PCR Based Molecular Characterization of Cyanobacteria with Special Emphasis on Non-Heterocystous Filamentous Cyanobacteria

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Abstract

Cyanobacteria have an ancient history dating almost 3.5 billion years and diversified extensively to become one of the most successful and ecologically significant organisms on earth, with respect to longevity of lineage and impact on earth’s early environment. Despite the availability of various monograph based on morphological and ecological variants, the identification and classification of cyanobacteria remain a difficult and confusing task leading to uncertain identifications. Therefore, molecular approaches based on PCR techniques and DNA fingerprinting have been adopted for taxonomical studies. Molecular markers such as RAPD, RFLP and AFLP are used for the PCR techniques.

Keywords: Non-heterocystous, cyanobacteria, molecular characterization.

Introduction

Cyanobacteria are an ancient group of prokaryotic microorganisms exhibiting the general characteristics of gram-negative bacteria. They are unique among the prokaryotes in possessing the capacity of oxygenic photosynthesis. Cyanobacteria are a morphologically diverse group ranging from unicellular to colonial and filamentous forms. Taxonomically, cyanobacteria are grouped into unicellular forms that divide by binary or multiple fission and filamentous forms that are non heterocystous or differentiate heterocysts in non branching or branching filaments. Unicells cyanobacteria may divide in one, two or three planes. Some unicells dividing in one plane show asymmetric division; in Chamaesiphon the smaller cells glide down to the base of the larger cell and repeats of this process eventually give rise to a colony of many cells. The resulting colonies can often be seen as brownish spots on submerged rocks. Maintenance of the colonial structure in many of these forms is aided by the presence of exopolysaccharides such as mucilage and / or a firm sheath. The presence or absence of a heterocyst is an important feature separating genera. However, gas vacuoles, structures that aid buoyancy, are found in species of many different genera. Rather surprisingly, the starting point for the morphologically less complex forms is much earlier, although there was of course little understanding of their diversity at that time. A number of “floras” summarizing the known species of cyanobacteria in particular regions have been published during the 20th century. Several of these provide a lot of information about species occurring elsewhere in the world. A review by Castenholz (1992) and the sister volume (Bryant, 1994) to the present one make clear how important is an understanding of cyanobacterial molecular biology not just as an aid to taxonomy, but for interpreting ecological phenomena in general. Suboptimal light and nutrient conditions result in a number of responses
that strongly influence the physiology of the cell. The responses can be striking or subtle and subsequent changes take place rapidly or very slowly. Cyanobacteria tend to show resistance to multiple environmental stresses, and it is probable that the response pathways to the different stimuli overlap. The ability of some cyanobacteria to withstand extremes of UV radiation and desiccation is aided by their capacity for efficient DNA repair. It will be of interest to understand if, and how, such repair plays a role in other responses to environmental stimuli such as nutrient limitation and cyanophage infection.

**Origin and early evolution of cyanobacteria**

**The evolution of the cyanophytes:**

The evidence for the time and manner of origin of cyanobacteria is still meager, and most of it subject to varying interpretation. The cyanophytes arose between 3-4 billion years ago (Schopf, 1970) probably from a photosynthetic bacterium. After the discovery of gliding photosynthetic bacterium *Chloroflexis* (Pierson and Castenholz, 1971) is a reasonable candidate as a forerunner of the cyanophytes. Cyanophytes were probably responsible for a marked increase in the O$_2$ concentration of the atmosphere during the Precambrian. Another reason why a cyanophyte is the best candidate for the first O$_2$-evolving photosynthetic organism is that at least some cyanobacteria are able to grow aerobically, a property not normally associated with eukaryotic algae (Stewart and Pearson, 1970). If the bulk of the atmospheric O$_2$ is indeed of biogenic origin, the organisms first involved in O$_2$ formation would of course have developed in an anaerobic environment and would have had to be able to live an aerobically.

