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Induction of non protein thiols by chromium in cyanobacteria isolated from polluted areas

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Two cyanobacterial strains *Synechocystis* "AHZ-HB-MK" and "AHZ-HB-P2A" were isolated from tannery effluent form Lahore, Pakistan and their tolerance against K_2CrO_4 was examined. Although growth was completely inhibited at 10 µg mL^{-1} in AHZ-HB-P2A strain, cell growth of AHZ-HB-MK strain was observed even at a concentration of 100 µg mL^{-1}. To show reasons for different tolerance between the two strains, we compared changes in nonprotein thiols, glutathione (GSH and GSSG), cysteine and cystine, after K_2CrO_4 treatment in both strains. After K_2CrO_4 treatment GSH completely disappeared in both strains, suggesting that GSH was involved in detoxification. GSSG content in former strain remarkably increased almost three and six times after 7-days treatment with 10 and 100 µg mL^{-1} K_2CrO_4, respectively while it decreased in the later strain. Cysteine content in the former strain increased almost three and eleven times after 1 day and 7 days treatment with 100 µg mL^{-1} K_2CrO_4, respectively, such increase in cysteine content was not observed in later strain. These data revealed that former strain could induce glutathione and cysteine but later strain could not. The difference in the ability to induce the nonprotein thiols between two strains must be responsible for differential tolerance in K_2CrO_4 observed in the growth inhibition.

Key words: Chromium, cysteine, cyanobacteria, glutathione, nonprotein thiol, *Synechocystis*.

INTRODUCTION

Chromium is an essential trace element for organism (Anderson 1998; DiBona et al., 2011; Ghosh et al., 2002; Vincent, 2000) however, high concentrations of chromium are toxic (Cheng and Dixon,1998; Kim et al., 2002; Medeiros et al., 2003) carcinogenic (Singh et al., 1998) genotoxic (Godet et al., 1996) and teratogenic (Asmatullah and Shakoori, 1998). Chromium is used in the manufacture of alloys, corrosion inhibitory paints, wood preservatives, mordants and fixatives for dyes and tanning, photographic sensitizers and pigment for rubber and ceramics and as an anticorrosive in cooking systems and boilers (Cotman et al., 2004) and chromium pollution in aquatic environments is now threatening ecological systems and human health in several regions in the world (Leghouchi et al., 2009; Shankar 2009; Tziritis et al., 2012; Yang and Liu 2012).

Bioremediation is one of expected method to remove toxic chemicals from polluted areas. Autotrophic organisms such as plants and algae suit for bioremediation of inorganic chemicals, the process is known as "phytoremediation" (Ghosh and Singh, 2005; Jadia and Fulekar, 2009; Kumar and Goyal, 2009). Since cyanobacteria are

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Abbreviations: GSH, Glutathione reduced form; **GSSG,** glutathione oxidized form.

ubiquitous and their physiological and genomic backgrounds are well known as compared to other algae, hence they may be potential candidates for phytoremediation. In the present study, we isolated two strains of *Synechocystis* species from chromium polluted areas in Pakistan, and examined tolerance against chromium and changes of nonprotein thiols after chromium treatments.

Glutathione, the tripeptide γ-glutamylcysteinylglycine, is a major source of nonprotein thiols in most organisms. The major roles of glutathione are to protect cells from environmental stress such as oxidation which results in the production of reactive oxygen species, and from xenobiotic electrophiles and heavy-metal ions having high electronegativity by combining with the sulfhydryl groups (Meister, 1975; Meister and Anderson, 1983; Marrs 1996; Coleman et al., 1997). There is possibility of glutathione involvement in Zn, Ni and Cr uptake (Gharieb and Gadd, 2004). Cysteine is also a major source of nonprotein thiol, but its roles in detoxification and antioxidant activities have been paid less attention. However, since cysteine is usually more abundant than glutathione in higher plants (Duke, 1992), it is known that addition of cysteine together with chromium alleviate the toxicity of chromium in a cyanobacterium *Anabaena doliolum* (Duke, 1992). Thus, cysteine must act as the first line of defense against chromium. The aim of this study was to determine whether glutathione and cysteine are involved in tolerance against chromium in the isolated strains. Understanding of the mechanism of their tolerance is expected to give useful information on how to utilize them for phytoremediation of chromium.

