific binding and auto-fluorescence of the cells hybridisation buffer was applied with or without the probe. The controls were performed with each hybridisation.

Tab. I. Oligonucleotides used in this study.

Probe	Sequence 5' to 3'	Target site (*E. coli* position)	Tm
Control			
Eub 338	GCTGCCTCCCGTACCAGT	38-355	60.5
N-Eub (Bn1253r)	ACGTCACCGTCCAGCCTCT		61
Cyanobacterial			
CYA(c) 664	G**A**AATTCCCTCTGCCCC[1]	664-680	55.2
CYA(c) 762	CGCTCCCCT**G**GCTTTCGTC[1]	762-780	63.1
CYXM 411	CCCAAAGGCCTCCAT	411-425	50.6
CYXM 602	GAGCTCCACGATTTAACAA	602-620	52.4
CYXM 629	AACAGTTTCCATTGCCGTG	629-647	54.5

1 Bold letters indicate the changes made in the original sequence adapted from SCHÖNHUBER *et al.* (1999)

RESULTS

Sectioning

The peripheral tissue of *Xestospongia muta* contains large quantities of unicellular rod-shaped cyanobacteria (2.5 by 1.2 μm). The cyanobacteria are found scattered in the mesohyl either individually or in clumps and some are dividing by binary fission (Fig. 1A). No close association with the sponge cells was observed; however, cyanocytes (Fig. 1B) containing more than 24 cyanobacteria were found. The cyanobacteria (c) have a distinctive spiral thylakoid, which consists of 5 - 6 turns (Fig. 1C).

Phylogenetic analysis of *CyXm*

The amplified 16S rRNA gene resulted in a 388-bp length fragment. The likelihood ratio test in MODELTEST estimated the GTR+G+I model as the most appropriate for this matrix.

Independent from the two applied reconstruction methods (Bayesian and ME) the *CyXm* sequence clusters close to the *Prochlorococcus* and *Synechococcus* spp. sequences and is well supported with the Caribbean *Aphanocapsa feldmanni* sequence isolated from *Aplysina archeri*. The *CyXm* sequence is clearly distant from *Synechocystis* sequences and the *A. feldmanni* sequence isolated from the Pacific *Theonella swinhoei* in this representative cyanobacterial data set (Fig. 2).

However, we hesitate to use these molecular results to assign *CyXm* / Caribbean *Aphanocapsa* to either *Prochlorococcus* or *Synechococcus* as both reconstruction methods used show different patterns regarding this particular question: The Bayesian tree favours a sister-group relationship of *CyXm* / Caribbean *A. feldmanni* to *Synechococcus* spp, (Fig. 2) which is not supported by the ME-tree. This ME-tree (not shown here)

groups *Prochlorococcus*, *Synechococcus*, *CyXm*, / Caribbean *A. feldmanni* in a well supported (BP=99) but polytomous monophyletic clade.

Although the exact phylogenetic position remains unclear, it is clearly evident that *CyXm* and the *A. feldmanni* isolated from *A. archeri* from the Caribbean is related to *Synechococcus* and *Prochlorococcus*, and unrelated to *Synechocystis*.

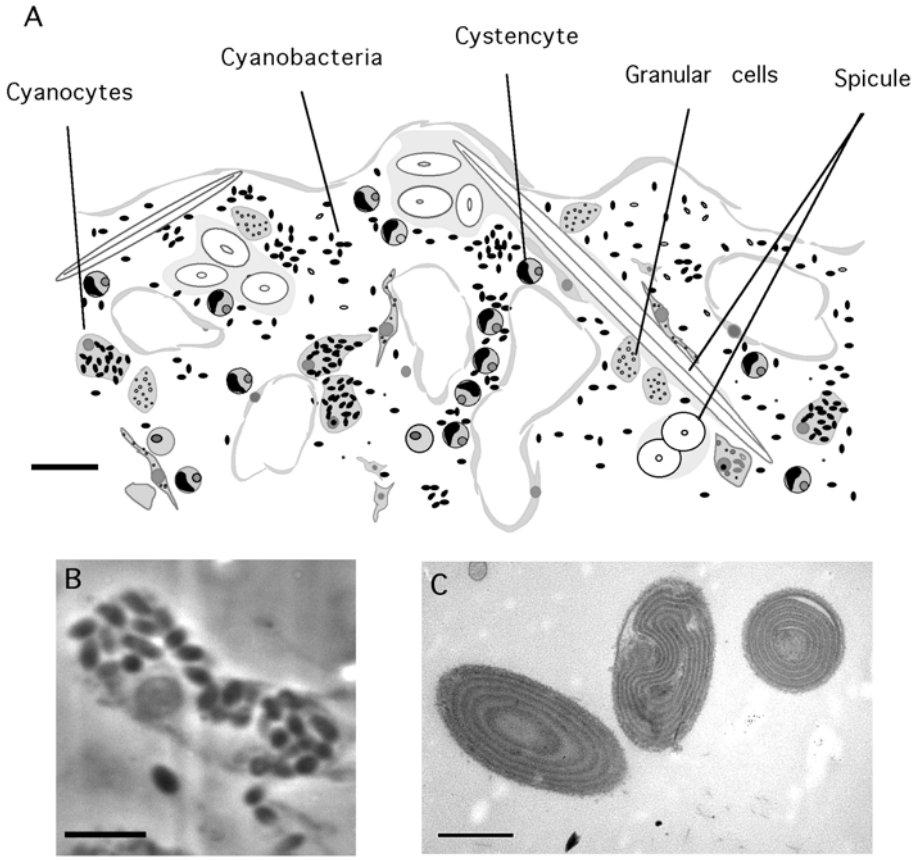

Fig. 1. Microscopy: **A,** Schematic drawing of a cross section through the peripheral tissue of *Xestospongia muta;* Scale bar 10 μm. **B,** Phase-contrast image of a cross section through the peripheral tissue of the sponge. A cyanocyte containing cyanobacteria and a nucleus is visible; scale bar 5 μm. **C,** TEM image of a cross section through three cyanobacteria, showing spirals of the thylakoid. Scale bar 1 μm.

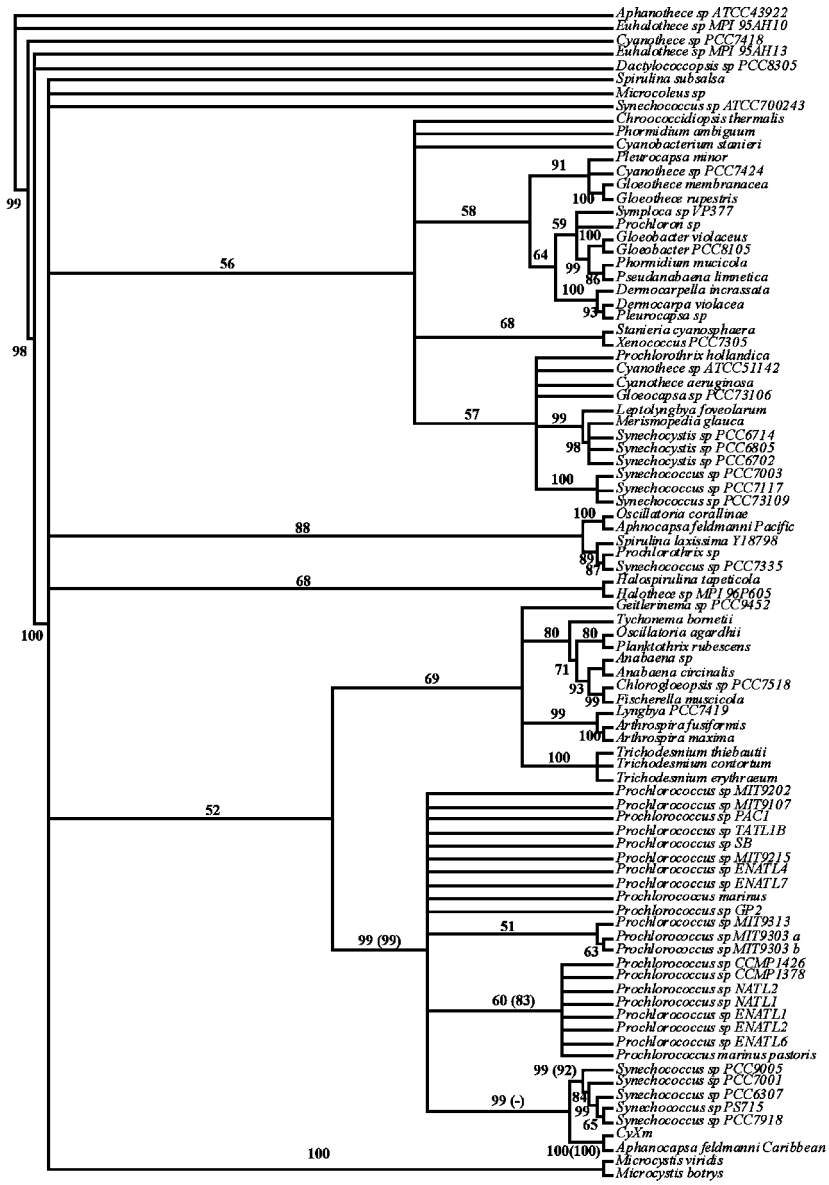

Fig. 2. Bayesian 16SrDNA gene tree of the sequenced *X. muta* cyanobacteria (*CyXm*) and additional cyanobacterial sequences. Numbers at the branches are Bayesian posterior probabilities (shown as percentages). Additional bootstrap support values of minimum evolution analyses based on maximum likelihood distances are given for selected clades of interest for this paper (in brackets). Branches with a support < 50 are collapsed. No outgroup was specified.

