African Journal of Microbiology Research Vol. 6(3), pp. 648-652, 23 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1517
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Isolation and molecular identification of metal resistant *Synechocystis* from polluted areas

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Accepted 27 December, 2011

The 16S rRNA genes were amplified after using cyanobacterial primers from two *Synechocystis* strains isolated form tannery effluents areas, near Lahore, Pakistan. Prior to ribotyping, their measured growth and chromium reduction potentials were checked. The amplified 16S rRNA genes were 700 base pairs approximately in both strains. Only forward primer gave sequence and reverse primer failed to give proper sequence in both strains. The obtained sequence was of 630 bases in *Synechocystis* "MK (S)" strain and 631 bases in *Synechocystis* "P2A" strain. The 16S rRNA gene partial sequence of *Synechocystis* strain "MK (S)" obtained from the sequenced PCR product showed 98% and *Synechocystis* Strain "P2A" showed 97% which is similar to reference strain *Synechocystis* PCC 6803, thus confirming their cyanobacterial identity and relationship with *Synechocystis* PCC 6803 through BLAST analysis. The DNA sequence was submitted and the accession number of *Synechocystis* "MK (S)" strain 16S rRNA gene was DQ381960; and its identity is now known as *Synechocystis* sp. AHZ-HB-MK, while that of *Synechocystis* "P2A" strain 16S rRNA gene was DQ398589 and its identity is now known as *Synechocystis* sp. AHZ-HB-P2A.

Key words: *Synechocystis*, cyanobacteria, chromium, ribotyping.

INTRODUCTION

Identification of metal resistant cyanobacterial isolates has traditionally been accomplished morphologically, biochemically and physiologically by determining the shape, size and assimilation patterns of a number of carbohydrates and nitrogen sources (Victor et al., 2011). A more accurate method for genotype determination is that of the molecular biology approach of ribotyping by comparing similarities in the rRNA gene sequences. The sequence data for the 16S rRNA gene is highly conserved for different organisms and has also been shown to be very accurate for genus and species identification of eubacteria. Carl Woese and his colleagues were responsible for the initial use of rRNA sequence data to examine evolutionary relationships among bacteria by comparing ribonuclease T_1-generated oligonucleotides (Woese et al., 1976). Subsequently, a number of authors have identified constructed and published phylogenetic trees based on 16S rRNA gene sequence (Shapiro et al., 2011). Comparisons of the sequence between different species suggest the degree to which they are related to each other. A relatively greater or lesser difference between two species suggests a relatively earlier or later time in which they shared a common ancestor. 16S rRNA gene sequence has properties, which predestine it as a universal phylogenetic marker in prokaryotes. There are regions on the 16S rRNA gene that are quite conserved and others, which are variable. Comparing the differences in the base sequence of 16S rRNA gene, therefore, is an excellent means to study evolutionary changes and phylogenetic relatedness of organisms (Lucia et al., 2011). Hence genotypic classification based on nucleotide sequence comparison of 16S rRNA gene has become available as

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additional taxonomic tool. Using this new standard, phylogenetic trees based on base differences between species, are constructed and cyanobacteria are classified and reclassified into new genera. The advantage of 16S rRNA gene analysis is that it can potentially be applied to the identification of all cyanobacteria. Molecular identification techniques provide two primary advantages to phenotypic identification: a more rapid turnaround time and improved accuracy in identification (Woo et al., 2008; Cloud et al., 2010). Molecular phylogeny increasingly supports the understanding of organismal relationships and provides the basis for the classification of microorganisms according to their natural affiliations.

Some of the molecular methods used for taxonomic studies of bacteria have also been applied to cyanobacteria (Allewalt et al., 2006), sometimes with modifications applicable to phototrophs. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has by far been the most common housekeeping genetic marker used for a number of reasons. These reasons include:

(i) Its presence in almost all bacteria, often existing as a multigene family, or operons.
(ii) The function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution) and
(iii) The 16S rRNA gene (1,500 bps) is large enough for informatics purposes (Patel, 2001).

MATERIALS AND METHODS

Cyanobacterial isolation and characterization

Two strains of *Synechocystis,* Strain "P2A" Synechocystis and strain "MK (S)" Synechocystis were isolated from chromium-polluted effluents samples collected from Muridkey and Kasur near Lahore, Pakistan. Strain P2A is from tannery effluent of Kasur and strain MK (S) is from tannery effluent of Muridkey. Cells were grown under a 12 h:12 h light-dark cycle at 28^{o}C in BG11 medium (Rippka et al., 1979). Cyanobacteria were examined at ×1000 magnification. Description of taxa was based on morphological descriptions following Komárek (2003).

Growth measurements

Two methods were tried for the determination of comparative growth of both strains in equal time intervals (days).

I) *Chlorophyll-a estimation*

Following the method of Sartory (1982) for chlorophyll-a concentration, 15 days old culture growth measurements were taken. A known volume of culture media was filtered with Whatman GF/C filter paper. The residue was placed along with filter paper in scintillation bottle with 10 ml 95% industrial ethanol in water bath at 78°C for five min, and the contents were cooled down to room temperature in the dark. Absorbance was monitored at 665 and 750 nm. 0.1 ml HCl with a concentration 0.3 mol l^{-1} was added to the sample, and after 2 min, the absorbency was observed at 665 and 750 nm.

II) *Cell density measurement*

This was carried out according to Song et al. (1998). The thoroughly washed and dried haemocytometer was used. The volume taken each time was 50 µl and this procedure was repeated three times.

Number of cell ml^{-1}= Average number of cell x5000 ($0.2x10^{-3}$) x dilution factor.

Chromate reduction experiments

Cyanobacterial chromate reduction was checked at three initial K_2CrO_4 concentrations (50, 100 and 200 µg ml^{-1}) using BG11 medium. Cultures were kept at 28ºC. After 15 and 30 days respectively, samples were taken aseptically and were analysed for Cr (VI) reduction (Clesceri et al., 1998). Reduction of Cr (VI) by cyanobacterial strains was monitored in the culture supernatant using the classical spectrophotometer method by reaction with diphenylcarbazide in acid solution. The absorbance was measured at 540 nm. Cr (VI) concentration was determined according to a calibration curve established under the same experimental condition by using a standard Cr (VI) solution ranging from 1 to 100 µg ml^{-1}.

16S rRNA sequencing

The 16S rRNA gene was amplified and the primers used were designed following Nubel et al. (1997). The forward primer 5'CGGACGGGTGAGTAACGCGTGA'3 and reverse primer 5'GACTACAGGGGTATCTAATCCCATTT'3 were used for amplification of 16S rRNA genes. Gel extraction of amplified DNA was done with QIAquick Gel Extraction Kit (Cat. # 28704, Qiagen, Inc.). The eluted contents were concentrated to approximately 25 µl, and checked on gel by running up to 5 µL. DNA was stored at –20°C till further use. All sequencing attempts were made in Beckman Coulter® (CEQ 8000) Genetic Analysis System sequencer and data was analysed by the software, CEQ System (Ver.9.0.25). Out of the total obtained data, the meaningful data was further processed. The sequences obtained were compared with known sequences using BLAST (Altschul et al., 1990). The sequenced data was then submitted to GenBank, in order to obtain the accession numbers for sequenced gene fragments.

RESULTS

Growth and morphology

The cells of both strains are solitary and mostly in pairs. It has no mucilage or very fine narrow colorless sheath. In both strains, the cells are pale in color and their diameter is 2.95 to 3 µm. Both strains belong to same genus; however, there is a distinct difference in growth performance. *Synechocystis* strain MK (S) showed rapid and better growth (maximum growth was 7.8 mg l^{-1}

