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Evidence on the Presence of tRNA^{fMet} Group I Intron in the Marine Cyanobacterium *Synechococcus elongatus*

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Self-splicing group I introns in tRNA anticodon loops have been found in diverse groups of bacteria. In this work, we identified tRNA^{fMet} group I introns in six strains of marine *Synechococcus elongatus*. Introns with sizes around 280 bp were consistently obtained in all the strains tested. In a phylogenetic analysis using the nucleotide sequence determined in this study with other cyanobacterial tRNA^{fMet} and tRNA^{Leu} intron sequences, the *Synechococcus* sequence was grouped together with the sequences from other unicellular cyanobacterial strains. Interestingly, the phylogenetic tree inferred from the intronic sequences clearly separates the different tRNA introns, suggesting that each family has its own evolutionary history.

Keywords: Group I intron, marine cyanobacteria, *Synechococcus elongatus*, tRNA^{fMet}

Cyanobacteria represent one of the major eubacterial groups. They are unique among the prokaryotes in possessing the capacity of oxygenic photosynthesis. In addition, some cyanobacteria also have the capacity of fixing atmospheric nitrogen. These qualities make cyanobacteria the most successful and widespread group among the prokaryotes, dominating other organisms to form blooms in various aquatic environments [1, 32]. *Synechococcus* is one member genus belonging under the order Chroococcales, being characterized as a unicellular, rod-shaped to coccoid organism, less than 3 μm in diameter, with division by binary fission into equal halves in one plane, occupying an important position at the base of the marine food web: they are among the most abundant members of the picoplankton in the open ocean, and their contribution to primary production has been estimated to be 5% to 30% [27]. Marine *Synechococcus* species possess a number of unique biological properties not found in any other cyanobacterial group. These include the ability of some strains to swim by a novel mechanism [28], the ability to acquire major nutrients and trace metals at the submicromolar concentrations found in the oligotrophic ocean, and the ability to synthesize unique photosynthetic pigments [2, 17].

The *Synechococcus* group has been divided into six clusters based mainly on the habitat (marine or freshwater) and the mole percentage G+C content of DNA, named the *Cyanobacterium* cluster, the *Cyanobium* cluster, the *Synechococcus* cluster, and marine clusters A, B, and C [26]. Furthermore, unlike *Synechococcus* species isolated from brackish waters or coastal areas, these oceanic *Synechococcus* species are obligately marine, having elevated growth requirements not only for Na^+ but also for Cl^-, Mg^{2+}, and Ca^{2+} [27]. The unique properties of this group of marine cyanobacteria have been the subject of a number of physiological and biochemical studies, including studies of motility and chemotaxis [27, 29, 30], growth and nutrient dynamics [9, 13], and photosynthesis [10, 31]. Furthermore, these organisms have become the subjects of a number of molecular biological analyses [reviewed in 5, 12].

Self-splicing group I introns in tRNA anticodon loops have been found in diverse bacteria; *i.e.*, in the β-purple bacterium *Azoarcus* sp. tRNA^{Ile} (CAU), the α-purple bacterium *Agrobacterium tumefaciens* tRNA^{Arg} (CCU) [18], and in cyanobacteria tRNA^{Leu} (UAA) and tRNA^{fMet} (CAU) [3, 4, 7, 18, 21, 22]. The tRNA^{Leu} (UAA) intron represents the most likely example of an ancient intron, because a similar intron is known to be present in the corresponding gene of many plastids at the same position as those recently discovered in some cyanobacteria. Since the plastids arose from a cyanobacterial endosymbiont [6], it has been suggested that this intron was inherited from a common ancestor. The intron inserted in the tRNA^{fMet} genes was found in only 8 of 33 cyanobacteria, five of which did not have the tRNA^{Leu} intron [3, 18]. On the basis of the observation that one of the tRNA^{fMet} introns contains an open reading frame (ORF), which presumably assists the

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intron in its own mobility, and the absence of correlation between relatedness of the intron sequences and relatedness of the rRNAs of the three strains in which they were found, Biniszkiewicz *et al.* [3] suggested that the $tRNA^{fMet}$ intron arose recently in cyanobacteria by horizontal transfer. Earlier studies showed the absence of $tRNA^{fMet}$ genes in the tested *Synechococcus* strain R2 [3, 18]. The aim of this study was to determine the presence or absence of $tRNA^{fMet}$ group I introns in marine *Synechococcus elongatus* strains available at the germplasm of the National Facility for Marine Cyanobacteria, Bharathidasan University, Tiruchirappalli, India.