MATERIALS AND METHODS

Plant material and growth conditions

Two strains of *Synechocystis* sp. were isolated from chromium-polluted effluents in the North Northeast of Pakistan. Strain AHZ-HB-P2A is from tannery effluent of Kasur district and strain AHZ-HB-MK is from tannery effluent in Muredkey locality near Lahore (Hameed and Hasnain, 2005) and their molecular identification was also performed (Hameed and Hasnain, 2012). The chromium concentration was 0.50 µg mL^{-1} in "AHZ-HB-P2A" strain isolation site (it was isolated from a pond of treatment plant) and 2.20 mg g^{-1} in AHZ-HB-MK strain isolation site (as it was isolated from moist soil of polluted site). The cells of both strains are solitary and mostly in pairs, have no mucilage or very fine narrow colorless sheath, cells are pale green in color and diameter is about 3 µm. The fluorescent tube lights used were of light intensity 5.8 to 7.8 µE m^{-2} s^{-1}. Cells were grown under a 12 h:12 h light-dark cycle at 28°C in BG11 medium (Rippka et al., 1979). Chromium was supplemented as K_2CrO_4 under same growth conditions for controls.

Extraction of nonprotein thiols

Procedure of extraction of nonprotein thiols was carried out following Satoh et al. (2002) with modification in which GSH was retained in its reduced form as per Anderson (1985). Nonprotein thiols were extracted at 6 h after starting the light period in the culture. Cells were collected from 300 mL of culture by centrifugation at 940 x*g* for 10 min and suspended in glass distilled water. Trichloroacetic acid was added to the cell suspension to final concentrations of 5%. The cells were completely disrupted by sonication with a Bioruptor (COSMO BIO, Tokyo, Japan) at 0°C and then separated by centrifugation at 11,000 x*g* for 15 min at 4°C. The supernatant was used for assays. In each extraction the number of cells in cultures was calculated from cell density measured using a hemacytometer.

Analysis of nonprotein thiols

Total GSH and GSSG were measured by the GSSG recycling method (Anderson, 1985) with GSSG as the standard. The final concentration of glutathione reductase (Boehringer Mannheim GmbH, Mannheim, Germany) was 0.5 unit mL^{-1}. Cysteine and cystine were measured using the acid ninhydrin technique of Gaitonde (1967). Nonprotein thiol recovery was determined by comparing GSH and GSSG contents between two samples from the same cell culture, one of which was externally added GSH prior to disruption of cells by sonication. Data were obtained from a minimum of three independent experiments. A *t*-test was used to determine statistical significance.

Estimation of cell biovolumes

Biovolumes of the strains AHZ-HB-MK and AHZ-HB-P2A were calculated to be 14.3 and 13.9 μm^3 respectively, according to the formulae of Hillebrand et al. (1999) considering the cell shapes of both strains to be sphere or spheroid. More than fifty cells were estimated from each strain. Cellular concentrations of non-protein thiols were calculated by dividing the non-protein content per cell by biovolume.

RESULTS

Figure 1 shows inhibition of growth by K_2CrO_4 in both *Synechocystis* strains AHZ-HB-MK and AHZ-HB-P2A. Cell of AHZ-HB-MK strain could grow under the conditions at 10 and 100 µg mL^{-1} of K_2CrO_4 although cell densities after 7 days were 1/14 and 1/23 as compared to the culture without K_2CrO_4. However, cell growth of AHZ-HB-P2A strain almost completely inhibited by 10 µg mL^{-1} K_2CrO_4, and even cell density slightly decreased after two days at 100 µg mL^{-1}. Interestingly, cell density of both strains slightly decreased after one day but increased after two day in the condition of 100 µg mL^{-1} K_2CrO_4, but the strain AHZ-HB-P2A could not grow the following days while the AHZ-HB-MK strain continued to grow. These results determine that AHZ-HB-MK strain is much more tolerant to K_2CrO_4 than AHZ-HB-P2A strain.

