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Chromium induced stress conditions in heterotrophic and auxotrophic strains of *Euglena gracilis*

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ABSTRACT

Oxidative stress parameter and antioxidant defense compound as well as enzyme activity were studied in relation to different Cr(VI) concentrations (0, 10, 20, 40 μM) in two strains of *Euglena gracilis*, one isolated from a polluted river (MAT) and the other acquired from a culture collection (UTEX). Chromium toxicity was measured in the auxotrophic and obligated heterotrophic variants of the two strains. Chromium uptake was higher in auxotrophic cultures, reflected by their higher cell proliferation inhibition and lower IC_{50} levels compared to heterotrophic ones. In the Cr(VI) treatments a reduction of chlorophyll *a* and *b* ratio (Chl *a*/Chl *b*) was observed, the ratio of protein to paramylon content was augmented, and total lipid content increased, having the auxotrophic strains the highest values. TBARS content increased significantly only at 40 μM Cr(VI) treatment. Unsaturated fatty acids also increased in the Cr(VI) treatments, with the higher storage lipid (saturated acids) content in the heterotrophic cells. The antioxidant response, such as SOD activity and GSH content, increased with chromium concentration, showing the highest GSH values in the heterotrophic cultures and the SOD enzyme participation in chromium toxicity. The MAT strain had higher IC_{50} values, higher carbohydrate and saturated acid content, and better response of the antioxidant system than the UTEX one. This strain isolated from the polluted place also showed higher GSH content and SOD activity in control cells and in almost all treated cultures. SOD activity reached a 9-fold increase in both MAT strains. These results suggest that tolerance of MAT strain against Cr(VI) stress is not only related to GSH level and/or biosynthesis capacity but is also related to the participation of the SOD antioxidant enzyme.

1. Introduction

It is known that chromium can be found in several oxidation states that have different chemical properties. The most stable and common forms are trivalent [Cr(III)] and hexavalent [Cr(VI)] chromium (Bagchi et al., 2002). The hexavalent form is the most toxic; it usually associates with oxygen to form chromate (CrO_4^{2-}). This molecule can easily go through cell membranes as an alternative substrate on the sulfate transport system (Cieslak-Golonka, 1996; García-García et al., 2009).

Effects of heavy metals in algae have been subjected to extensive research for many years (Küpper et al., 2002; Mendoza-Cozatl and Moreno-Sánchez, 2005; Pinto et al., 2003; Sabatini et al., 2009). Most of the damages produced by these metals are caused by the strong inhibition of photosynthesis (Küpper and Kroneck, 2005; Rocchetta and Küpper, 2009).

It has been postulated that an important contribution to heavy metal stress is the increased production of reactive oxygen species (ROS) which consequently results in occurrence of oxidative stress (e.g., Pinto et al., 2003). Increased ROS levels produce oxidative damage to macromolecules such as proteins, nucleic acids, and lipids, leading to damage of different cellular organelles. Algae chloroplasts are formed by a complex system of membranes rich in polyunsaturated fatty acids, which are potential targets for peroxidation (Halliwell and Gutteridge, 1999). Increased ROS levels can also cause changes in lipid composition, damage to DNA, and ultra-structural disorganization (Watanabe and Suzuki, 2002; Pinto et al., 2003; