CHAPTER 4

Molecular Identification of Cyanobacteria using 16s rRNA Sequence Analysis
4.1 INTRODUCTION

The art of classification is known as taxonomy and phylogeny helps to draw evolutionary relationships between the organisms classified. So a phylogenetic system of classification serves a better purpose for classification as well as inferring evolutionary relationships. The earliest taxonomic treatises considered cyanobacteria as an algal group under the general name blue-green algae. Traditionally thus, the blue-green algae have been classified along with eukaryotic algae under the International Code of Botanical Nomenclature (ICBN). As per rules of ICBN, a type species has to be deposited in a recognized Herbarium either in a dried state or fixed algal material. Due to the limitation in the availability of pure culture strains and the paucity of information on their biochemical and genetic features, the earlier workers depended mostly on morphological characteristics.

Due to the absence of membrane-bound cell organelles, the blue-green algae were considered closer to bacteria (Cohn, 1872, 1875). Based on the recommendations of the subcommittee on Phototrophic Bacteria of the International Committee on Systematic Bacteriology (ICSB) of the International Association of Microbiological Societies (IAMS), the cyanobacteria have been included under the 1st Division followed by the bacteria in the 2nd division in the eighth edition of Bergey’s Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). However, looking into the advances made in the physiology, biochemistry and genetic characterization of cyanobacteria based on pure culture techniques, Stanier et al. (1978) proposed the inclusion of cyanobacteria under the provisions of International Code of Nomenclature of Bacteria. In doing so, they emphasized that properties common to and distinctive to all bacteria may be extended to cyanobacteria.

Rippka et al. (1979) suggested a classification based on bacteriological code and divided the cyanobacteria into five sections. In Section I and Section II members belonging to the orders Chroococcales; Chamaesiphonales and Pleurocapsales as suggested in the Botanical Code have been redistributed. Section III entirely consists of non-heterocystous filamentous cyanobacteria.
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and Sections IV and V comprise heterocystous, unbranched and branched representatives, respectively.

At one time, the occurrence of chlorophyll b in cyanobacteria was used as a criterion to place the organisms in a separate group, the Prochlorophyta. Modern nucleic-acid sequencing, however, has shown that chlorophyll b evolved a number of times within the cyanobacteria and the term Prochlorophyta has been discarded (Palenik and Haselcorn, 1992; Urback et al., 1992).

Molecular biology has provided new tools to decipher genetic information and can be used in attempts to reconstruct the evolution of organisms and improve their taxonomy. In the cyanobacteria, the use of molecular methods to study the genotypic relationships is underway, and initial results are promising. Different chemotaxonomic and macromolecular techniques are reviewed and their usefulness is evaluated. The most complete phylogenetic scheme of the cyanobacteria which is presently available is based on 16S rRNA sequence analysis. With this method, controversial taxonomic problems have been solved, such as the relationships among Pseudanabaena sp. strains or between the genera Arthrospira and Spirulina. In other cases, additional 16S rRNA sequences are necessary to obtain a clear picture.

Peptidoglycan (murein, mucoprotein) is the only cell wall polymer found in both gram-positive and gram-negative bacteria. It is also present in the cell walls of cyanobacteria (Weckesser et al., 1979). There are only a few prokaryotes, such as the mycoplasma (mollicutes) and the archaebacteria, which lack peptidoglycan. It is a heteropolymer consisting of glycan strands that are cross-linked through short peptides. The glycan strand is made up of alternating $\beta$-1,4-linked residues of N-acetylglucosamine and N-acetylmuramic acid, a derivatives of glucosamine and unique constituent of peptidoglycan. The peptidoglycan of cyanobacteria is chemically quite similar to that of gram-negative bacteria, but it forms a thicker layer than is found in most gram-negative bacteria (Schleifer et al., 1983).

The biochemical diversity in the biosynthesis of aromatic amino acids may be also helpful in the classification of cyanobacteria (Hall, et al., 1982). Studies
by Weitzman and co-workers have demonstrated that the structural and functional patterns of two enzymes of the citric acid cycle are highly conserved and can be used for the separation of gram-positive and gram negative bacteria. These two enzymes are citrate synthase and succinate thiokinase. Many bacterial citrate synthases have been studied. Based on the molecular weight, "large" enzymes (250,000 daltons) found in gram-negative bacteria and cyanobacteria can be distinguished from the "small" citrate synthases (100,000 daltons) present in gram-positive bacteria and eukaryotes.