We examined changes in nonprotein thiols after K_2CrO_4 treatment in the two strains (Figures 2 and 3). Nonprotein thiols per intact cell of AHZ-HB-MK strain are 6.15 amol (corresponding to 0.43 mM) for GSH 10.7 amol (0.75 mM) for GSSG 17.3 amol (1.2 mM) for cysteine and 40.2 amol (2.8 mM) for cystine. Those for AHZ-HB-P2A strain are 6.74 amol (0.48 mM) for GSH and 6.59 amol (0.47 mM) for GSSG, 24.4 amol (1.76 mM) for cysteine and 13.0 amol (0.94 mM) for cystine. Content and concentration of each molecule is similar between the two strains

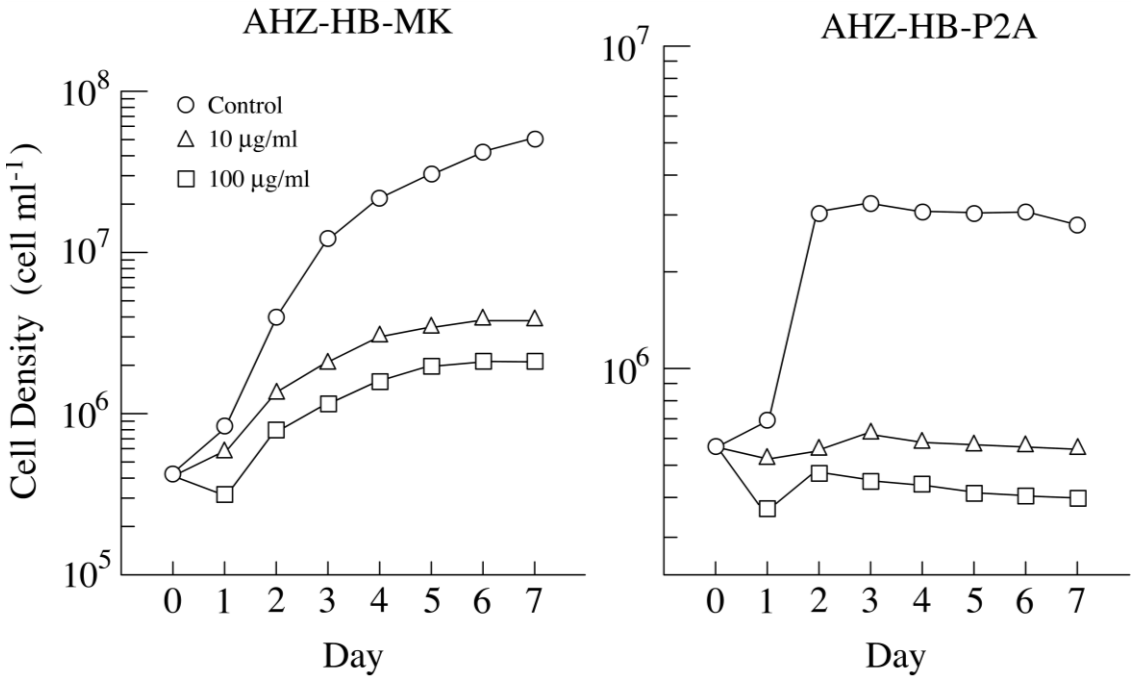

Figure 1. Growth inhibition by K_2CrO_4 in two strains of *Synechocystis* sp. AHZ-HB-MK and AHZ-HB-P2A. Cell density was measured using a hemacytometer after inoculating the cells from a full grown culture into medium containing 0, 10 and 100 µg mL^{-1} of K_2CrO_4.

