Evaluation of a new DNA amplification technique to screen cyanobacteria isolated from drinking water sources for microcystin toxins

Emina Atikovic

Faculty Mentors:
Miriam Steinitz-Kannan, PhD (Department of Biological Sciences)
Patrick Schultheis, PhD (Department of Biological Sciences)
Northern Kentucky University
Highland Heights, KY 41099

Abstract

The quality of drinking water may be significantly reduced by the presence of cyanobacteria capable of producing toxins, taste, and odor. Several different genera of cyanobacteria are capable of producing microcystin toxins. Moreover, toxin-producing cyanobacteria share specific ribosomal RNA (rRNA) sequences not found in non-toxin-producing strains. Consequently, an rRNA gene probe can be a useful tool for distinguishing potential toxin-producing strains of cyanobacteria from nontoxin-producing strains, regardless of genus. This study involved eight strains of cyanobacteria belonging to three different genera, which were isolated from drinking water sources at the Metropolitan Water District of Southern California. The cyanobacteria were cultured in Carolina Spring Water and Alga-Gro® concentrate. Enzyme Linked Immunosorbent Assay (ELISA) was set up to evaluate the level of microcystin toxin present. Although all isolates tested negative by ELISA, the organisms may still have the genetic capacity to produce toxin. To address this issue, the polymerase chain reaction was used to amplify 16S rRNA sequences specific to toxin-producing strains. Based on the data, the conclusion is that the cyanobacterial isolates tested from the Metropolitan Water District of Southern California do not have the genetic capability of producing microcystin toxins.

Background

Cyanobacteria are a diverse group of algae found in aquatic ecosystems, often forming blooms when nutrients are high. These blooms can form in drinking water reservoirs or other source water and interfere with water quality by producing toxins, tastes, and odors (Drikas et al 2001).

From the chemical and toxicological standpoint, cyanobacterial toxins are an abundant group of natural toxins (Drikas et al 2001). Chemically they can be divided into three groups: cyclic peptides, alkaloids, and lipopolysaccharides. Toxicologically they are classified as hepatotoxins (liver), neurotoxins (nerve), and irritants or allergic substances (Drikas et al 2001).

Several cyanobacteria, including Microcystis aeruginosa, have the potential to produce toxins commonly known as microcystins. Microcystins are hepatotoxins known to promote liver tumors and to inhibit specific enzymes, causing selective liver damage in humans and poisoning in livestock (Carmichael 1997). They belong to the family of cyclic heptapeptides, but the actual composition of microcystins varies between species and strains within the genus Microcystis and other cyanobacterial genera, including Anabaena, Nodularia, Nostoc, Oscillatoria, and Synetococcus (Carmichael 1992).

Methods to detect microcystins include nuclear magnetic resonance (NMR) spectroscopy and immunological procedures such as ELISA. All of these methods require a large sample size, are labor intensive, and are time consuming. Application of molecular biology techniques could make it easier to identify toxin-producing organisms without the need to culture them. Recently, Neilan et al (1997) compared the 16S rRNA gene sequences of several toxin- and non-toxin-producing strains of Microcystis. Based on this information they were able to design oligonucleotide prim-
ers that allowed them to use the polymerase chain reaction (PCR) to identify potential toxin-producing strains of cyanobacteria. In this procedure a pair of oligonucleotide primers is used to amplify a specific DNA sequence from a complex mixture.

During spring 2001 a preliminary study was conducted using the above technique. DNA was isolated from a toxin-producing strain of *Microcystis aeruginosa* obtained from Lake Inferior on the Northern Kentucky University campus. PCR was then performed using the strain-specific primers designed by Neilan. Representative data from this study are depicted in Figure 1. The 400 base pair (bp) control band was amplified using primers 27F1 (forward) and 409R (reverse). These primers hybridize (or bind by complementary base pairing) to 16S rRNA gene sequences that are conserved among all cyanobacteria whether they produce toxin or not. The 200 bp test band, which is specific for microcystin producing strains, was amplified using primers 209F (forward) and 409R (reverse). Thus, as did Neilan et al. (1997), I was able to correlate toxin production with specific rRNA gene sequences.

The Metropolitan Water District of Southern California has been having serious problems with algal blooms in their source water. One of its scientists, Dr. George Izaguirre, has isolated and cultured several cyanobacteria that he suspects are producing taste and odor compounds and potential toxins. Although none of these algae belong to the genus *Microcystis*, the fact that several belong to genera reported to produce microcystins suggests that they should possess the genes for its production, even when no toxins are detected by ELISA methods. The genes may be there but turned off. The present study was undertaken to use the new gene amplification technique to test Dr. Izaguirre’s cultures for the microcystin-producing genes. To obtain the DNA from the cultures, a sufficient quantity of algae must be available. Dr. Izaguirre provided only a few milliliters of culture material, some of it not pure. A large part of this project included finding the best way of growing these algae and keeping them alive until the completion of the project.