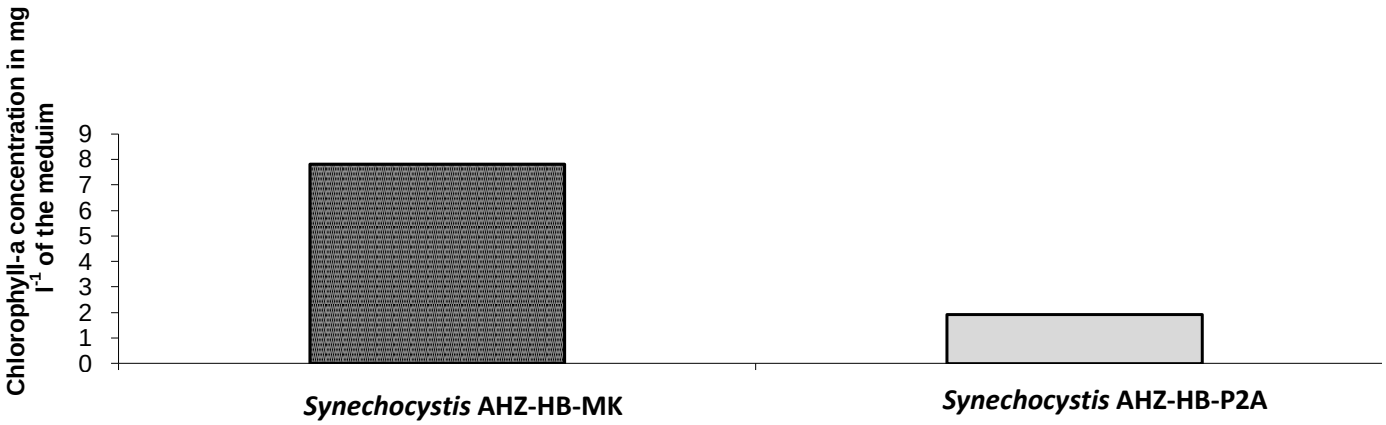

Figure 1. Growth measurements of *Synechocystis* strains (Chlorophyll-a estimation). *Synechocystis* strain MK (S) showed rapid (maximum growth was 7.8 mg L^{-1} chlorophyll-a concentration in BG 11 medium) growth when compared with *Synechocystis* P2A (the maximum growth was 1.91 mg L^{-1} chlorophyll-a concentration in BG 11 medium).

chlorophyll-a concentration in BG 11 medium) when compared with *Synechocystis* sp P2A (the maximum growth was 1.91 mg l^{-1} chlorophyll-a concentration in BG 11 medium) (Figure 1). Same results of rapid growth were also obtained through cell counting measurements (Figure 2), which showed *Synechocystis* strain MK (S) as 4.98 million, while *Synechocystis* sp P2A as 0.22 million increase in cell numbers per ml of medium within seven days time period.

Chromium reduction

Synechocystis sp MK (S) tolerated more chromium (up to 250 µg ml^{-1}) than *Synechocystis* sp P2A (up to 100 µg ml^{-1}). Both have good Cr (VI) reduction potential with little difference as *Synechocystis* sp MK (S) has 62.14% and *Synechocystis* sp P2A has 60.98% at an initial chromium concentration of 100 µg ml^{-1}. This reflect their physiological adaptation for chromium stress (Hameed and Hasnain, 2005) (Table 1).

16S rRNA sequencing

The amplified 16S rRNA gene is 700 base pairs approximately. Only forward primer (5'CGGACGGGTGAGTAACGCGTGA'3) gave sequence for both strains and reverse primer failed to give proper sequence. The obtained sequence has 630 and 631 bases in *Synechocystis* sp MK (S) and *Synechocystis* sp P2A, respectively. The 16S rRNA partial sequence of *Synechocystis* strain "MK (S)" obtained from the sequenced PCR product showed 98% similarity to *Synechocystis* PCC 6803. The 16S rRNA partial sequence of *Synechocystis* strain "P2A" obtained from the sequenced PCR product showed 97% similarity to *Synechocystis* PCC 6803, thus confirming their cyanobacterial identity and relationship with *Synechocystis* PCC 6803 through BLAST analysis. The DNA sequence was submitted and the accession number of *Synechocystis* strain "MK (S)" which was DQ381960 and that of *Synechocystis* Strain "P2A" which was DQ398589 were obtained.