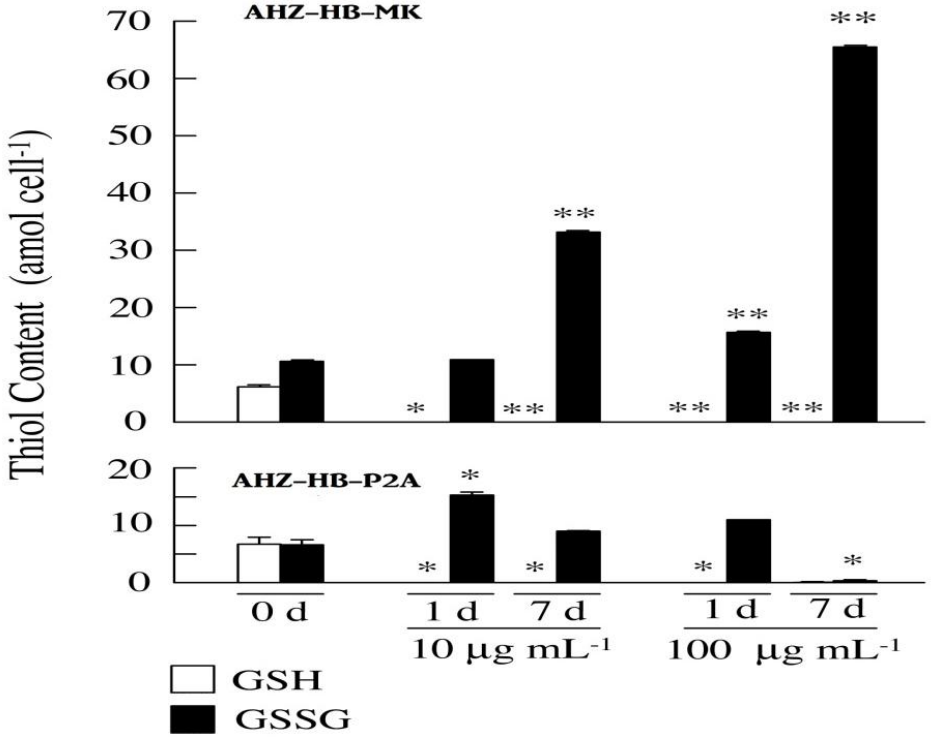

Figure 2. Changes in nonprotein thiol (Glutathione) content per cell after treatments with K_2CrO_4 in strains AHZ-HB-MK and AHZ-HB-P2A. Nonprotein thiols were extracted 0, 1 and 7 day after treatments with 10 and 100 µg mL^{-1} of K_2CrO_4. Data are means ± standard deviation from a minimum of three independent experiments. Significant differences between control (0 d) and chromium-treated one are indicated with asterisks (*t*-test; *$P < 0.01$; **$P < 0.001$).

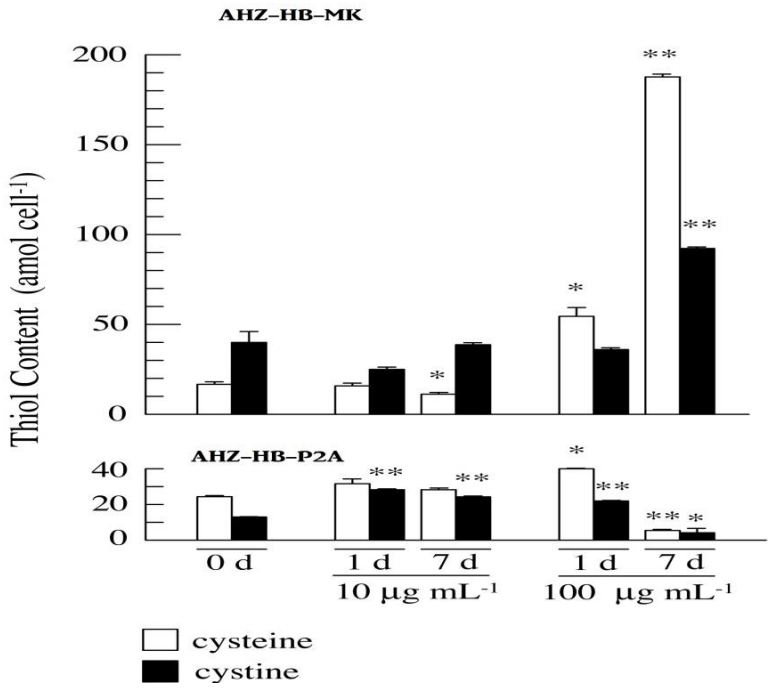

Figure 3. Changes in nonprotein thiol (cysteine and cystine) content per cell after treatments with K_2CrO_4 in strains AHZ-HB-MK and AHZ-HB-P2A. Cysteine and cystine were extracted 0, 1 and 7 day after treatments with 10 and 100 µg mL^{-1} of K_2CrO_4. Data are means ± standard deviation from a minimum of three independent experiments. Significant differences between control (0 day) and chromium-treated one are indicated with asterisks (*t*-test; **P* < 0.01; ***P* < 0.001).

except for cystine. Cysteine content in AHZ-HB-MK strain increased to almost three and 11 times after 1 and 7 days treatment with 100 µg mL^{-1} K_2CrO_4, respectively, whereas at 10 µg mL^{-1} some decrease or no change in its level was observed. Large increase in cysteine content was not observed in AHZ-HB-P2A strain; instead it remarkably decreased after 7 days at the concentration of 100 µg mL^{-1}.