Oligonucleotide design

Three different regions were selected on the 16S rDNA to develop *CyXm*-specific oligonucleotides probes for *in situ* hybridisation. Regions on the 16S rDNA that have the most mismatches with *Prochlorococcus* were used as target sites. Probe CYXM 629 was selected as these are targeted on regions, which are specific to *CyXm* and have no match with any *Prochlorococcus* or *Synechococcus* species (BLAST and RDP). Probe CYXM 411 was selected on the basis that this target site is specific to the *CyXm* but also to the symbiont found in *T. swinhoei* (GenBank Accession No. AF186426 and AF186425). Probe CYXM 602 was selected on the basis that it is strictly specific to the *CyXm*. Both have one mismatch with different *Synechococcus* species but not with any *Prochlorococcus* species. Probes CYA 762 and CYA 664 developed by SCHÖNHUBER *et al.* (1999) were altered to match the *CyXm* sequence. Probe CYA (c) 762 targets a region which is specific to *CyXm* and to *Prochlorococcus*, *Synechococcus* species and species in the *Oscillatoria*-group (GenBank and RDP 6/6/01). Probe CYA (c) 664 is completely specific to our cyanobacteria and only matches with chloroplasts of plants but with no other cyanobacteria (GenBank & RDP 6/6/01) (Tab. II).

Tab. II. Specificity of developed probes and FISH results with the different stringency applied on *CyXm* and reference strains.

		10 %	35 %	50 %	65 %
	CYA (c) 664				
	G G G G C A G A G G G A A T T T C				
CyXm	. . . . .	++	++	++	- -
Prochlorococcus PCC 9511	. . . . . C .	++	++	++	- -
Synechococcus PCC 7001	. . . . . C .	++	+ -	- -	- -
	CYA (c) 762				
	A C G A A A G C C A G G G G A G C G				
CyXm	. . . . .	++	++	++	+ -
Prochlorococcus PCC 9511	. . . . .	++	++	++	+ -
Synechococcus PCC 7001	. . . . .	++	- -	- -	- -
	CYXM 411				
	A T G G A G G C C T T T G G G				
CyXm	. . . . .	+ -	- -	- -	
Prochlorococcus PCC 9511	. C . A . . . . . C	+ -	+ -	- -	
Synechococcus PCC 7001	. . . A . . . . . C	- -	- -	- -	

Fluorescent *in situ* hybridisation

We were unable to obtain a positive FISH signal with probes CYXM 602 and CYXM 629 on the *CyXm*. This was also the case when these probes were applied to *Prochlorococcus* and *Synechococcus*. As probe CYXM 602 has only one mismatch with *Synechococcus* it was expected that when using a low stringency (10 % formide) these cells would be labelled. We therefore conclude that the target site selected is not accessible for this specific probe. Probes CYA (c) 664 and CYA (c) 762 did bind to

the cyanobacteria isolated from *X. muta* and to *Prochlorococcus* and *Synechococcus*. CYXM 411 did bind to *CyXm* and *Prochlorococcus* when applying a low stringency (Tab. II).

DISCUSSION AND CONCLUSIONS

Microscopic observations of the peripheral tissue of *Xestospongia muta* confirm the presence of the unicellular rod-shaped cyanobacteria located either inter- or intracellularly. The morphology is consistent with the cyanobacteria described by RÜTZLER (1990), WILKINSON (1992) and SARÀ (1971) as *Aphanocapsa feldmanni*: the cells have a spiral thylakoid (Fig 1C), no sheath, are relatively large (2.5 by 1.2 μm), and divide by binary fission. Although many authors have described morphological characteristics of the *A. feldmanni*-like cyanobacteria in different sponge species, their phylogenetic relationships were unknown until now. This is the first genetic characterisation of the *A. feldmanni*-type cyanobacteria in *X. muta*.

KOMÁREK & ANAGNOSTIDIS (1998) have discussed the possibility that *A. feldmanni* belongs to the genus *Synechocystis*. Formerly known *Aphanocapsa* (PCC6714 and PCC6701) species have been renamed as *Synechocystis* according to data obtained from the Pasteur collection. However according to RIPPKA *et al.* (1979) scheme the *X. muta* cyanobacteria may be better morphologically classified as *Synechococcus*. These unicellular ovoid cyanobacteria, reproduce by binary transverse fission, have a thylakoid and no sheath.

