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Molecular phylogeny of nitrogen-fixing unicellular cyanobacteria

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Abstract. Molecular phylogenetic study was conducted using maximum likelihood tree inference methods with small subunit ribosomal RNA sequence data to ascertain the evolutionary relationships among sheathless, single-cell cyanobacteria capable of nitrogen fixation. Cyanobacterial strains of the genus *Cyanothece* (circumscribed by Waterbury and Rippka, 1989) fall into at least three independent lines of descent within a larger assemblage previously designated the SPM sequence group. No strong correlation between aerobic versus anaerobic nitrogen-fixing activity and phylogenetic relationships was observed. The results support a hypothesis of multiple gains and/or losses of nitrogen-fixation abilities among the sheathless, unicellular cyanobacteria.

Keywords: Aerobic; Anaerobic; Cyanobacteria; Nitrogen fixation; Phylogeny; Ribosomal RNA; Sheathless; Small subunit.

Introduction

The unicellular cyanobacteria exhibit a great diversity of physiological properties. Few of them are able to fix nitrogen, either aerobically or anaerobically. Those capable of fixing nitrogen can be grouped into two types: sheathed and sheathless, the former being enclosed in a glycoprotein sheath (glycocalyx, capsule) external to the cell wall (Castenholz and Waterbury, 1989). The first sheathed species was classified in the genus *Gloeocapsa* (Wyatt and Silvey, 1969); however, it was later assigned to the genus *Gloeothece*, based on patterns of cell division (Rippka et al., 1979). On the other hand, the sheathless isolates were primarily attributed to the genus *Synechococcus* (Huang and Chow, 1986; León et al., 1986) or *Aphanothece* (Singh, 1973; Ni et al., 1988). More recently, Reddy et al. (1993) treated their sheathless isolates as members of *Cyanothece*, in agreement with the newer taxonomic criteria proposed by Waterbury and Rippka (1989).

Due to the fact that both botanical and bacteriological criteria have been used by various authorities to classify cyanobacteria, the systematics of these organisms has undergone numerous changes. (For a review of the problematic nature of cyanobacterial systematics, see Turner, 1997). The genus *Cyanothece* was first proposed by Komárek (1976) to accommodate some species previously placed in *Synechococcus*, the major feature distinguishing the former from the latter being that *Cyanothece* are present as single cells or in pairs, but never grouped into chains as are some *Synechococcus*. Subsequently, Waterbury and Rippka (1989) reserved the genus *Cyanothece* for unicellular cyanobacteria that lacked sheaths, divided in a single plane, and were larger than 3 μm in diameter. They also noted that, with one possible exception, the seven strains they originally placed in this genus were able to express nitrogenase activity either aerobically or anaerobically. The physiologies of these two kinds of nitrogen-fixing cyanobacteria are quite distinct. The aerobic type has developed a strategy to protect nitrogenase from oxygen generated by photosynthesis, but the anaerobic type either has not or has subsequently lost the ability to do so.

Because of the unique capability of fixing nitrogen and carbon dioxide within the same cell under aerobic conditions, the sheathless aerobic type has attracted more attention and has been better characterized than the anaerobic type (Mitsui et al., 1986; Huang et al., 1990; Colón-López et al., 1997). Therefore, our knowledge of the anaerobic type is very limited, and more studies are needed to justify whether the accommodation of the above two types of unicellular, nitrogen-fixing cyanobacteria in the same genus are reasonable. In this report, we present a phylogenetic analysis of small ribosomal subunit RNA (SSU rRNA) sequences to evaluate the phylogenetic relationships among sheathless, unicellular cyanobacteria with nitrogen-fixation abilities.

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Materials and Methods

Bacterial Growth and Gene Sequencing

Isolates of *Synechococcus* RF-1 (syn. *Cyanothece* PCC 8801) and *Cyanothece* PCC 7418 were obtained from the laboratory of T.-C. Huang and Institut Pasteur (Paris, France), respectively. They were grown in nitrate-free BG-11 medium (Stanier et al., 1971) supplemented with 0.01M HEPES buffer (pH 8.0). The cultures were incubated in continuous light at 28°C, and the cells were harvested during log phase.