Axenic culture of *Synechococcus elongatus* strains BDU 30312, BDU 70542, BDU 130912, BDU 140431, BDU 141741, and BDU 142311 were obtained from the germplasm of the National Facility for Marine Cyanobacteria (NFMC), a publicly accessible culture collection center set up by the Department of Biotechnology, Government of India at Bharathidasan University, Tiruchirappalli, India. All strains were identified morphologically at the species level. All *S. elongatus* strains shared common morphological and biochemical traits and had been previously characterized [24]. The cultures were grown in constant light intensity (50 $\mu E\ m^{-2}\ S^{-1}$) for up to 7 days at 25°C in ASN III medium [20]. Total genomic DNA extractions were done as described previously [16].

Primers SM1 (5'-GCGGGGTAGAGCAGCCTGGTAC-CTCGTCGGG-3') and SM2 (5'-GCGGGATCCGGATTT-GAACCACCGACCTTCGGG-3') [3] were used to amplify $tRNA^{fMet}$. All PCR reactions were carried out in a 50-µl volume containing 1 µl (50 pmol) of each primer, 1 µl of 1.25 mM dNTPs, 1 µl (50 ng) of cyanobacterial DNA, and 1 U of DyNAzyme DNA polymerase (Finnzymes, Espoo, Finland). The buffer supplied with the enzyme was used according to the manufacturer's directions. The amplification was performed with a DNA thermal cycler (Eppendorf Matercycler Gradient, Germany) with the following parameters: one cycle at 94°C for 5 min, 30 cycles at 92°C for 1 min, 52°C for 1 min, and 72°C, 2 min, followed by a single final extension step at 72°C for 10 min. After the reaction was completed, 10 µl of amplified DNA was separated on 1.5% low melting agarose (Sigma, U.S.A.), stained with ethidium bromide, and recorded using a CCD camera in an Alpha Imager (Alpha Innotech, U.S.A.). A ready-to-use DNA size standard supplied with the DyNAzyme II DNA polymerase kit (Finnzymes, Espoo, Finland) was included. One of the tested strain *Synechococcus elongatus* BDU 142311 PCR product was purified using the Quiaquick PCR purification kit (Qiagen, Valencia, CA, U.S.A.), cloned into the pGEM-T vector (Promega), and transformed into competent *E. coli* JM109 cells. Sequencing reaction was performed with selected clones by using universal sequencing primers (T7 and SP6). The sequence of the PCR product was determined by using the BigDye Terminator Cycle Sequencing v2.0 kit on an ABI 310 automatic DNA sequencer (Applied Biosystems, CA, U.S.A.).

The new $tRNA^{fMet}$ sequence was multiple-aligned using CLUSTAL W, version 1.7 [25], with a selection of published $tRNA^{Leu}$ and $tRNA^{fMet}$ cyanobacterial sequences. The alignment was corrected manually and a distance matrix was calculated from the Kimura two-parameter model [11] and the tree was built by a neighbor-joining (NJ) algorithm [23] by using MEGA 3.1 [15]. The statistical significance of the tree branches was assessed by bootstrap analysis involving the construction of 1,000 trees from resampled data [8]. Nucleotide sequence generated for this study has been deposited in GenBank under the accession no. DQ080029.

The characterization of $tRNA^{fMet}$ group I introns in marine *Synechococcus elongatus* strains was done by screening for the presence or absence of introns by PCR. The PCR primer pair (SM1 and SM2) complementary to the $tRNA^{fMet}$ (CAU) gene generated PCR products of ~280 bp in size (Fig. 1) in all the strains tested. No size variation among the tested strains was observed. Amplification of an uninterrupted gene should give a product of ~70 to 85 bp, whereas an intron-containing tRNA gene is expected to produce a fragment of 275 to 365 bp. Products longer than 400 bp could indicate an ORF-containing intron [3, 18]. In addition to the eight strains from which the $tRNA^{fMet}$ intron was already characterized [3, 18], it was found in all the six strains of marine *Synechococcus elongatus* tested. Previously characterized *Synechococcus* strain R2 (also known as PCC7942 or *Anacystis nidulans* R2) [14] resulted in a single band of ~70 bp, confirming the absence of