DISCUSSION

Two strains belonging to genus *Synechocystis* were isolated from polluted areas and checked for chromium resistance, reduction and production of non-protein thiols as a possible mechanism of chromium detoxification. It was found that one strain *Synechocystis* sp. AHZ-HB-MK showed more chromium resistance, reduction and production of non-protein thiols, when exposed to chromium stress, as compared to other strain such as *Synechocystis* sp. AHZ-HB-P2A which has a probable mechanism of chromium detoxification (un-published data). Protein profile bands resolved on SDS-PAGE showed high similarity of these strains with each other (presence of only one extra protein band of about 51 KDa in *Synechocystis* sp. AHZ-HB-P2A).

Identification and classification are important steps in investigating whether a cyanobacterium has potential use in bioremediation/biotechnology (Dubey et al., 2011; Geoffrey, 2010). The identified strains can be studied further and their characteristics can be compared to other organisms of the same specie to find any unique characteristics that might make the newly identified organisms better suited for use in biotechnology and their genetic manipulation can be done in order to get more desirable results. Cr (VI) reduction by microbes has been

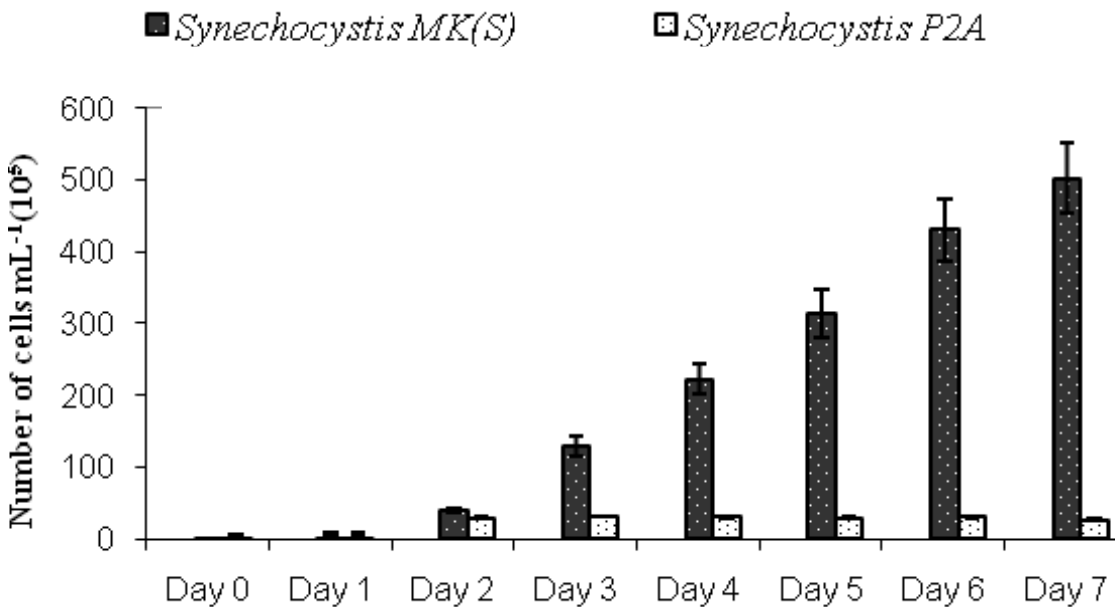

Figure 2. Growth measurements of *Synechocystis* strains (cell density measurement-Cell counting). Cell density was measured using a hemacytometer after inoculating the cells from a full grown culture. The increase in cell numbers from the respective previous period of 1-7 days in *Synechocystis* strain MK (S) were (96.98, 381.10, 228.30, 71.54, 41.25, 36.83 and 16.70%) and in *Synechocystis* strain P2A were (23.10, 334.10, 5, -3.14, -2.60, 3.30 and-11.30%) respectively

studied extensively (Thengodkar and Sivakami, 2010; Abed et al., 2009) but relatively, natural microbial communities were rarely used for Cr (VI) reduction (Arias and Tebo, 2003; Marsh et al., 2000). Cyanobacteria of local habitat have been seldomly screened for biological effects (Mundt et al., 2001), but now more frequently (Hussain and Hasnain, 2011; Victor et al., 2011; Hameed and Hasnain, 2005). Therefore, in this study, the main theme was to investigate cyanobacterial role especially from industrial effluents in their local environment in being a potential candidate for Cr (VI) detoxification in wastewaters. Strain *Synechocystis* AHZ-HB-MK, was a better reducer of hexavalent chromium to trivalent chromium (>60% reduction potential).