The results obtained from the 16S rRNA gene analysis show that *CyXm* bears phenetic similarities to *A. feldmanni* isolated from *Aplysina archeri* in the Caribbean (DIAZ, 1997). The 16S gene tree further suggests a closer relationship of the *CyXm* / *A. feldmanni* sequences to those of *Synechococcus* and *Prochlorococcus* species than to *Synechocystis* species. Based on these results we have compared the morphological characteristics of *CyXm* with those of *Prochlorococcus marinus* (PCC 9511) and *Synechococcus* sp. *P. marinus* cells are much smaller (0.5 to 0.8 μm long and 0.4 to 0.6 μm wide) compared to *CyXm*. The thylakoid in *P. marinus* has a "horseshoe arrangement" (PARTENSKY *et al.,* 1999; RIPPKA *et al.,* 2000) a weak chlorophyll fluorescence (red). Although *Synechococcus* has a similar spiral thylakoid (CHISHOLM *et al.,* 1988; OLSON *et al.,* 1990) their size is much smaller (0.5 - 1 μm). Some *Synechococcus* species fluoresce bright orange due to their phycobilins. Based on these characteristics and our own observations *CyXm* seems to be more similar to *Synechococcus* species than to *Prochlorococcus*. The sequence data cannot assign *CyXm* unambiguously to *Prochlorococcus* or *Synechococcus*. However, our data do not exclude that *CyXm* shares a common ancestry with the *Synechococcus* lineage. The large genetic diversity in *Synechococcus* is evident in WEST *et al.* (2001). We have to point out that our phylogenetic analysis is based on a considerably short rDNA fragment and additional base pairs are needed to provide robust support.

As there is a small possibility that the DNA extracted and sequenced is not from the cyanobacteria predominantly present in the cyanobacterial cell fraction it was our aim to confirm their presence in the sponge material by FISH using specific probes. Due to the auto-fluorescence of the cyanobacteria detection of the fluorescence hybridisation signal is hampered. Therefore SCHÖNHUBER *et al.* (1999) applied horseradish peroxidase-labelled probes to amplify the probe signal. Although we initially intended to use the same protocol we found that the auto-fluorescence of

CyXm could be repressed with osmium tetroxide enabling us to clearly see the probe signal. This technique was also applied to *Prochlorococcus* and *Synechococcus* cells with the same results; a repressing of the auto-fluorescence and clear probe signal. However, depending on size and auto-fluorescence of cyanobacteria, different percentages of osmium tetroxide need to be applied. Another advantage of this technique is that the auto-fluorescent particles in sponge material, which can also hamper the visualisation of positively labelled cells, is also weakened but does not influence the FISH signal (data not presented).

Unfortunately we were not able to develop a probe specific for *CyXm* with our obtained sequence. Based on our results we think that this is due to the accessibility of the selected target sites on the 16S rRNA molecule. We have found no reference where the selected target site in this study has been successfully hybridised before (WELLER *et al.,* 1991, AMANN, 1995; NELISSEN *et al.,* 1996; NÜBEL *et al.,* 1997; WORDEN *et al.,* 2000; WEST *et al.,* 2001). As we did obtain a clear signal with the probes CYA (c) 664 and CYA (c) 762 in *CyXm* there is sufficient cellular rRNA content and accessibility of the probe through the cell wall (AMANN *et al.,* 1995).

We did not detect any other cyanobacteria in the isolated fraction which could be the origin of the isolated DNA and only one specific sequence from this same fraction was found. The sequence we obtained also matches sequences isolated from cyanobacteria in other sponge species. This is the case for the *A. feldmanni* isolated from the Caribbean sponge *A. archeri* (GenBank Accession number AF497567), and a partial sequence from an uncultured sponge symbiont (cyanobacteria) in *Theonella swinhoei* (GenBank Accession number AF186426) has a 100 % match with the 3' part of this sequence (343-525). Furthermore, K. Usher (personal communication) found that the sequences from cyanobacteria obtained from the sponges *Chondrilla* (three species so far unnamed from Australia, Indian and Pacific Oceans, and *Chondrilla nucula* from the Mediterranean), *Ircinia variabilis*, *Petrosia ficiformis* and *Aplysina aerophoba* from the Mediterranean, and a *Haliclona* species from Australia are all closely related in the *Synechococcus* clade. We conclude that the cyanobacterium we isolated from *X. muta* shares a phylogenetic relationship with *Prochlorococcus* and *Synechococcus* and is found in association with at least one sponge specie from the Caribbean and maybe to other sponge species from different geographic regions.

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