**Hypothesis**

An rRNA gene probe can be a useful tool for distinguishing potential toxin-producing strains of cyanobacteria from non-toxin-producing strains, regardless of genus.

**Methods and Materials**

Dr. George Izaguirre from the Metropolitan Water District of Southern California provided the cultures obtained from drinking water sources for use in screening for the Microcystin gene. The cultures, shipped overnight in 5 ml flasks and refrigerated until the start
of the project, were *Pseudanabaena* SV996 from San Vicente Reservoir; *Synechococcus* BCR 1092 from Big Canyon Reservoir; *Pseudanabaena* from Castaic Lake; *Pseudanabaena* LW397 from Lake Whitehurst; *Pseudoanabaena* IN 620 and *Pseudanabaena* LS 1196a from Lake Skinner; *Anabaena lemmermannii* CL799 from Castaic Lake. All of these strains of cyanobacteria, except for the last one, are producers of methylisoborneol (MIB), a compound that causes tastes and odors in drinking water. *Anabaena lemmermannii* produces geosmin, another taste-and-odor compound. A sample of a *Microcystis aeruginosa* bloom that occurred in Lake Inferior at NKU and tested positive for toxin production by the ELISA method was used as a positive control. One culture of a non-toxic green alga, *Pediastrum*, obtained from Carolina Biological Supply Company, was used as a negative control.

**Algal Culture Procedure**

The eight samples of algae obtained were cultured by using Carolina Spring Water. First, 1200 ml of Carolina Spring Water was mixed with 24 ml of AlgaGro® concentrate. The solution was mixed thoroughly and the pH was adjusted to 7.8. For pH adjustment 0.5N NaOH and 0.5N HCl were used. After the pH was brought to 7.8, 150 ml of the prepared media was placed into each of eight 250 ml sterile tissue culture flasks, followed by the addition of algae concentrate. This mixture was incubated at 22 to 25 °C and a photoperiod of 12 hours light and 12 hours of dark using a shaker, for approximately 2 weeks.

**Microcystin Toxin Assay**

Strategic Diagnostics Inc. EnviroGard® Microcystin Plate Kit 75400 was used for detection of microcystin presence in all eight test samples.

**Algal Concentration and DNA Extraction**

A 1 ml aliquot of a late-log phase algal culture was concentrated by centrifugation (IEC HN-SII centrifuge-International Equipment Company) for 2 to 3 minutes, the medium was decanted, and the pellet was resuspended in a solution of 500 μl of 50 millimolar (mM) Tris-HCl (pH 8.0), 5 mM EDTA, and 50 mM NaCl. Lysozyme was added to obtain a final concentration of 10 mg/ml, and the solution was incubated at 55 °C for 30 minutes. After incubation, 10 μl of proteinase K (20 mg/ml) and 20 μl of 10% sodium dodecyl sulfate were added, and the mixture was incubated at 55 °C for 20 minutes or until the solution cleared (complete cell lysis). The solution was chilled on ice and then extracted with an equal volume of phenol-chloroform-isomylalcohol. The organic extraction was repeated, and the supernatant was added to an equal volume of 4M-ammonium acetate. Total genomic DNA was precipitated by the addition of 2 volumes of isopropanol followed by centrifugation for 10 minutes at room temperature. This procedure was adapted from Neilan et al (1995).

**PCR Amplification of DNA**

The DNA isolated from algae cultures was analyzed by PCR. The PCR reactions were carried out in a total volume of 20 μl containing 200 μM dNTPs, 2 mM MgCl₂, 1 unit of TAQ polymerase, 1×PCR buffer (Life Technologies), 1 μl DNA, and 1 pmol of each primer. Amplification was performed using a MJ Research PTC-100 Thermal Cycler. Samples were initially denatured at 94 °C for 4 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing (55 °C for 30 seconds), and extension (72 °C for 1 minute). The 400 bp test band, which is specific for cyanobacterial strains able to produce toxins, was amplified using the primers 209F (ATGTGC-GAGGTGAAACCTAAT) and 409R (TTACAA(C/T)CCAA(G/A) (G/A)CAG) and 409R (TTACAA(C/T)CCAA(G/A) (G/A)CCTTCCCTCCC). The 200 bp test band, which is specific for cyanobacterial strains able to produce toxins, was amplified using the primers 209F (ATGTGC-GCGAGGTAACCTAAT) and 409R.

**Gel Electrophoresis**

Following PCR, samples were analyzed by gel electrophoresis. The agarose gel was prepared by adding 2 grams of agarose to 20 ml of 5 x TBE and 80 ml of distilled water. The agarose was dissolved into solution by microwaving it for about 1 minute. 5 μl of Ethidium Bromide (5 mg/ml) was added to the solution after it was allowed to cool to about 50 °C. The gel was poured into a mold with the plastic comb in place to create wells. The gel was submerged in a buffer made of 160 ml of 5 x TBE and 640 ml distilled water. Next, with a micropipette, 1 μg of a 1 kilobase (kb) plus DNA ladder was placed in the first well. Then 1 μl of loading dye was added to each of the three samples of alga DNA. Each of the samples were transferred to a separate well in the gel. Electrophoresis was performed for 1 hour. After 1 hour, the gel was removed from the buffer and placed on a UV transilluminator for examination. Photographs from the gel were taken.
with a Kodak digital camera. If predictions are correct, these photographs should show a 400 bp control band. Also, DNA samples isolated from potential toxin-producing cultures should also show a 200 bp 16S rRNA gene band.