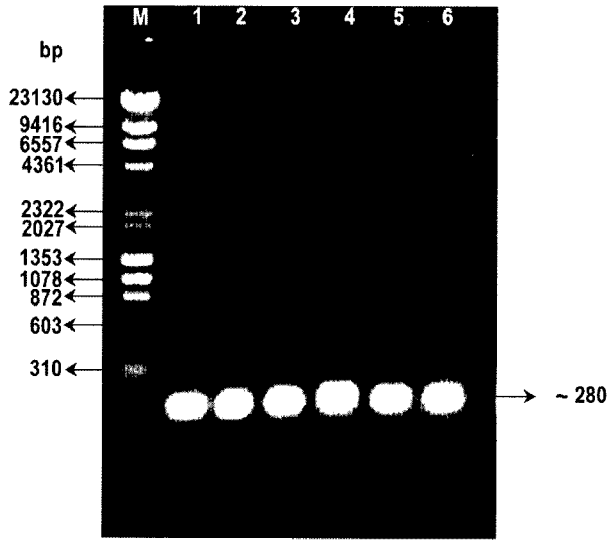

Fig. 1. PCR amplification products of cyanobacterial $tRNA^{fMet}$ genes.
Lanes 1 to 6 are *Synechococcus elongatus* strains BDU 142311, BDU 30312, BDU 140431, BDU 141741, BDU 70542, and BDU 130912 respectively. Lane M, Molecular weight marker (Finnzymes).

tRNA^{fMet} intron. Most of the bacterial introns do not contain an ORF, the only exceptions being two closely related *Synechocystis* isolates (PCC 6803 and PCC 6906) that have an ORF in their tRNA^{fMet} introns. As these ORFs do not resemble any proteins of known function in sequence databases, the role of homing endonucleases in the mobility of bacterial introns has remained speculative. Recently, it was demonstrated that the protein encoded in the tRNA^{fMet} intron from *Synechocystis* PCC 6803 displays a specific double-strand DNA endonuclease activity typical of an intron-encoded homing endonuclease [4].

The tRNA^{fMet} intron was sequenced from *Synechococcus elongatus* BDU 142311 and the sequence has been deposited in the GenBank database with the accession number DQ080029. An extended alignment of the intron sequences was produced by adding tRNA^{Leu} introns, tRNA^{fMet} introns from cyanobacteria, as well as the tRNA^{Arg} intron from *Anaplasma marginale* and the tRNA^{Ile} intron from *Azoarcus* [19]. The branches separating the different intron families are well supported. Phylogenetic reconstruction strongly suggests clustering of the *S. elongatus* with other unicellular cyanobacteria *Synechococcus* sp., *Prochlorococcus marinus* and *Gloeothece* sp. (Fig. 2). The tRNA^{fMet} intron subtree is relatively well supported in bootstrap analysis (bootstrap values between 56% and 98%) and consisted of *Oscillatoria williamsii*, *Scytonema hofmanii*, *Cylindrospermum*, *Dermocarpa*, and *Oscillatoria* sp. The topology of the intron tree (Fig. 2) is in general agreement with the tree inferred by other groups [18, 21]. However, a major discrepancy was found upon comparison of the tRNA^{fMet} intron subtree and SSU rRNA tree [3, 18].

There are different evolutionary patterns for group I introns located in the distinct clustered groups. It has been suggested that the evolution of tRNA^{Leu} group I introns is fundamentally different from that of tRNA^{fMet} introns. The tRNA^{Leu} introns were anticipated to be stable and of ancient origin, whereas the tRNA^{fMet} introns were suggested as recent invaders of the genomes [3, 18]. An argument for the intron stability view for tRNA^{Leu} introns is that the cyanobacterial ancestor of chloroplasts, engulfed by an

