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Assessment of Cyanobacterial Biodiversity Through Molecular Approaches and Possible Exploitation for Value Addition

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Abstract

Cyanobacteria a diverse group of photosynthetic prokaryotes often play central roles in carbon and nitrogen assimilation which makes their environment productive. Molecular approaches have been divided into 2 classes: PCR independent and PCR based approaches. The former includes guanine plus cytosine (G+C) content, nucleic acid re-association and hybridization and DNA microarrays, the latter basically uses DNA cloning and sequencing, DGGE (denaturing gradient gel electrophoresis), TGGE (Temperature gradient gel electrophoresis), SSCP (single strand conformation polymorphism) RFLP (restriction fragment length polymorphism), ARDRA (amplified ribosomal DNA restriction analysis), T-RFLP (terminal restriction fragment length polymorphism), RISA (ribosomal intergenic spacer analysis) ARISA (automated ribosomal intergenic spacer analysis) and RAPD (random amplified polymorphic DNA). Molecular assessment of cyanobacteria frequently uses markers like16S rDNA, phycocyanin locus, nif gene, rpo gene, ITS region etc. Genetic diversity was assessed using the banding patterns of repetitive DNA sequences including the short tandemly repeated repetitive (STRR) sequences and long tandemly repeated repetive (LTRR) sequences that are present in the cyanobacterial genome. The phylogenetic relationships inferred from comparison of the STRR sequences generally supported the traditional classification based on morphological criteria.

Keywords: Biodiversity, molecular characterization, value addition.

Introduction

Cyanobacteria are photosynthetic organisms of cosmopolitan distribution in terrestrial and aquatic habitats. (Schopf, 2000). Cyanobacteria are also involved in symbiotic associations with an exceptionally broad range of representative within the plant kingdom.Molecular systematic has been used to understand the phylogenetic divergence within the cyanobacteria (Rajaniemi et al., 2005). Cyanobacteria are also called as blue green algae because they contain the photosynthetic pigments-c phycocyanin (dominant pigment) c-phycoerithrin, and chlorophyll-a are responsible for their characteristic. In more recent years, DNA sequence has been used for the taxonomic and phylogenetic analysis of cyanobacterial isolates by laboratories. DNA base composition is also a very important genetic characteristic used in taxonomic studies of cyanobacteria (Wilmotte, 1994). Repetitive DNA sequences have been found in filamentous cyanobacteria (Holland and Wolk.,1990). Rapid and sensitive methods for the detection and genetic characterization of cyanobacteria have been developed based on DNA amplification techniques.

Natural Occurrence

The majority of cyanobacteria are aerobic photoautotrophs. Their life processes require only water, carbon dioxide, inorganic substances and light. Photosynthesis is their principal mode of energy metabolism. Cyanobacteria show a distinct ability for heterotrophic nutrition. The prominent habitats of cyanobacteria are liming and marine environments. They flourish in water that is salty, brackish or fresh, in cold and hot springs, and in environments where no other micro-algae can exist. Cyanobacteria have an impressive ability to colonise infertile substrate such as volcanic ash, desert sand and rocks. They are extraordinary excavators, boring hollows into limestone and special types of sandstone.