Traditional methods of classifying cyanobacteria rely heavily on morphological and biochemical characteristics. Taxonomists who were involved in these classification schemes during this time were limited by the absence of culture strains, advanced light microscopes and biochemical tests. Currently, there is an increased interest in applying molecular techniques to resolve many of the issues and problems created by the present state of cyanobacterial taxonomy. Within the last decade, a number of cyanobacteriologists began using molecular techniques to answer questions dealing with the taxonomy, population dynamics, and the evolution of cyanobacteria (Valerio et al., 2009). The 16S rRNA gene partial sequence of *Synechocystis* strain, “MK (S)” and “P2A” obtained from the sequenced PCR products showed 98 and 97% similarity to *Synechocystis* PCC 6803, thus confirming their cyanobacterial identity and relationship with it and they are now named as *Synechocystis* AHZ-HB-MK and *Synechocystis* AHZ-HB-P2A, respectively. Both strains showed 98% similarity when compared with each other through BLAST analysis. Members of genus *Synechocystis* are planktonic in fresh water reservoirs or in saline or sea waters. Others grow in metaphyton of pools, thermal and mineral springs, saline (brackish) swamps or in moors (Komárek, 2003). Foster et al. (2006) have used cyanobacterial 16S rDNA primers following Nubel et al. (1997) to investigate the phylogenetic diversity of cyanobionts associated with non-photosynthetic eukaryotic hosts. The phylogenetic tree analysis of both strains showed that although their homology/similarity is 98%, but still many other organisms are more phylogenetically identical with them than they are with one another. In this study, both cyanobacterial and bacterial communities exhibited noticeable changes concomitant with degradation of the compounds. Indeed, all or some of the populations might played a significant role in metabolizing the petroleum compounds. It may also be concluded that the microbial mats from Wadi Gaza, Palestine were rich in microorganisms with high biodegradative potential. Other closely related cyanobacterium, *Woronichinia naegeliana* (Rajaniemi-Wacklin et al., 2006) was isolated and

Table 1. Effect of different pH (6, 7, 8 and 9) and temperature (25, 30 and 35ºC) on chromium reduction by the cyanobacterial cells at an Initial chromate concentration of 100 µg .

Strains	Temperature (ºC)	pH			
		6	7	8	9
Synechocystis MK (S)	25	49.11±1.2	49.82±1.5	47.0±1.5	42.86±1.0
	30	57.77±1.8	62.15±1.2	56.52±1.6	59.28±1.9
	35	40.36±1.3	40.71±1.1	38.21±1.1	47.86±1.4
Synechocystis P2A	25	54.02±1.9	44.73±1.3	46.43±1.1	52.68±1.5
	30	56.25±1.8	60.98±1.9	52.06±1.3	58.30±1.9
	35	42.15±1.2	38.30±1.2	39.92±1.1	42.05±1.3

characterized among strains belonging to the genus *Woronichinia*. The results showed that *Woronichinia* and a monophyletic cluster of *Snowella* strains were grouped together with a high bootstrap values in the 16S rRNA gene analyses and their morphology were in agreement with their phylogeny. In addition, *Snowella* and *Woronichinia* were closely related to *Merismopedia* and *Synechocystis* strains.

The results of our present study indicate that biological Cr (VI) removal in wastewater might be very promising because

i) Both strains preferred a pH and temperature optima which is according to the environmental requirements.
ii) Strains were able to reduced significant amount of Cr (VI).

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