**Stromatolites and the evolution of the cyanophytes**

Stromatolites are rocks consisting of many layers, most commonly developing from the mats of cyanobacteria. The rocks may be either siliceous or calcareous, although the later are most common. The layers probably build as a result of sediment trapping and carbonate precipitation by unicellular and filamentous cyanobacteria. In shallow marine habitats, calcareous sediments deposit continuously on the surface of the mat, and the cyanophytes move up through the sediments during the day (Gebelein, 1969). In some cases the layers are diurnal, resulting from phototrophic movement of the cyanophytes. Stromatolitic formations became less and less frequent and diverse after the Cambrian, and the most common explanation for this is that the evolution in the Cambrian of microbe eating metazoans made it impossible for the large cyanophytic mats to build up.

**Ecology of cyanophytes**

The cyanophytes as a group adapted, in the Precambrian were found in a wide variety of habitats but today their distribution is more restricted, in many habitats they must compete with eukaryotes. There is only one known type of habitats on earth today where cyanophytes are the exclusive O$_2$-evolving photosynthetic organisms, and that is in thermal springs (Brock, 1970). However, even in the thermal springs cyanophytes are not always successful. In acid springs with pH less than 4.0, cyanophytes are never found, irrespective of temperature. In neutral and alkaline thermal springs, cyanophytes are the exclusive O$_2$-evolving photosynthetic organisms at temperatures above 55-60°C, and are far in the dominance at temperatures down to about 40°C. The upper temperature limit for cyanophytes is about 72-73°C and at temperatures above this only non photosynthetic bacteria are present. In other habitats, even in those in which cyanophytes are widely held to be successful, they are never exclusive and often not dominant. The fixation of nitrogen is found under aerobic conditions in heterocystous cyanophytes and under anaerobic condition also in some non heterocystous forms (Stewart and Lex, 1970). The ability of a variety of cyanophytes to fix nitrogen in natural aquatic systems is well established. The presence in cyanophytes of phycobilins as accessory photosynthetic pigments may confer a considerable ecological advantage to them under conditions of low light intensity. Perhaps one of the most important factors controlling cyanophytes development may be the ability of this group to
grow under conditions of low O₂ concentration. It might be in such a niche that the first eukaryotic alga could have developed. In such an alga, with its photosynthetic apparatus concentrated in a chloroplast, the cytoplasm surrounding the chloroplast could have provided an effective pH buffer which would have served to keep H⁺ from the very acid sensitive chlorophyll molecule. The cyanophytes, with its photosynthetic apparatus in the cell periphery, may be less well adapted for keeping H⁺ from chlorophyll.

**Mechanism of movements in cyanobacteria**

Motility in cyanobacteria is gliding in contrast to swimming is movement in contact with a solid or semi solid substrate, but without a visible change in the shape of the organism. A filament may have considerable passive flexibility, but there does not seem to be any possibility of steering. The relatively slow progress of gliding is accompanied in some species by a rotation of the trichome along its axis which is either right handed or left handed. This means simply that any point on the surface of the trichome will trace either a right handed or left handed helix, but whichever it is, it will be species specific and non interchangeable. In liquid microscope mounts with or without a cover slip, free ends of trichome are seen to jerk back and forth, wave to and fro, or oscillate by tracing a cone. It appears that all of these oscillations are passive responses to the rotation of the trichome when any part of it is able to bear against a substrate and glide. Consequently, the whole trichome rotates and, since they are seldom straight threads they will appear to be swinging their free ends around. Rotation and accompanying oscillations are the usual features of gliding trichome of most oscillatoriaceae, but not of most hormogonia and gliding trichome of heterocystous groups.

**Gliding behaviour:** Gliding is movement in contact with a solid or semi solid surface without flagella like propulsive organs. In most cases it is a smooth, non jerking movement resembling somewhat the progress of a snail. In many cases the movement is continuous in one direction for prolonged periods; in others frequent or regular reversals occur. Gliding of trichome in almost all circumstances probably occurs within a thin sheath casing or film of mucus which adheres to the substrate and is shed and left behind as a 'mucous' trail. When measured microscopically, the velocity of movement without reversals varies with species and environmental conditions. In the oscillatoriaceae rates of well over 2µm sec⁻¹ are command and range up to 11µm sec⁻¹ (Halfen and Castenholz, 1971). There is no consistent correlation between trichome diameter of different species and the velocity of gliding. Other non photosynthetic, filamentous prokaryotes and myxobacteria glide at rates usually less than 1.0 µm sec⁻¹. In contrast, flagellated rod and spirilla bacteria commonly attain speeds of 20-30 µm sec⁻¹ and vibrios apparently reach speeds as high as 50-200 µm sec⁻¹. When measurement are made on a macro scale, such as distance covered on an agar surface over a period of a few hours, less than maximum rates are usually recorded. This is presumably because of the more or less frequent stops and reversals of individual trichome together with the devious path followed by some on the surface of the substrate.