Organization, function and behaviour

The structure and organization of cyanobacteria are studied using light and electron microscopes. The basic morphology comprises unicellular, colonial and multicellular filamentous forms. Unicellular forms have spherical, ovoid or cylindrical cells. They occur singly when the daughter cells separate after reproduction by binary fission. The cells may aggregate in irregular colonies, being held together by the slimy matrix secreted during the growth of the colony. By means of a more or less regular series of cell division, combined with sheath secretions, more ordered colonies may be produced. Filamentous morphology is the result of repeated cell divisions occurring in a single plane. The multicellular structure consisting of a chain of cells is called trichome. The trichome may be straight or coiled. Cell size and shape show great variability among the filamentous cyanobacteria. Vegetative cells may be differentiated into heterocyst. The only means of reproduction in cyanobacteria is asexual. Filamentous forms reproduce by trichome fragmentation, or by special hormogonia. Hormogonia are distinct reproductive segments of the trichomes. They exhibit active gliding motion upon their liberation and gradually develop into new trichomes. In contrast to eukaryotic microalgae, cyanobacteria do not possess membrane- bound sub-cellular organelles; they have no discrete membrane- bound nucleus; they possess a wall structure based upon a peptidoglycan layer and they contain 70 S rather than 80 S ribosome's. The photosynthetic pigments of cyanobacteria are located in thylakoids that lie free in the cytoplasm near the cell periphery. The green of chlorophyll-a is usually marked by carotenoids (beta-carotene) and accessory pigments such as phycocyanin, allophycocyanin and phycoerythrin. The pigments are embodied in phycobilisomes which are found in rows on the outer surface of the thylakoids (Douglas 1994). The ability for continuous photosynthetic growth in the presence of oxygen, together with having water as their electron donor for CO_2 reduction, enables cyanobacteria to colonise a wide range of ecological niches. Dinitrogen fixation is a fundamental metabolic process of cyanobacteria, giving them the simplest nutritional requirement of all living organisms. By using the enzyme nitrogenase, they convert N_2 directly into ammonium ions and by using solar energy their metabolic and biosynthetic machinery, only N_2, CO_2, water and mineral elements are needed for growth in the light. Many species of cyanobacteria possess gas vesicles. These are cytoplasmic inclusions that enable buoyancy regulation and are gas-filled, cylindrical structures. Their function is to give the species an ecologically important mechanism enabling them to adjust their vertical position in the water column (Walsby, 1987).

Biological diversity

Although cyanobacteria probably evolved as a group of organisms about 2,000 million years before the advent of eukaryotes, they comprise fewer taxa than eukaryotic microalgae (Bisby, 1995). The diversity of cyanobacteria can be seen in the multitude of structural and functional aspects of cell morphology and in variations in metabolic strategies, motility, cell division, developmental biology, etc. A molecular approach to the systematics of cyanobacteria may be most fruitful for inferring phylogenetic relationships. Macromolecules, such as nucleic acids and proteins, are copies or translations of genetic information. The methods applied involve direct studies of the relevant macromolecules by

sequencing, or indirectly by electrophoresis, hybridization, or immunological procedures (Wilmotte, 1994).Nucleic acid technologies, especially the polymerase chain reaction (PCR), have advanced to the point that it is feasible to amplify and sequence genes and other conserved regions from a single cell. 16S r-RNA has given the most detailed information on the relationships within the cyanobacteria. However, the molecular results obtained should be integrated with other characteristics as the base for a polyphasic taxonomy (Vandamme, *et al*., 1996). The properties that make the cyanobacteria generally undesirable are also the qualifications for possible positive economic use. Blue-greens are the source of many valuable products and carry promising physiological processes, including light-induced hydrogen evolution by bio-photolysis (Skulberg, 1994).

Phosphorus and nitrogen

Because cyanobacterial blooms often develop in eutrophic lakes, it was originally assumed that they required high phosphorus and nitrogen concentrations. This assumption was maintained even though cyanobacterial blooms often occurred when concentrations of dissolved phosphate were lowest. Experimental data have shown that the affinity of many cyanobacteria for nitrogen or phosphorus is higher than for many other photosynthetic organisms. This means that they can out-compete other phytoplankton organisms under conditions of phosphorus or nitrogen limitation. In addition to their high nutrient affinity, cyanobacteria have a substantial storage capacity for phosphorus. However, if total phosphate rather than only dissolved phosphate is considered, high concentrations indirectly support cyanobacteria because they provide a high carrying capacity for phytoplankton. High phytoplankton density leads to high turbidity and low light availability, and cyanobacteria are the group of phytoplankton organisms which can grow best under these conditions. A low ratio between nitrogen and phosphorus concentrations may favour the development of cyanobacterial blooms.

PCR- based approaches

DNA cloning and sequencing

Initially, molecular methods for biodiversity studies relied on cloning of target genes isolated from environmental samples. In the initial step of this approach PCR products are first cloned and then sequenced. By comparing the sequences with those available in sequences databases (Gen Bank, EMBL, DDBJ), the information about the identity and relatedness of the new sequences to known species is obtained. (Giovannoni *et al*., 1998) first used the cloning sequencing approach by targeting 16S rDNA for the determination of bacterio-plankton diversity in Sargasso Sea. (Semenova *et al*., 2001) have analyzed nucleotide sequences of fragments of 16S rDNA of the Baikal natural populations and laboratory cultures of cyanobacteria by DNA cloning and sequencing. The cloning sequencing strategy has been used in various ecosystems for studying prokaryotic diversity (Zwart *et al*., 2002). (Svenning *et al*., 2005) studied the diversity of symbiotic cyanobacterial strains within the genus *Nostoc* based on 16S rDNA sequence analysis with the help of DNA cloning and sequencing.

Denaturing gradient gel electrophoresis and thermal gradient gel electrophoresis (DGGE/TGGE)

They are two routinely used almost similar techniques used in assessing the biodiversity of microbial communities from environmental samples as well as monitoring of their dynamics. (Muyzer et al., 1993) were the first to expand the use of PCR-DGGE to study microbial diversity. In most of the electrophoretic fingerprinting methods, nucleic acid fragments are separated by their size but DGGE/TGGE separates DNA strands by their sequence composition. The PCR-DGGE protocol consists of 6 major steps, sample collection, nucleic acid extraction, PCR amplification of target gene, separation of these PCR amplicons by DGGE, staining and visualization of profiles and data analysis. DNA extracted from the soil samples is amplified using PCR with primers targeting part of 16S rDNA

sequences. The PCR product is separated on a gel that is composed of a linear gradient of denaturant. In DGGE, the chemical denaturant urea and formamide are used while in TGGE, temperature is used (Lessa and Applebaum, 1993). Differential migration occurs as more denaturant is needed to separate sequences with high GC content. A high GC sequence (35-40 bp GC clamp) is attached to the 5 end of the forward primer to prevent complete strand separation. On denaturation, DNA melts in domains which are sequence specific and it migrates differentially through the polyacrylamide gel. The fingerprints obtained on the denaturing gradient gel represent the community structure, an approximation of number of population (represented by each band) and their relative abundance (represented by band intensity). More specific information of population composition can be obtained by secondary analysis of the DGGE/TGGE bands via sequencing or hybridization. DGGE/TGGE bands can be excised from gels, re amplified and sequenced or transferred to membranes and hybridized with specific probes to get more diversity information. One of the most general measures of cellular activity is the ribosome content. DGGE has been used to evaluate this ratio among different natural populations by comparing pattern and intensities of bands derived from using either rDNA or rRNA. Thus, DGGE/TGGE has the advantage of being reliable, reproducible and rapid.

Single strand conformational polymorphism (SSCP)

SSCP analysis is the electrophoretic separation of single-stranded nucleic acids based on subtle differences in sequence (often a single base pair) that results in a different 3 dimensional folded secondary structures. This brings about measurable difference in mobility through a gel. In fact the mobility of double-stranded DNA in gel electrophoresis is dependent on strand size and length but is relatively independent of the particular nucleotide sequence. However, the mobility of single strands is noticeably affected by very small changes in sequence, because of the relatively unstable nature of single-stranded DNA. Actually in the absence of a complementary strand, the single strand experiences intra-strand base pairing, resulting in loops and folds that give the single strand a unique 3D structure, regardless of its length. Therefore, even a single nucleotide change could dramatically affect the strand's mobility through a gel by altering the intra strand base pairing and its resulting 3D conformation. Single strand conformation polymorphism analysis takes advantage of this quality of single-stranded DNA and is a means of detecting DNA polymorphisms or sequence variations.