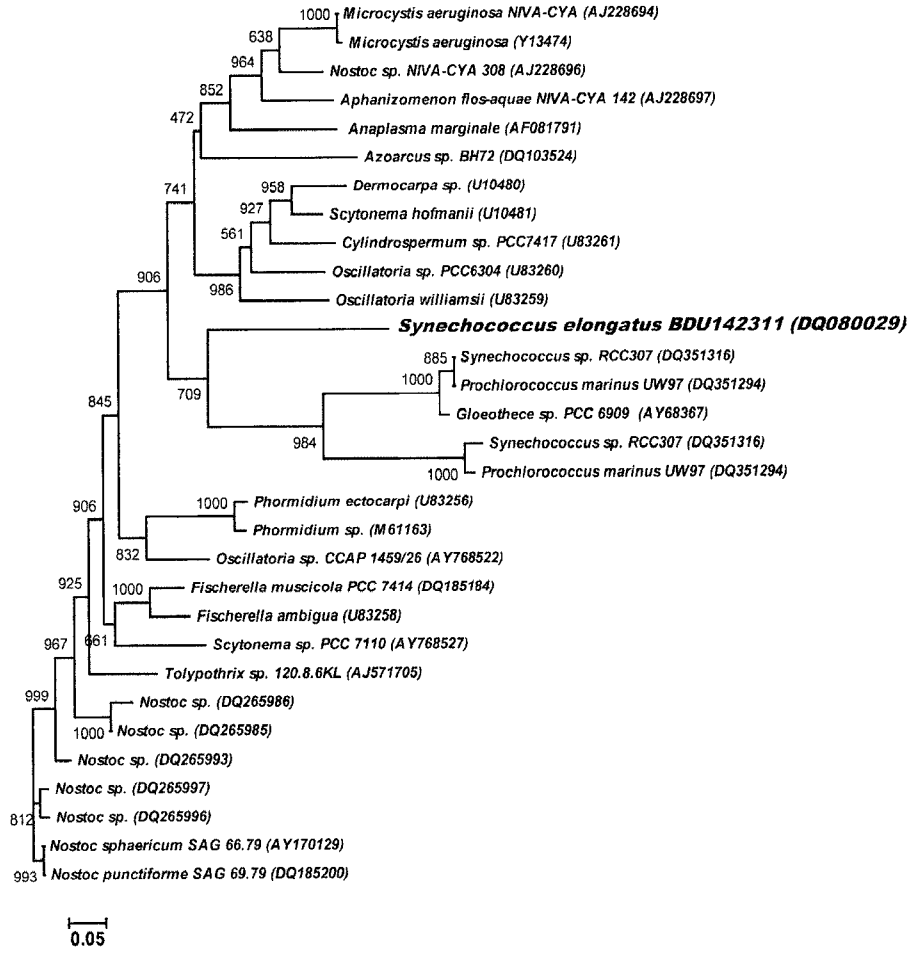

Fig. 2. Phylogenetic reconstruction of tRNA intron sequences.
Comparison of the different eubacterial tRNA genes containing group I introns. The tree was built with the neighbor-joining method, and a bootstrap analysis was performed. Numbers at the nodes indicate the fraction of the 1,000 bootstrap trees. The strain of which the tRNA^{fMet} genes were determined in this study is indicated in bold. Accession numbers in DDBJ, EMBL, and GenBank databases are in parentheses.

eukaryote more than 1 billion years ago, contained a $tRNA^{Leu}$ and not a $tRNA^{fMet}$ intron. There is fairly good evidence for a monophyletic origin of chloroplasts. Thus, the presence of $tRNA^{Leu}$ introns and the absence of the intron in $tRNA^{fMet}$ genes in chloroplasts may simply be explained by that the chloroplast ancestor coincidently contained a $tRNA^{Leu}$ gene with an intron and a $tRNA^{fMet}$ gene without an intron, as seen for many of the current cyanobacterial species. It was suggested that an evolutionary model involving both lateral transfer and differential loss of the intron should be considered [21].

This study is the first step in a more extensive survey of the presence or absence of $tRNA^{fMet}$ introns in cyanobacteria. Our data suggest the possible presence of $tRNA^{fMet}$ introns in other cyanobacterial phyla. However, experimental data demonstrating the properties of the introns are probably needed to resolve the intron stability-versus-intron mobility controversy.

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