**The mechanism of motility:** One of the earliest suggested mechanism for gliding motility (Doetsch and Hageage, 1968) has been supported and modified by Halfen and Castenholz (1971) that the Oscillatoria is that numerous microfibrils strung uninterrupted in helices around the exterior portion of the trichome are the propulsive organelles which move the chain of cell against an elastic sheath or other suitable substrate by a rapid succession of unidirectional waves with submicroscopic amplitude. The origin of wave propagation may shift from one pole to the other, reversing the direction of wave movement and of gliding. Unicellular species or those with trichome of more disjointed cell could have a similar system but continuous for only a single cell length. Species that do not rotate as they glide should have longitudinal microfibrils which are not helically strung.

**Orientated movements:** Phototaxis is only tactic response of cyanobacteria that has received much attention. Photo topotaxis is the orientation and movement towards (+) or away from (-) the...
incident light. Photo phototaxis is the reversal of direction of movement following a sudden change from high to low light intensity (+) or from low to high light (-) (Nultsch 1965). The general term phototaxis can mean any of these responses. It is often difficult to distinguish topotactic and phobotactic reactions.

Gas vacuoles in cyanobacteria

Fine structure of gas vesicles: The fine structure of gas vacuoles has been investigated in a total of eleven species from the genera *Microcystis*, *Oscillatoria*, *Trichodesmium*, *Anabaena*, *Aphanizomenon*, *Nostoc* and *Gloeotrichia* and in each case they have been found to comprise gas vesicles of the same basis morphology originally described by Bowen and Jensen (1965). The vesicles have the form of hollow, cylindrical tubes with conical caps at each end. The tubes are possibly of uniform diameter, usually given as being about 70 nm the reported extreme being 65 nm for *Oscillatoria rubescens* and 75-85 nm for *Trichodesmium erythraeum*. Various synonyms have been used to describe gas vesicles in cyanobacteria but it is suggested that the original term should be retained. Gas cylinder aptly described these structures in cyanobacteria but is inapplicable to homologous structure of different shape found in various bacteria (Walsby 1972). In many species the gas vacuole are irregular in form and distributed throughout the cell but in some algae they have a characteristic shape and position.

Chemical composition of gas vesicles: Chemical analyses have now been made on gas vesicles isolated from cyanobacteria and the results have confirmed the idea, suggested by electron microscopy, that the gas vesicles membrane is fundamentally different from typical unit membrane. Quantitative preparations of highly purified intact vesicles have been obtained by Walsby and Buckland (1969) from the filamentous alga *Anabaena flosaquae*. To avoid collapsing the gas vesicles, the cell were lysed by method which did not involve exposing the preparations to pressure, the filamentous alga being disrupted by osmotic shrinkage in hypertonic sucrose solution and the unicellular form by osmotic shock after weakening the cell wall by penicillin treatment. The intact vesicles were then purified by a process of repeated, centrifugally accelerated flotation, followed by combination of membrane filtration, molecular sieving and liquid polymer partitioning (Buckland and Walsby, 1971).

Functions of gas vesicles: Of the three functions that gas vacuoles might fulfill that of storing gas is no longer tenable. The gas vesicle membranes are far too permeable to retain any particular gas. It has been pointed out that the two other functions, providing light shielding and buoyancy do not depend on the presence of gas in the vesicles, but rather on these structures being kept free of liquid or solids.

Light shielding: The peculiar optical quantities of gas vacuoles have suggested (Waaland et al., 1971) that they might provide light shielding for cells exposed to high light at water surfaces. Fuhs (1968) has pointed out that in light microscope gas vacuoles are poor amplitude objects on account of their low absorptivity, but the large refractive index difference between their contents and the surrounding cytoplasm makes them excellent phase objects. The path length of light passing through a gas vacuole may differ from that passing through the neighbouring cytoplasm by more than ¼ wavelength and result in ‘false reversal’ so that the vacuole looks brighter instead of darker than the background. With high resolution optics, gas vacuoles appear almost invisible under bright field illumination but with low numerical apertures they are characterized by diffraction fringes.