Terminal restriction fragment length polymorphism (T-RFLP): It is a quantitative molecular technique that was developed by Liu *et al*., (1997) for rapid analysis of microbial community diversity in various environments. The technique employed PCR in which one of the 2 primers used was fluorescently labeled at the 5'end and was used to amplify a selected region of bacterial genes encoding 16S rRNA from total community DNA. The PCR product was digested with restriction enzymes, and the fluorescently labeled terminal restriction fragment was precisely measured by using an automated DNA sequencer. Computer simulated analysis of T-RFLP shows that with proper selection of PCR primers and restriction enzymes, DNA sequences could be PCR amplified and classified into unique terminal restriction fragment lengths or ribotypes. Terminal restriction fragment length polymorphism analysis is a commonly used fingerprinting technique that is basically an improvement of amplified fragment length polymorphism (AFLP) (Tiedje *et al*., 1999). This involves restriction digestion of double stranded fluorescently end labeled PCR fragments as one primer is labeled at 5'terminus with a fluorescent dye. Thies (2007) has suggested that T-RFLP can be used to analyze communities of bacteria, archaea, fungi, other phylogenetic groups or subgroups, as well as functional genes.

Ribosomal intergenic spacer analysis/ automated ribosomal intergenic spacer analysis (RISA/ARISA)

DNA fingerprinting techniques used to assess microbial diversity and is similar in principle to RFLP and T-RFLP. The intergenic spacer region (IGS) located between the 16S and 23S ribosomal

subunit in the rRNA operon is amplified by PCR using primers, denatured and separated on a polyacrylamide gel under denaturing conditions. Primers are designed to target the conserved regions in the 16S and 23S genes. Amplification products differing in length are separated on polyacryamide gels because of heterogeneity of the IGS length and sequence.

Restriction fragment length polymorphism (RFLP)

It is a powerful molecular tool in studies of biodiversity. RFLP is generated by the presence and absence of a recognition site for the same restriction endonuclease in same region of a chromosome from different individuals of a species. As a result the concerned restriction enzyme produces fragments of different length representing the same chromosome region of different individuals. These differences are detected by gel electrophoresis combined with hybridization with a labeled probe specific for that chromosome region. Thus, RFLP marker results from a combination of a specific restriction endonuclease and a specific DNA sequence used as probe. The lanes of the different strains/ selected species are compared and RFLPs are detected due to differential movement of a band on their gel lanes each of such band is regarded as a single RFLP locus. Lyra *et al*., (1997) demonstrated the significance of this technique in a study based on 7 enzymes, which separated a number of hepatotoxic heterocystous cyanobacterial strains.

Randomly amplified polymorphic DNA (RAPD)

RAPD polymorphism is detected by using oligonucleotide usually 10 bases long of random sequences as primer in a PCR reaction. The strains, which have their genomic DNA sequences complementary to the primer oligonucleotide, will be amplified in PCR. Stringency of paring between primers and the template DNA is reduced to ensure amplification as some of these bands are present in some and absent in the other strains. These bands constitute the RAPD loci. Genetic variability of the cosmopolitan, ubiquitous fresh water cyanobacterium *Phormidium retzii* was assessed using RAPD markers (Eskew *et al*., 1993). In the case of unicellular marine cyanobacteria from the Baltic Sea, a unique band obtained from the RAPD pattern was cloned and finally used to compare the strains by dot-blot hybridization using this sequence as probe.

16S rRNA gene

16S rRNA gene sequencing is the most widely applied strategy for assessing cyanobacterial biodiversity in nature. For broad phylogenetic studies, sequence data from the 16S rRNA gene are the most commonly used due to their efficacy for distinguishing higher-level taxonomic groups, as well as traditional species. Diversity assessment of *Phormidium retzii*) has been done with the help of 16S rRNA gene sequence analysis. DNA sequence information for the small subunit rRNA gene obtained from cyanobacterial culture has been used to investigate the presence of cyano-bacteria and their abundance in natural habitat (Rudi *et al*., 2000). One of those is that the size of the genes for the 16S molecules is extremely constant with the total variation of about 200bp for a mean length of 1500 bp (Linton *et al*., 1994) and therefore different genes cannot be easily separated by size.