In order to avoid the amplification of pseudogenes or otherwise nonfunctional genes, or any post-transcriptionally processed intervening sequences potentially present, total RNA was extracted from fresh cells using the modified method of Raha et al. (1990) in which genomic DNA was removed by DNase I (Boehringer Mannheim). The method of Goodman and MacDonald (1979) was then used to synthesize the first strand cDNA with the AMV reverse transcriptase (Promega) and the primer 16SR (AGAAAGGAGGTGATCCAGCC) to prime the 3' end. The reverse transcription reaction contained (per µl) 20 ng RNA template, 0.5 U AMV reverse transcriptase, 1 pmol primer, 5 nmole $MgCl_2$ and 1 nmole dNTPs. The reaction was incubated at 42°C for 30 mins. Three independent cDNAs were synthesized and used as templates for PCR amplification following the method of Chaw et al. (1995) except that primer 16SF (AGAGTTTGATCCTGGCTCAG) and 16SR were used. A negative control, in which template was replaced by double distilled water, and an additional control, in which total RNA served as template in the absence of reverse transcriptase, were also run during all PCR amplifications. The former was used to detect any contamination in the PCR components, and the latter to determine if there was any trace of undigested DNA present in the RNA preparation.

PCR products were purified using Promega WizardTM PCR Preps DNA Purification System, and cloned into a pGEM T-Easy vector (Promega, WI). Plasmid DNAs were purified using Qiaprep Spin Miniprep Kit (Qiagen, Hilden, Germany), and sequenced with additional internal primers on an ABI 373 automated sequencer (Applied Biosystem, CA). Independent PCR clones were sequenced using universal (M13, M13 reverse, and/or T7) primers plus additional sequencing primers (530F, TGCCAGCAGCCGC GGTA; 1170F, CGTGTCGTGAGATGTTG; 630R, TTCCGG ATAACGCTTGC; 1100R, GGGTTGCGCTCGTTGC).

The sequences generated in this study have been deposited in the GenBank/ EMBL/DDBJ databases under the accession numbers AF296872 and AF296873.

Phylogenetic Analysis

The SSU rRNA sequences of *Synechococcus* RF-1 and *Cyanothece* PCC 7418 were aligned with others in a cyanobacterial SSU rRNA database maintained by one of us (S. Turner). Initial alignment was done by eye and adjusted as necessary following secondary structure analysis of the new sequences using the structure of *Synechococcus* PCC 6301 as a template (Gutell, 1993). A total of 1,377 unambiguously aligned sequence positions were used in phylogenetic analyses (Turner et al., 1999). A preliminary maximum likelihood analysis (not shown) indicated that both sequences fell within the *Synechocystis/ Pleurocapsa/Microcystis* (SPM) SSU rRNA sequence group as previously defined (Turner, 1997; Turner et al., 1999). This sequence group corresponds to Group 5 of Honda et al. (1999) and Branch G of Wilmotte (1994).

A more refined analysis limited to the SPM sequence group was carried out using all available, approximately full-length, unique SSU rRNA sequences for strains of cyanobacteria known to fall within this group, except that representation of the genus *Microcystis*, for which over 70 full-length sequences are available, was limited to the single suggested type strain *Microcystis aeruginosa* PCC 7941. The taxa and accession numbers of the sequences in this analysis are given in Table 1. The transition/transversion (ti/tv) ratio was estimated using the program TREE-PUZZLE (Version 4.0.1, formerly named PUZZLE) under

Table 1. Taxa and corresponding sequence accession numbers.