Light intensity in cyanobacteria: Within certain limits, an inverse correlation between light intensity and photo pigment content is general among photosynthetic organisms. As might be expected, the ratio of phycocyanin to chlorophyll can vary widely in response to simultaneous change in light intensity, temperature and carbon dioxide partial pressure. In chromatically adapting strains, growth in green light stimulates the synthesis of the red coloured phycoerythrin whereas when growth occurs in red light it is the blue protein, phycocyanin, which is the dominant biliprotein. After a brief ‘red’ suspension immediately,
phycocyanin alone is synthesized whereas after a brief ‘green’ irradiation, phycoerythrin and phycocyanin are both synthesizes. These effects are repeatedly reversible; the last 6 irradiation determines the pattern of biliprotein synthesis in the dark.

**Modern approaches and concepts on oscillatoriales**

It includes all filamentous cyanobacteria that undergo binary fission in a single plane and that produce “vegetative” cells only and heterocysts and akinetes do not occur. The terminal cell of some species may be distinctly shaped but is apparently still capable of photosynthesis. Sometimes the terminal cell is tapered and with a cap or calyptra, but in a few forms the taper may include several sub terminal cells as well. The terminal cell in some may never divide and trichomes may be flexible or semi rigid. In some cases, the entire trichome is wound into a loose or tight spiral; in others, only terminal portions of the trichome may be openly spiraled. An apparent sheath may be present, but even species without an easily visible sheath leave behind at least a very thin, gossamer sheath when moving by gliding. When short fragments of a few cells separate from the remainder of the trichome near the free, open end of a sheath, these free trichomes may glide out, eventually forming new sheaths. Although trichomes without apparent sheaths also fragment, a separable, migrating, hormogonial phase is difficult to distinguish, since all lengths of trichome are generally motile. Movement forward or backward may or may not be accompanied by a right or left handed rotation of the trichome. Fragmentation of trichomes occurs in some forms where a cell loses much of its contents and dies. In some cases, there appears to be an orderly sacrificial death of these cells (necridial cells) which determines the sites of trichome breakage (Ciferri, 1983).

The range in mole % G + C of the DNA is large (40-67) and the range of genome sizes is also great (2.14-5.19 x 10^9 daltons), but all sizes are generally less than those in the Nostocales or Stigonematales (Herdman et al., 1979b). The range in DNA base composition and in genome size indicates a probable artificiality in the grouping of oscillatoriales, a conclusion further supported by the degree of disparity in some sequences of 800-900 continuous nucleotides of 16S rRNA from 11 strains of 7 genera of this group (Giovannoni et al., 1988). The triviality of some generic distinctions used here should also be emphasized. Often only one characteristic is used, a characteristic that may be the result of a slight difference in genetic code. In the “Geitlerian” system, generic distinction in the oscillatoriales is based primarily on the diversity of sheaths or their absence. Although still used as a characteristic in the present system, knowledge of physiology, biochemistry, and nucleotide base sequence homologies will eventually determine degree of relatedness. Members of oscillatoriales occur in an enormous diversity of habitats: freshwater and marine, both as plankton, mats, and periphyton. Terrestrial crusts, mats, and turfs are also common. The order oscillatoriales is characterized into eight genera namely, *Spirulina*, *Arthrospira*, *Oscillatoria*, *Lyngbya*, *Pseudanabaena*, *Starria*, *Crinalium* and *Microcoleus*.

**Spirulina**

Filamentous organisms that divide exclusively by binary fission and in one plane but that grow in the form of a tight to nearly tight coiled right or left handed helix. The cross walls are thin and are invisible or nearly so with light microscopy. The trichome does not truly rotate but moves along the outer surface of the helix. Free ends not in contact with substrate may oscillate. Variations in the tightness of the trichome helix occur in both *Spirulina* and *Arthrospira* (Jeeji Bai and Seshadri, 1980; Hindak, 1985). The mole % G + C of the DNA of the reference strain (PCC 6313) is 54, and the genome size is 1.53 x 10^9 daltons. The members of this genus have a worldwide distribution in freshwater, marine, and brackish waters.