Phycocyanin locus

Genetic diversity of cyanobacteria has been further characterized by determining the DNA polymorphism within the phycocyanin (PC) locus (Neilan *et al*., 1995). The distribution of PC in aquatic microorganisms especially in cyanobacteria makes the study of PC gene sequence heterogeneity ideal for the classification of fresh water cyanobacteria. The entire PC operon contains genes coding for 2 bilin subunits and 3 linker polypeptides .The intergenic spacer (IGS) between the 2 bilin subunit genes, designated (cpcB) and (cpcA) of the PC operon is selected as a potentially highly variable region of DNA sequence useful for the identification of cyanobacteria to the strain level.

RNA polymerase

DNA-dependent RNA polymerase is a potential molecule for examining organizational diversity because it exists in single copies in prokaryotic genome (Bergsland and Haselkorn, 1991). PCR is used to amplify DNA dependent RNA polymerase gene sequences specifically from the cyanobacterial population to assess biodiversity. The various polymerase subunits contain highly conserved regions that can be used to develop PCR primers. Two such regions have been used to develop primers that will amplify specifically a fragment of the *rpo*C1 subunit gene from the cyanobacterial lineage. The sequences obtained have been valuable for probing cyanobacterial evolution (Palenik and Haselkorn, 1992). In addition, the nucleotide sequence divergence between closely related species is much greater for the *rpo*C1 gene fragment than for the 16S rRNA sequences.

Biodiversity among cyanobacterial communities from diverse habitats

Fresh water cyanobacteria: The cyanobacteria are found in a wide variety of habitats. Most of the species are fresh water inhabitants. They are unicellular, colonial or form chains of cells in trichomes. In colonial forms all cells of a colony occur in a common gelatinous matrix. The cells of the colony may divide to form definite or indefinite and irregular assemblage of cells. In filamentous form each trichome has its own gelatinous sheath. The filaments are usually unbranched and uniseriate or may sometimes show pseudobranching. Garcia-Pichel *et al.,* 2001) used PCR in combination with DGGE to probe the diversity of oxygenic phototrophic cyanobacteria in cultures, complex microbial communities and those in association with lichens. Sequences of 16S rRNA genes are independent of growth conditions and can be retrieved by PCR from small amount of DNA extracted from laboratory cultures or natural environments. The *rpo*C1 gene encoding the subunit of RNA polymerase has been described as an alternative target for the analysis of cyanobacterial phylogeny (Bergsland and Heselkorn, 1991) and community structure. Gugger *et al*., (2002) reported that the planktonic *Anabaena* strains were not distinguishable from *Aphanizomenon* strains by morphological analysis. DNA fingerprinting has been found to yield significant diversity among nitrogen-fixing cyanobacteria in soil samples collected from different ecosystems. PCR methods using DNA dependent RNA polymerase (*rpo*C1) have been found quite useful in understanding molecular phylogeny of *Anabaena circinalis*. 16S rRNA gene analysis revealed that the cyanobacterial phylotypes observed in dolomite were related to known diazotrophs including *Anabaena, Calothrix, Scytonema* and *Nostoc*. Svenning *et al*., (2005) studied the phylogeny of symbiotic cyanobacteria within the genus *Nostoc* based on 16S rDNA sequence analysis. Analysis of the genetic diversity of symbiotic *Nostoc* strains using molecular methods revealed heterogeneity reflecting high genetic diversity (West and Adams, 1997). Sequence heterogeneity between strains at a higher taxonomic level has also been demonstrated using 16S- RFLP and ITS-RFLP combined with DGGE analyses of the functional *het*R gene (Svenning *et al.,* 2005). Phylogenetic analysis revealed that *Nostoc* strains are intermixed within the genus *Anabaena* and vice-versa.Ten 16S rDNA probes to identify the cyanobacteria genera, *Microcystis, Plankothrix, Anabaena* and *Aphanizomenon*; in addition a probe which corresponds to the *Nostoc* (which includes *Nostoc, Anabaena* and *Aphanizomenon* sp.) has been reported by Rudi *et al*., 2000).