Climacodium frauenfeldianum symbiont	AF193247
Cyanobacterium stanieri PCC 7202[a] (syn. *Synechococcus cedrorum* SAG 88.79)	AF132782
Cyanothece ATCC 51142	AF132771
Cyanothece PCC 7418[b]	AF296872
Cyanothece PCC 7424[b]	AF132932
Dactylococcopsis salina PCC 8305[a]	AJ000711
Euhalothece MPI 95AH10	AJ000709
Euhalothece MPI 95AH13	AJ000710
Euhalothece MPI 96N304	AJ000713
Gloeocapsa PCC 73106[b]	AF132784
Gloeothece membranacea PCC 6501[a]	X78680
Halospirulina tapeticola CCC Baja-95 C1.2[a]	Y18791
Halospirulina CCC Baja-95 C1.3	Y18790
Halospirulina MPI S3	Y18789
Halothece MPI 96P605	AJ000724
Merismopedia "*glauca*" B1448-1	X94705
Microcystis aeruginosa PCC 7941[a]	AJ133171
"*Oscillatoria rosea*" IAM M-220	AB003164
Pleurocapsa PCC 7516[b]	X78681
Prochloron didemni	X63141
Spirulina major PCC 6313[a]	X75045
Spirulina "*subsalsa*" IAM M-223	AB003166
Spirulina P7	AF091109
Spirulina CCC Snake P. Y-85	Y18793
Spirulina MPI S4	Y18792
Stanieria cyanosphaera PCC 7437[a]	AF132931
Synechocystis PCC 6803	D64000
Synechococcus PCC 7002	AJ000716
Synechococcus PCC 7003[b]	AB015059
Synechococcus PCC 7117	AB015060
Synechococcus PCC 73109	AB015061
"*Synechococcus*" RF-1 (syn. *Cyanothece* PCC 8801)	AF296872
Xenococcus PCC 7305[b]	AF132783

Epithets in quotes are provisional.

[a]Suggested type strain (Rippka and Herdman, 1992, Nübel et al., 2000).

[b]Suggested reference strain (Rippka and Herdman, 1992).

two models of evolution, the first being the Tamura-Nei model under the assumption of a uniform rate of change among sequence sites, and the second under the assumption that site-to-site variability in evolutionary rate follows a Gamma distribution approximated by 16 rate categories (Tamura and Nei, 1993; Strimmer and von Haeseler, 1996; Strimmer et al., 1997). Settings for the TREE-PUZZLE program were as follows: approximate quartet likelihood, exact parameter estimates, parameters estimated from data set, and parameter estimation uses set to "quartet sampling + NJ tree." Initial maximum likelihood trees were inferred with the program fastDNAml (Versions 1.0 and 1.1. 1a) using the ti/tv ratio estimated by TREE-PUZZLE under a uniform rate of change (ti/tv = 1.39) (Olsen et al., 1994). Trees were inferred 40 times with random sequence addition, localized branch swapping across a maximum of five branches during tree building, and global branch swapping after addition of the last taxon. A total of nine unique topologies were found with log likelihood scores ranging from -9068.16948 (best) to -9071.49465 (worst). Each of these trees was subsequently input into the program DNArates (Version 1.0.3). This program uses the tree topology and branch lengths in conjunction with the aligned sequences to make a maximum likelihood estimate of the evolutionary rate of change for each site in the sequence alignment. The program then parses the sites into a user-defined number of rate categories, in this case 16, the rate of each category being an average of the rates of the individual sites within it (Pracht, S., Overbeek, R., and Olsen, G.J., personal communication). The ti/tv ratio used in this step and subsequent ones was that estimated by TREE-PUZZLE under a model of Gamma-distributed rates (ti/tv = 2.00).

For each set of rate categories estimated from the nine initial trees, phylogenetic trees were inferred de novo using the "categories" option of fastDNAml, the same branch-swapping scheme as before, and multiple random additions of sequences until the best tree had been found at least five times. This step resulted in a total of three tree topologies, two of which had not been found in the first tree-inference step, with log likelihood scores ranging from -7231.59110 to -7237.83215. Rates were calculated de novo using these three trees as input to the DNArates program and trees were subsequently inferred de novo as before, resulting in two new unique topologies with log likelihood scores of -7219.3025 and -7226.46717. A third round of rates determination and tree inference resulted in no change in tree topologies, but slight improvement in log likelihood scores due to further optimization of branch lengths (log likelihood = -7215.47021 and -7221.79242, respectively).