**Arthrospira**

Filamentous organisms that divide exclusively by binary fission and in one plane. The entire trichome is arranged as an open helix in which transverse walls may be seen via light microscopy. Cells are
generally shorter than broad to quadrate but are occasionally elongate. Constrictions at cross walls may be present or absent. The mole % G + C of the DNA of the reference strain (PCC 7345) is 44.3. The life cycle of Arthrospira in laboratory culture involves the breaking up of trichomes at the sites of a necridium at intervals of every 4-5 cells.

**Oscillatoria**

Filamentous organism that divide exclusively by binary fission and in one plane. The trichomes are straight to loosely sinuous near apices; flexible or semiflexil. Transverse septa are generally visible under light microscopy. Constrictions may or may not occur at cross walls, but the total indentation never exceeds one eighth of the trichome diameter. During fission the cytoplasmic membrane invaginates, with a thinner peptidoglycan layer separating the new membranes of the daughter cells. Usually, sheaths are nearly invisible, gossamer tubes that are shed as flattened trials when the trichome moves on solid substrates. Occasionally, more visible sheath may build up on some trichomes, particularly during periods of immobility in liquid culture (Chang, 1977). Several species show "chromatic adaptation" (Tandeau de Marsac, 1977). Some species, almost black in colour, contain abundant C-PE and c-phycocyanin.

**Lyngbya**

Filamentous organism that share the entire range of cellular types with Oscillatoria but which produce a distinct and persistent sheath. The sheath may be thin but can be seen with phase contrast optics, particularly when it extends beyond the terminal cell of the trichome. The trichome diameters range from 1 im to about 80 im. The mol% G + C of the DNA is 43.4 and the genome size is about 4.58 x 10⁹ daltons.

**Pseudanabaena**

Filamentous organism that divide exclusively by binary fission and in one plane and that has conspicuous constrictions at the cross-walls; in most strains, constriction cuts into about half or more of the diameter of the trichome. Cells are longer than broad to iso diametric and are often barrel shaped. The trichomes are usually straight and quite frequently short, consisting of only a few to several cells. Single, detached cells are frequent in most culture populations. Gliding motility occurs in trichomes and unicells, probably without rotation. The mol% G + C of the DNA ranges from about 42 to 47 (Guglielmi and Cohen-Bazire, 1984) and the genome size is from 2.14 to 5.19 x 10⁹ daltons.

**Microcoleus**

These Oscillatorian type trichomes are characterized by the presence of a common, homogeneous sheath. It surrounds several parallel trichomes that are often spirally and tightly interwoven.

**Importance of cyanobacterial germplasm**

A germplasm is a collection of genetic resources for an organism. Genetic material, specially its specific molecular and chemical constitution that comprises the physical basis of the inherited qualities of an organism and that is transmitted from one generation to another. There are several reasons that could justify a collecting mission: the species is in danger of extinction or genetic erosion, users have expressed a need at the national or international level, and the diversity is missing from existing ex situ collections. The more diversity is conserved and made available for future use, the better the chances of meeting tomorrow’s needs.

**Conventional/ traditional methods for systematics**

Traditionally, the classification of cyanobacteria has been based on morphological characters such as trichome width, cell size, division planes, shape and arrangement, pigmentation and the presence of characters such as gas vacuoles and a sheath (Baker, 1992; Komarek and Anagnostidis, 1989). The importance of structural and developmental characters for classification of both field and cultured material makes it essential for researchers to isolate and purify the specimen of interest under optimal cultural conditions and provide it with a taxonomic identity. Many cyanobacteria are conspicuous in nature and
collection of samples is therefore greatly facilitated.

Direct Observation

This is most suitable for samples that contain good number of organisms. Rice field floodwater carrying suspended soil particles are filtered on membrane filters and the population is smeared on the slide and observed under microscope. For proper enumeration of small cells of cyanobacteria, microscopy as well as epifluorescence microscopy is also used to distinguish between cyanobacteria and small sized bacteria. This method is based on the chlorophyll-a fluorescence of algae that distinguishes them from bacteria. Combination of excitation and suppression filters may be used to differentiate between phycocyanin and phycoerythrin (Hawes and Davey, 1989). Since cultured samples give better performance, there is a need to improve it for field application.