Marine cyanobacteria

The range of habitats and conditions occupied by cyanobacteria as a group, however, is wider than the most eukaryotic phototrophs. Cyanobacteria proved themselves successful in occupying freshwater, brackish, marine and hypersaline environments. Marine forms employ both halophily and halotolerance as survival strategies. The open ocean, the largest and ecologically most stable marine environment is the home of picophytoplaktonic unicells (Waterbury *et al*., 1979), classified within the genera, *Synechocystis, Synechococcus* and *Prochlorococcus*. These tiny microorganisms

contribute significantly to the primary production of lakes, oceans and lagoon waters. *Anacystis nidulans* axenically cultured marine strain proved worth separating from freshwater counter parts on the basis of nucleotide sequencing (Giovannoni *et al*., 1998). Species of *Trichodesmium* are characterized by distinctive mostly clonal colony formation with cell differentiation occurring along individual trichomes and thylakoid separation (Keratomization) intensified distal portions of trichomes. Function differentiating along multicellular trichomes has been shown recently by localizing the position of nitrogenase gene.Use of molecular technique demonstrates much more diversity among marine cyanobacteria sequences retrieved directly from the Greazt Sippewissett Salt Mars, MA, USA (Dillon and Wilmotte, 1999). However, *Prochlorococcus* is the dominant phototroph in the temperate oceans. Sequencing and analysis of cloned fragment suggested that the population in the sample consisted of two distinct clusters of *Prochlorococcus* like cyanobacteria and four clusters of *Synechococcus* like cyanobacteria.

Industrial application of cyanobacteria

Cyanobacteria as food: Several microalgae have established as protein sources of good quality (Anusuya *et al*., 1981). Some strains of *Anabaena* and *Nostoc* are consumed as human food in Chile, Mexico, Peru and Philippines and employed as green fertilizer in Far East (Guerrero *et al*., 1990). *Nostoc commune* contains only a moderate amount of protein and a high amount of fibre which suggested its potential as a new dietary fibre source which plays important physiological and nutritional role in human diets (Jeraci and Van Soest 1986). *Spirulina* is gaining popularity in recent years as a food supplement because of its excellent nutrient composition and better digestibility due to the thin cell wall. This feature is important for people suffering from intestinal malabsorption or older people unable to digest complex proteins. *Spirulina* powder has the highest protein content of any natural food.

Pharmaceutical properties

Secondary metabolites from cyanobacteria have reported to have pharmaceutical potential belonging to a wide range of structural classes like alkaloids aromatic compounds, peptides, terpenes, all of which exhibit some biological activity (Konig and Wright, 1993).

Therapeutic feeding and effects

Spirulina has an effect on lowering cholesterol levels in human as well as alleviating premenstrual syndrome (Richmond, 1986). An extract from *Spirulna platensis* is a selective inhibitor of Herpes simplex virus Type1 (Hayashi *et al*., 1993). Moore *et al.*, 1987 reported an alkaloid which possessing antibacterial and antimycotic action in *Hapalosiphon fontinalis* isolated from soil. They described the structure of hepalindole A, a novel chlorine and isonitrile containing indole alkaloid which is responsible for most of the antibacterial and antimycotic activity. Antiviral activity has also been reported by the extracts of *Microcystis aeruginosa*.*Nostoc commune*, a new dietary fibre source, is suggested to be involved through various undefined complex processes in the prevention of certain pathologies (Mc Pherson, 1986). The fibre extracted form *Nostoc commune* was referred to as oxalate oxalic acid soluble substances. Another physiological action from this alga is the ability to provide protection against amaranth toxicity.

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