DISCUSSION

Nonprotein thiols such as glutathione and cysteine are involved in detoxification against heavy metals, chromium (VI) for example, forms thiolate complex with reduced form of glutathione (GSH) and cysteine (Brauer et al., 1996; Suzuki, 1990; Wiegand et al., 1985). Furthermore, addition of cysteine together with chromium alleviates the toxicity of chromium in a cyanobacterium *Anabaena doliolum* (Duke, 1992). After K_2CrO_4 treatment GSH completely disappeared under all conditions in both strains, suggesting that GSH was involved in detoxification. GSSG content in AHZ-HB-MK strain remarkably increased to about three and six times after 7-days treatment with 10 and 100 µg mL^{-1} K_2CrO_4, respectively. However, the change in GSSG content in AHZ-HB-P2A strain showed a different pattern from AHZ-HB-MK strain. GSSG content of AHZ-HB-P2A strain slightly increased after 1 day at the both concentrations, but it decreased after 7 days, especially at the concentration of 100 µg mL^{-1}.

The data revealed that AHZ-HB-MK strain could induce glutathione and cysteine but P2A strain could not. Difference in the ability to induce nonprotein thiols between the two strains might be responsible for the difference of the tolerance against K_2CrO_4 observed by the inhibition of growth. Although GSH was completely depleted after K_2CrO_4 treatment but cysteine was not, rather the ratio of cysteine/cystine became higher after their induction by 100 µg mL^{-1} K_2CrO_4 in AHZ-HB-MK strain. It has also been reported that time-dependent decrease of reduced glutathione (GSH) with an increase of oxidized glutathione (GSSG) level suggested GSH was involved in detoxification of MC-LR in the liver (*Jinlin* et al., *2011*). There are possibilities of activation of cysteine reductase, the synthesis of new cysteine or both. It has also been reported that redox of cysteine and glutathione must be controlled independently in marine phytoplankton (Satoh et al., 2002).

In this study, we measured intracellular contents and concentration of nonprotein thiols in two strains of *Synechocystis* genus. Concentrations of glutathione in plants, algae, bacteria, and animal tissues range from 0.1 to 12 mM (Meister, 1975; Satoh et al., 2002; Coppellotti,

1989). Our results showed that the glutathione concentrations of the two strains of *Synechocystis* are within this range under the condition without chromium. Information about intracellular concentration of cysteine in organisms is limited, but it is known that it ranges from 0.6 to 12 mM in several species of eukaryotic algae (Satoh et al., 2002; Coppellotti, 1989). Cysteine concentrations of the two strains of *Synechocystis* are also within this range. Although concentrations of nonprotein thiols are similar before chromium treatment in two strains, AHZ-HB-MK and P2A, the concentrations become very different after chromium treatment, with large increase in AHZ-HB-MK strain and decrease in AHZ-HB-P2A strain. It indicates that glutathione and cysteine are involved in detoxification of K_2CrO_4 and that the control mechanism of levels of glutathione and cysteine are more sensitive in AHZ-HB-MK strain than that in AHZ-HB-P2A strain. Involvement of detoxification mechanisms other than glutathione and cysteine against K_2CrO_4 related to the difference of the tolerance is uncertain. Cyanobacteria are known to have a cysteine-rich metal-binding protein, zinc metallothionein (SmtA), in response to elevated concentrations of zinc, cadmium and copper (Cavet et al., 2003). Mutants of *Synechococcus* PCC7942 lacking *smtA* showed reduced tolerance to elevated zinc levels (Turner et al., 1993), and conversely, mutants over-expressing SmtA showed increased zinc tolerance (Turner et al., 1995). However, no evidence has been reported that SmtA is induced by chromium nor involved in chromium detoxification. Furthermore, since the two strains shows the same tolerance against $ZnSO_4$ (data not shown) SmtA must have no relation with the different tolerance level against K_2CrO_4. AHZ-HB-MK strain will be a candidate for bioremediation of chromium pollution if cells are found to accumulate chromium in a high level or if they modify chromium to less toxic compounds, from chromium (VI) to chromium (III) for example, even if they release chromium to the environment.

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