Isolation and purification

Many cyanobacteria present in axenic culture can be isolated by the liquid enrichment technique, which imposed a positives selection on those members of the population most of them to proliferate in the medium and under the culture conditions (light, temperature) provided.

Direct isolation

The field samples are examined under the microscope immediately on arrival in the laboratory to evaluate the composition of cyanobacterial species. Depending on the consistency of the crude material, the sample for examination is placed on the slide with the platinum loop or pasteur pipette. Once the sample has been thoroughly examined and its composition recorded, aliquot are transferred directly with proper precautions, to solid media. After streaking the deposited crude material on solid media, the plates are examined under binocular microscope in order to isolate cyanobacterial forms without inoculation.

Culture media

For enumeration of algae in the soil, the following procedure is adopted. There are many recipes for media for cultivation of cyanobacteria under laboratory conditions. Most of them are the modifications of previously published formulae and some are derived from analysis of water in the native habitat and ecological considerations.

Enrichment media

Most of the defined culture media are of known chemical composition and are suitable for a particular community and can be used for enriching additional algal groups by adding a variety of components like lake or sea water, soil extracts and other special substance. Such enrichment media stimulate diverse nutritional groups of species and have advantage of supporting the growth of large number of algal species.

Plating techniques

In this method, selection of medium and its concentration is very important. Care should be given to pH and temperature of the culture procedure because some cyanobacteria prefer to grow above 30°C. Best comparisons can be made by counting the colony forming units (CFUs).

Purification

a. Repeated liquid subculture: This technique has been successfully used when a natural collection is particularly rich in specific cyanobacteria.

b. Fragmentation: Homogenization of filaments with a glass homogenizer for 5-10 minutes allows short filaments of 4-8 cells long to be obtained. Individual colonies can be obtained when suspension is streaked on agar plates containing suitable medium.

c. Antibiotics: These can be used to kill certain contaminants which cannot be removed by other means.

d. Ultra violet radiation: This method has been widely used to obtain some strains including those frequently used culture of cyanobacteria.

e. Higher temperature incubation: Thermophilic forms can be isolated by enrichment at about 40°C.

Maintenance

All strains are maintained by sub culturing in liquid media and on agar slants. Some strains including
those frequently used in the inoculation of paddy fields are maintained on suitable carriers. Strains properties may change during repetitive sub culturing after long term cultivation under laboratory conditions. Therefore, following methods can be adopted for the conservation of cyanobacteria.

**Frequency of transfer**

Cultures are transferred at different intervals depending on the maintenance conditions and species. Unicellular and filamentous non motile may be transferred once every three to six months. Flagellated species required more frequent transfers.

**Preservation**

The primary purpose of preserving cultures is to maintain cyanobacterial population in a viable state for considerably longer period. During preservation, all the physiological processes of an organism are considerably slowed down, without affecting viability. A preserved culture can be reactivated whenever desired by providing suitable growth conditions. For preservation, most algae do well at less temperature (15-20°C), with few exceptions where algae prefer higher temperatures for survival.

**Lyophilization**

Freeze drying or lyophilization is a technique where drying is achieved by avoiding the liquid state, through sublimation. In this process, the ice crystals forms in cells are directly converted into water vapours through evacuation at low temperature. Since the lyophilized culture are hygroscopic and show loss in viability when exposed to molecular oxygen, they should be preserved in evacuated sealed glass ampules such sample show very little metabolic activity and remain viable for many years.

**Cryopreservation**

Preservation of an organism at low temperature in a deep freeze at -20°C to -80°C or in liquid nitrogen is good and effective methods.

**Immobilization**

Immobilization can be carried out either by physical means such as adsorption or entrapment of the cells in a gel or foam matrix, or by chemical methods such as covalent binding. The advantages of immobilization are, stabilization of the catalytic activity resulting in increased product formation, ease of its separation from the medium and re-use of catalysts for extended periods of time.