Shortly after this analysis was completed, five new sequences that also fall into the SPM sequence group became available from the public DNA databases (accession numbers Y18789 to Y18793) (Nübel et al., 2000). These were added to the best current phylogenetic tree using the "restart" option of fastDNAml and the previous ti/tv value, rate categories, and branch-swapping scheme. The combined data set of 33 taxa was also reanalyzed with TREE-PUZZLE as before again assuming 16 Gamma-distributed rate categories. The newly estimated ti/tv ratio (2.28) was then used with the new tree as input to DNArates, and these latest rate categories along with the new ti/tv ratio were used to infer a 33 taxon tree de novo as before.

Confidence values for groupings of the final, best tree were determined by using it in conjunction with fastDNAml with the "global," "restart," and "keep" options. Based on log likelihood scores, the best 1000 trees out of 3540 topologies examined were retained and used as input to the program TreeCons (version 1.0) (Jermiin et al., 1997). Of these, 480 were found not to be significantly different from the best tree at the 1% significance level using the Kishino-Hasegawa test (Kishino and Hasegawa, 1989). These 481 trees were used to compute a majority-rule consensus tree with associated relative likelihood support (RLS) scores under a standardized, exponential weighting scheme (Class V in the TreeCons program). For presentation purposes, trees were graphically modified using the programs TreeView (Version 1.5) (Page, 1996) and MacClade (Version 3.05) (Maddison and Maddison, 1992).

Results and Discussion

Sequences that were generated by the RT-PCR method were of the same length as those of other cyanobacteria generated by DNA-based PCR in other studies. In the case of *Cyanothece* strain PCC 7418, the sequence is essentially identical to that generated by PCR amplification of genomic DNA (accession AJ000708) (Garcia-Pichel et al., 1998). From these results, we conclude that neither post-transcriptional modification, such as the excision of transcribed intragenic intervening sequences, nor the fidelity of RT-PCR is an issue in this study.

The optimal unrooted maximum likelihood tree is shown as a phylogram in Figure 1 with RLS scores indicated for the internal branches. The same tree is shown as a cladogram in Figure 2. Branches with RLS <90% are considered to be poorly supported and are truncated in Figure 2 (Turner et al., 1999). For purposes of discussion, RLS scores ranging from 90% to 95% will be considered indicative of weak support, from 95% to 98% indicative of moderate support, and 98% to 100% indicative of strong support.

Of the two taxa examined in this study, *Cyanothece* PCC 7418 falls within a clade of halophilic cyanobacteria containing strains assigned to the genera *Dactylococcopsis* and *Euhalothece*, a grouping that receives strong support (RLS = 100%). In particular, *Cyanothece* PCC 7418 appears most closely related to *Dactylococcopsis salina* PCC 8305 with moderate support (RLS = 97.6%). *Halothece* MPI 96P605 is the sister taxon to this clade, also with strong support (RLS = 98.5%). These results are in agreement with those of an earlier study (Garcia-Pichel et al., 1998).

Synechococcus RF-1 groups with weak support with two other cyanobacteria, *Cyanothece* ATCC 51142 and an unidentified unicellular endosymbiont of the marine diatom

Climacodium frauenfeldianum (RLS = 94.9%). The association of strains RF-1 and ATCC 51142 is in agreement with their highly similar ultrastructures, particularly the presence of polymorphic bodies (inclusion granules) throughout the cytoplasm under aerobic nitrogen-fixing conditions (Chou and Huang, 1991; Reddy et al., 1993).