Results
The use of Carolina Spring Water along with Carolina Alga-Gro® concentrate as sterile enrichment algal medium, resulted in a vigorous growth in all isolates after only 2 weeks.

Table 1, Figure 2, and Figure 3 show results of ELISA testing. None of the eight test samples showed a concentration gradient higher than 0.2 parts per billion (ppb). The positive control, *Microcystis aeruginosa*, (S10 on Table 1) had a concentration gradient of 0.995 ppb.

Table 1: Results from ELISA testing of *Microcystis aeruginosa*, *Pediastrum*, and 8 samples of cyanobacteria

<table>
<thead>
<tr>
<th>Samples</th>
<th>Average Absorbance</th>
<th>Concentration (ppb)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1**</td>
<td>2.020</td>
<td>0.001</td>
</tr>
<tr>
<td>S2</td>
<td>2.038</td>
<td>0.000</td>
</tr>
<tr>
<td>S3</td>
<td>2.024</td>
<td>0.000</td>
</tr>
<tr>
<td>S4</td>
<td>1.930</td>
<td>0.041</td>
</tr>
<tr>
<td>S5</td>
<td>1.972</td>
<td>0.022</td>
</tr>
<tr>
<td>S6</td>
<td>1.975</td>
<td>0.021</td>
</tr>
<tr>
<td>S7</td>
<td>1.926</td>
<td>0.043</td>
</tr>
<tr>
<td>S8</td>
<td>1.884</td>
<td>0.062</td>
</tr>
<tr>
<td>S9</td>
<td>1.948</td>
<td>0.033</td>
</tr>
<tr>
<td>S10***</td>
<td>0.656</td>
<td>0.995</td>
</tr>
</tbody>
</table>

*Concentration gradient (ppb). **Negative control sample. ***Positive control sample.

As seen in the Figure 4 the first wells represent negative and positive controls. The negative control (*Pediastrum*) is represented in wells 1 and 2 and, as a result, had no detectable bands. Wells 3 and 4 contained samples from the toxin-producing strain of *Microcystis aeruginosa*, which were used as a positive control. The resulting migration from electrophoresis shows one band at 200 bp (well 3), common for toxin producing strains. It also shows the band at 400 bp (well 5), which is present in all cyanobacteria, whether they are toxin producers or not.

Samples obtained from Dr. Izaguirre are present in wells 5 through 20. None of these samples had observable bands at 200 bp, but all of them (except well 6) had the 400 bp control band.

Discussion
The results of this investigation show that cyanobacterial isolates from the Metropolitan Water District of Southern California did not possess the rRNA sequences found in toxin-producing strains of microcystis. As mentioned before, these algae could possess the genetic capacity to produce toxins even though these toxins were not detected by ELISA methods (see Table 1). All eight test samples had concentration gradients for toxins lower than 0.2 ppb. The genes may be there but simply not expressed.

Having the positive and negative controls as points of reference made it possible to test eight samples for the Microcystin gene. As expected, the negative control (lines 1 and 2), *Pediastrum*, did not show any 200 bp and 400 bp bands because this particular algae is not a cyanobacteria. In contrast, *Microcystis aeruginosa* is a highly toxic cyanobacterium that, like all other members of this group, shows the control band at 400bp (line 4). In addition, this isolate also had the band appearing at 200 bp (line 3), which indicates microcystin toxin genes. As a consequence, this sample was used as a positive control.

As shown in Figure 4, seven samples (*Synechococcus LP1181, Synechococcus BCR 1092, Pseudanabaena Cast. ’93, Pseudanabaena LW397, Pseudanabaena LS 1196a, Pseudanabaena IN 620, and Anabaena lemmermanii, CL799*) have a control band appearing at 400 bp, but no detectable 200 bp test bands. Sample 3 (*Pseudanabaena SV 996*) had no 400 bp bands, because the procedure described by Neilan...
et al (1995) at one point required that the DNA be extracted with two volumes of isopropanol.

This made it necessary to divide the sample, and it most likely reduced the yield of precipitated DNA.

Lack of the 200 bp band in all of the tested samples suggests that these algae do not have the genetic capacity for potential production of microcystin.
The attempt to test eight cyanobacterial cultures for their microcystin production led to the conclusion that none of these algae have the genetic capability for toxin production. Nevertheless, this study should be repeated using other strains of different genera of cyanobacteria that may have the genes for microcystin and do test positive for the toxin. That will validate this method for assaying the “potential” of toxicity in drinking water samples. In addition, the present study opened possibilities for further research that could be done on detecting other toxins that these algae could potentially produce.

Acknowledgements

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References