**Biochemical and physiological characterization**

Photosynthesis, \( \text{N}_2 \) fixation, \( \text{NH}_3 \) excretion, pigment profile, carbohydrates, total soluble proteins and N-assimilatory enzymes like nitrogenase, nitrate reductase and glutamine synthetase activity plays key role for characterization.

**Measurement of pigments**

This is useful in quantifying surface growth dominated by cyanobacteria (Davey, 1988; Whitton and Roger, 1989) but less suited for sparse populations spread over the upper part of the soil column. The organisms can be frozen also to avoid degradation of pigments. The use of television image analysis with epifluorescence microscopy for cyanobacteria and other microorganisms has also been described (Wynn-Williams, 1990). Satellite sensor images offer a completely different approach to quantifying surface crusts. This method is similar to those used for studying semiarid vegetation index (Karnieli et al., 1996).

**Molecular approaches for classification**

Limitations of phenotypic characters have highlighted the requirement for more reliable methods and promoted molecular approaches in cyanobacterial taxonomy, including DNA base composition (Kaneko et al., 2001), DNA hybridizations (Kondo et al., 2000), gene sequencing (Nubel et al., 1997) and PCR fingerprinting (Rasmussen and Svenning, 1998; Versalovic et al., 1991). Cyanobacterial specific methods not requiring axenic cultures are of utmost importance since such cultures are difficult to obtain (Choi et al., 2008). PCR based techniques
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proved to be more reliable than the conventional ones in various aspects of identification of the cyanobacterial population and to uncover cryptic variations of strains or closely related species. The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by in vitro enzymatic replication. As PCR progresses, the DNA generated is used as a template for replication. With PCR it is possible to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece. PCR can be extensively modified to perform a wide array of genetic manipulations. Developed in 1984 by Kary Mullis, PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications.

The purpose of PCR is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing. There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time. During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop. The primers are jigging around, caused by the brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore. Elongation is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match get loose again (because of the higher temperature) and don’t give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3’ side (the polymerase adds dNTP’s from 5’ to 3’, reading the template from 3’ to 5’ side, bases are added complementary to the template).

PCR amplification and sequencing of 16S rRNA and rpoC1 genes

Since universal primers for direct sequencing of 16S rRNA genes are usually designed to be used with axenic cultures, and available primers for rpoC1 amplification and sequencing are highly degenerate, specific primers were selected or designed in order to obtain clean sequences for both genes without the need for a cloning step.

Restriction fragment length polymorphisms (RFLPs)

The restriction fragment length polymorphisms (RFLPs) of particular PCR products can provide signature profiles specific to the genus, species, or even strain. Genetic characterization of cyanobacterial strains has been undertaken using RFLPs of the 16S rRNA gene (16S-ARDRA) (Lyra et al., 1997) and of the intergenic transcribed spacer region (ITS-ARDRA) (West and Adams, 1997). Furthermore, amplification of the 16S–23S rRNA ITS, which has been shown to be variable in length (Rocap et al., 2002; Iteman et al., 2002; Neilan, 2002, Laloui et al., 2002) and number in cyanobacteria, can also be used as an identification tool. Neilan et al., (1995) have also found heterogeneity in the cluster containing mostly heterocystous planktonic strains of Anabaena and Aphanizomenon genera by using PCR/RFLP of the phycocyanin locus with intergenic spacers. RFLP has revealed the genetic proximity of the Aphanizomenon with Anabaena and Nodularia with Nostoc. Also, it was found that the neurotoxic starins of Anabaena were identical while the hepatotoxic strains formed a heterogenous group.

Random amplified polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence. Unlike traditional PCR analysis, RAPD
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does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers’ sequence. Furthermore, by RAPD, polymorphisms can be easily analyzed by small amounts of template DNA. RAPD was used to generate unique and identifying profiles for members of cyanobacterial genera *Anabaena* and *Microcystis*. This method is based on the combination of the two 10-meroligonucleotides in a single PCR and provided specific and repeatable DNA fingerprints which made it possible to discriminate among all toxigenic cyanobacteria studied, to the taxonomic ranks of genera, species and strains. Analysis of DNA typing results obtained by this method clearly discriminates between genera *Anabaena* and *Microcystis*. Several amplified DNA fragments which were expected to be markers for a particular taxon with identical allozyme genotype were also observed on RAPD patterns. The good accordance between the RAPD and allozyme divergency, indicate the high reliability of RAPD analysis for the easy and rapid discrimination of cyanobacteria.

**Amplified fragment length polymorphism (AFLP)**

AFLP-PCR is a highly sensitive method for detecting polymorphisms in DNA. The procedure is divided into three steps: i) Digestion of total cellular DNA with one or more restriction enzymes and ligation of restriction half site specific adaptors to all restriction fragments. ii) Selective amplification of some of these fragments with two PCR primers that have corresponding adaptor and restriction site specific sequences. iii) Electrophoretic separation of amplicons on a gel matrix, followed by visualisation of the band pattern. The AFLP technology has the capability to detect various polymorphisms in different genomic regions simultaneously. It is also highly sensitive and reproducible. As a result, AFLP has become widely used for the identification of genetic variation in strains or closely related species of plants, fungi, animals, bacteria and cyanobacteria. There are many advantages to AFLP when compared to other marker technologies including randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and microsatellites. AFLP not only has higher reproducibility, resolution, and sensitivity at the whole genome level compared to other techniques, but it also has the capability to amplify between 50 and 100 fragments at one time. In addition, no prior sequence information is needed for amplification (Meudth and Clarke 2007). As a result, AFLP has become extremely beneficial in the study of taxa including bacteria, fungi, and plants, where much is still unknown about the genomic makeup of various organisms. AFLP analysis is based on selective amplification of DNA restriction fragments. It is technically similar to restriction fragment length polymorphism analysis, except that only a subset of the fragments are displayed and the number of fragments generated can be controlled by primer extensions. The advantage of AFLP over other techniques is that multiple bands are derived from all over the genome. This prevents over interpretation or is interpretation due to point mutations or single locus recombination, which may affect other genotypic characteristics. The main disadvantage of AFLP markers is that alleles are not easily recognized (Majer et al., 1998). The utility, repeatability and efficiency of the AFLP technique are leading to broader application of this technique in the analysis of cyanobacterial populations.

**DNA fingerprinting based on STRR and LTRR sequences**

Repetitive sequences constitute an important part of the prokaryotic genome (Van Belkum et al., 1998). Despite their unknown function, and lack of knowledge about how they are maintained and dispersed, the presence, widespread distribution and high conservation of these sequences make them methodologically important for DNA fingerprinting and allow their use as an alternative for the identification of species or strains and in diversity studies among related prokaryotes and for identification (fingerprinting) of microorganisms in general. However it has been suggested that they may regulate transcription termination or be
the target of DNA binding proteins responsible for chromosomal maintenance in the cell. In the particular case of cyanobacteria, a family of repetitive sequences, the short tandemly repeated repetitive sequences (STRRs), has been described (Mazel et al., 1990). These heptanucleotide sequences have been identified in several cyanobacterial genera and species, so far mostly in heterocystous cyanobacteria (Zheng et al., 1999; Wilson et al., 2000; Teaumroong et al., 2002; Lyra et al., 2005) but also in some non heterocystous ones. Furthermore, a 37 bp long tandemly repeated repetitive sequence (LTRR) has also been identified in some cyanobacterial species (Prasanna et al., 2006). Analysis of STRRs and LTRRs has been described as a powerful tool for taxonomic studies. Moreover, the specificity of these sequences has made STRRs useful even for non axenic cyanobacterial cultures.

As it can be difficult and time-consuming to establish axenic cultures of cyanobacteria, the developed PCR method with STRR or LTRR primers provides a useful method for studying the diversity of cyanobacteria in the natural environment, whether free living or symbiotic. A universal marker for DNA fingerprinting is the oligonucleotide csM13. It has already been tested in a small number of cyanobacteria (Valerio et al., 2005), and has a demonstrated ability even to discriminate strains of the same species. Techniques based on the enterobacterial repetitive intergeneric consensus (ERIC) have also been used for identification and discrimination purposes in some cyanobacteria (Lyra et al., 2001; Bruno et al., 2006). However, the method based on STRR and LTRR sequences is accurate in distinguishing and classifying even closely related strains of cyanobacteria.

References