Based on the strains examined so far, it can be concluded that gram-negative bacteria and cyanobacteria contain a "large" succinate thiokinase (140,000--150,000 daltons), whereas a "small" enzyme (70,000-75,000 daltons) is found in gram-positive bacteria and eukaryotes (Schleifer et al., 1983).

Isoprenoid quinones are constituents of the cytoplasmic membrane of bacteria and play important roles in electron transport. They are terpenoid lipids. Two major structural groups can be recognized: the benzoquinones (e.g. ubiquinones, plastoquinones) and the naphthoquinones (e.g. menaquinones, diethylmenaquinones, phyloquinones). The usefulness of these compounds as chemotaxonomic markers has been discussed in detail by Collins & Jones. The majority of the strictly aerobic gram-negative bacteria produces only ubiquinones; exceptions to this are some gliding bacteria that produce only menaquinones. Most members of gram-negative facultatively anaerobic rods contain ubiquinones, menaquinones, or demethylmenaquinones, or a combination of these compounds (Collin et al, 1981). Cultivation of some of these facultative organisms at a low oxygen tension increases the level of menaquinones and reduces the amount of ubiquinones. Gram-negative obligate anaerobes usually produce menaquinones or completely lack isoprenoid Quinones. Cyanobacteria are rather unusual since they possess neither ubiquinones nor menaquinones but contain plastoquinones and phyloquinones which are normally associated with green plants. Gram-positive bacteria only synthesize menaquinones or demethylmenaquinones but never ubiquinones. Therefore, strains producing ubiquinones should no longer be classified in gram-positive taxa. (Collin et al, 1981).
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The variety of phenotypes of cyanobacteria is accompanied by morphological plasticity changing accordingly to different environmental or culture conditions. This can result in misidentifications when using morphological analysis alone (Lyra et al., 2001). Thus, the inclusion of other characteristics, such as genetic information, is an important complement for the accurate identification and classification of cyanobacteria. The analysis of genes encoding the small subunit ribosomal RNA, the 16S rRNA, is currently the most used approach for the phylogenetic classification of cyanobacteria, because their sequences are independent from culture or growth conditions. Furthermore, the universality and conservation of this gene makes it suitable for broad phylogenetic studies (Nubel et al., 1997). Nevertheless, phylogenetic studies have revealed contradictory results with the morphological classification of several Cyanobacterial taxa. That is the case of Oscillatoria and Microcoleus that are described as morphologically different genera, but 16S rRNA analyses grouped them within the same genera (Wilmotte et al., 1992).

4.2 METHODS AND MATERIALS

4.2.1 DNA Extraction

Purelink Plant DNA extraction kit (Invitrogen, USA) was used to obtain high quality genomic DNA samples from cyanobacterial isolates, according to the manufacturer’s protocol. Electrophoresis was done in 0.8% agarose gel at 5 V/cm for 30 min.

4.2.2 DNA quantification and dilution

Quantity of the genomic DNA was estimated spectrophotometrically by measuring absorbance at 280nm while the quality was determined by taking ratio of absorbance at 260 and 280 nm in a UV 1800 Shimadzu UV Vis Spectrophometer by measuring unknown sample at 260nm and 280nm by taking 3μl of DNA and 2997μl of deionized water.

\[ A_{260} = \text{measurement of concentration of DNA (1.0 } A_{260} = 50\mu g/ml) \]

\[ A_{260}/A_{280} = \text{Estimate of DNA purity} \]
Amount of DNA $\mu$g/$\mu$l = O.D. at 260nm x 50 x dilution factor

1000

Working concentration of DNA was 1ng/$\mu$l

**4.2.3 PCR amplifications**

In order to get the sequence of 16S rRNA from cyanobacterial isolates two sets of oligonucleotide sequences were used (Garcia-pichel et al., 1997). The sequence of the primer and the approximate length of DNA amplified by them are given in table 1.