Strain RF-1 has been deposited in the Pasteur Culture Collection of cyanobacteria, where it has been given the designation *Cyanothece* PCC 8801 on the basis of phenotypic criteria. However, the results shown in Figures 1 and 2 indicate that those strains assigned to the genus *Cyanothece* are polyphyletic with four strains (including RF-1) falling into three distinct sequence clusters containing strains assigned to other genera. Besides *Cyanothece* PCC 7418 falling within the *Euhalothece/Dactylococcopsis* cluster and strain RF-1 grouping with *Cyanothece* ATCC 51142 and the *Climacodium* symbiont, *Cyanothece* PCC 7424 is related to *Gloeothece membranacea* PCC 6501 with strong support (RLS = 98.1%). This result suggests that strain PCC 7424 may be a sheathless

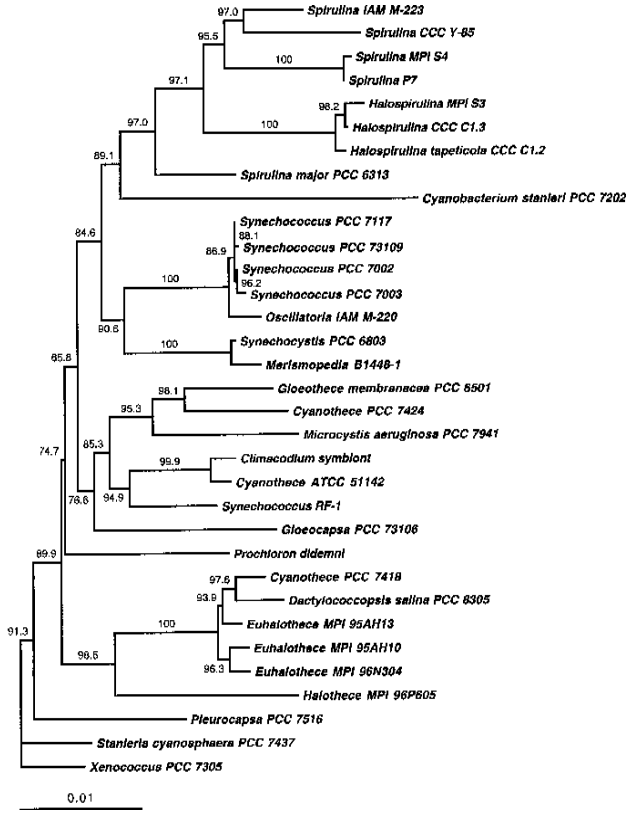

Figure 1. Unrooted phylogenetic tree of the SPM sequence group of cyanobacteria. The tree was inferred from SSU rRNA sequences by maximum likelihood analysis with correction for site-to-site variation in evolutionary rates. RLS scores are given above, below, or alongside their corresponding internal branches. Species epithets are limited to type strains; complete names are given in Table 1. The scale for horizontal branch lengths corresponds to the number of fixed point mutations per sequence position. For further details, see text.