<table>
<thead>
<tr>
<th>Set</th>
<th>Primer Sequence</th>
<th>Length of PCR amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CYA106F-5′CGGACGGGTGAGTAACGCGTGA3’ CYA781R-5′GACTACTGGGGGTATCTAATCCCATT 3’</td>
<td>675</td>
</tr>
<tr>
<td>2</td>
<td>CYA106F-5′CGGACGGGTGAGTAACGCGTGA3’ CYAN1281R-5′GCAATTACTAGCGATTCCTCC3’</td>
<td>1175</td>
</tr>
</tbody>
</table>

**Table 1: Primer used for the 16S rRNA amplification**

PCR were performed by GeneAmp PCR system 9700. The PCR reaction mixture (20µL total volume) consisted of 100 ng genomic DNA, 1.5 mM MgCl$_2$.10mM of each dNTP, 1xTaq buffer, 10µM of each primer, and 1 unit of Taq polymerase. Samples were subjected to the following thermal profile: 3 min of denaturing at 94°C and 30 cycles of three steps: 30 sec of denaturing at 94°C, 30 sec of annealing at 58°C, and 1 min of elongation at 72°C followed by final extension of 10 minutes at 72°C. A digital gel image was obtained utilizing Gel Doc TM XR system (Bio-Rad) & QUANTITY ONE® 1-D V 4.6.7 analysis software.

**4.2.4 Sequencing**

After purification [PCR purification kit (Genei, Bangalore)] the PCR amplicons were directly sequenced at 1st BASE DNA sequencing Services, Malaysia. The sequence obtained using both set of primers were checked for overlaps and a final sequence of 1175 base pair was obtained. The sequences
obtained were submitted to NCBI (KC140132- KC140134 and KC248207-KC248211)

4.2.5 Phylogenetic analysis

DNA sequence data was analyzed by BLAST. Multiple alignments were generated using the CLUSTALW program (Thompson et al., 1994). Phylogenetic distance trees were inferred by Neighbour-Joining analyses (Saitou and Nei, 1987), using MEGA5.10 (Tamura et al., 2011). Confidence in topologies was assessed using bootstrapping (1,000 replicates).

4.3 Result and Discussion

4.3.1 Amplification of 16S rRNA gene by PCR

The 16S rRNA gene was amplified using hot start PCR with two set of primers, CYA106F and CYA781R; CYA106F and CYA1281R. A PCR product of 675 bp was obtained using the first set and an amplification product 1175 bp was obtained using the second set of primer. The sequence obtained using both primers were aligned to get the complete sequence of 1175 bp as sequencing gave a read only up to 700 bp. The complete sequences of 1175 bp were submitted to NCBI.
4.3.2 Phylogenetic Tree

A phylogenetic tree based on 16S rRNA sequences was constructed using MEGA 5.10 software (figure 4.2). The isolates grouped into two clusters, the first cluster, supported by 100% bootstrap value, included isolates Cyanobacterium sp. POKH1, Cyanobacterium sp. PSGH, Cyanobacterium sp. PBJ1, and Cyanobacterium sp. PBJ2 while the other cluster which was not supported by a sufficient bootstrap value included Cyanobacterium sp. PRJT, Cyanobacterium sp. POKH2, Cyanobacterium sp. PPST and Cyanobacterium sp. MTP.
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Figure 4.2: Phylogenetic tree based on an alignment containing the partial 16S rRNA sequences from 8 cyanobacteria constructed by neighbour-joining with *Beggiatoaalba* as outgroup

The 16S rRNA gene sequence analysis grouped these eight morphologically distinct isolates into two distinct clusters, highlighting the importance of both morphological and genetic methods in studying cyanobacterial diversity. The cyanobacteria has been reported to exhibit pronounced polymorphism with changing environmental conditions, and thus studying diversity just based on morphological characteristics is not sufficient [Dor et al., 1987]. Garcia-Pichel et al. (1998) also demonstrated that morphology is not a phylogenetically reliable character for the taxonomy of cyanobacteria and it has to be substantiated with physiological and genetic characteristics [Garcia-Pichel et al., 2001]. 16S rRNA gene analyses have been used to study the diversity of cyanobacteria. Analysis of diversity based on comparison 16S rRNA gene sequences is a reliable way of studying cyanobacterial diversity.
4.4 REFERENCES