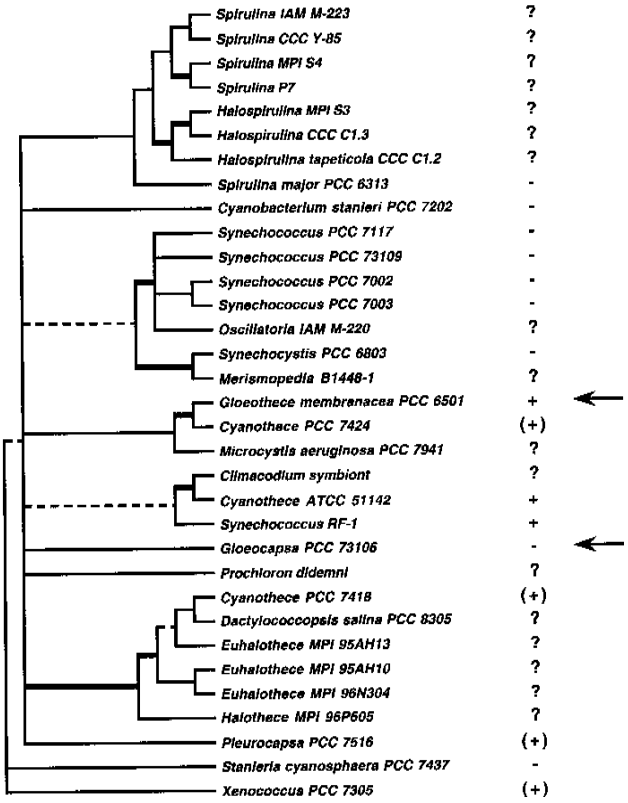

Figure 2. Unrooted phylogenetic tree as in Figure 1 but represented as a cladogram with truncation of internal branches having less than 90% RLS scores to yield polytomies. Support for internal branches is indicated by a thick horizontal line for strong support (98% ≤ RLS ≤ 100%), by a thin horizontal line for moderate support (95% ≤ RLS < 98%), and by a broken line for weak support (90% ≤ RLS < 95%). Arrows point to the strains with prominent sheaths. Nitrogenase activity is indicated as follows: +, aerobic activity; (+) anaerobic activity; -, no activity; ?, no information. Data on nitrogenase activity are from Reddy et al. (1993), Rippka et al. (1979), and Waterbury and Rippka (1989). For further details, see Figure 1 legend.

variant of *Gloeothece*, a possibility raised previously (Rippka and Cohen-Bazire, 1983). This pair groups in turn with *Microcystis aeruginosa* PCC 7941 with moderate support (RLS = 95.3%).

Irrespective of the polyphyly of the genus *Cyanothece*, strain RF-1 is clearly not a close relative of other strains in the SPM sequence group that have been assigned to the genus *Synechococcus*, specifically PCC strains 7002, 7003, 7117, and 73109. These four strains appear to be closely related to one another and with the filamentous cyanobacterium *Oscillatoria* IAM M-220 (RLS = 100%). In the PCC Catalogue of Strains, they have been assigned on the basis of phenotypic properties to *Synechococcus* Cluster 3, composed mostly of marine *Synechococcus*, and which generally corresponds to Marine-cluster C of Waterbury and Rippka (1989). Moreover, as indicated in Figure 2, these strains of *Synechococcus* are negative for nitrogenase activity.

Cyanobacterium stanieri PCC 7202, another strain originally assigned to the genus *Synechococcus* (Cluster 4), also appears to be unrelated to RF-1 or to the Cluster 3 strains of *Synechococcus*. This contradicts an earlier proposal that strains RF-1 and ATCC 51142 may be related to strain PCC 7202 (cf. *Synechococcus cedrorum* SAG 88.79) based on ultrastructural similarities, specifically the distribution pattern of thylakoids within the cytoplasm (Komárek and Cepák, 1998).

In a recent microscopic and ultrastructural study of cyanobacteria assigned to the genus *Cyanothece* sensu Komárek, *C. aeruginosa* SAG 87.79 was selected as an exemplar for this species and, by extension, the genus (Komárek and Cepák, 1998). For this strain, the cellular distribution of both thylakoids and DNA, as well as other features, are distinctly different from the other strains assigned to *Cyanothece* discussed in the work presented here. At present, there are no DNA sequence data for strain SAG 87.79, so the extent of its molecular phylogenetic relationship with these other strains is unknown. However, given the generally good correlation between ultrastructural and phylogenetic differences, it is unlikely to be closely related to any of them. In conclusion, no strong correlation between aerobic versus anaerobic nitrogenase activity and phylogenetic relationships was observed. The results support a hypothesis of multiple gains and/or losses of nitrogen-fixation abilities among the unicellular cyanobacteria. Future investigations are necessary to further illuminate the similarities and differences among the unicellular, nitrogen-fixing cyanobacteria in order to better understand the relationship between their phenotypes and genotypes.

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固氮單細胞藍綠藻的分子親緣

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我們利用最大可能法的親緣演繹方法分析藍綠藻的次小單位核醣體 RNA 序列，以確認無鞘的、能固氮的單細胞藍綠藻間之演化關係。發現 *Cyanothece* 屬【依據 Waterbury and Rippka (1989) 之界定】的藍綠藻品系可分為至少三種獨立演化的血緣路線，他們都歸在一個較大的聚集內 — 即以前所鑑定出的 SPM 序列群。需氧對厭氧的固氮活性和親緣關係間並無強烈的相關。以上結果支持單細胞藍綠藻的固氮能力有多次的得與（或）失之假說。

關鍵詞：藍綠藻；無鞘的；固氮；需氧；厭氧；親緣關係；次小單位；核醣體RNA。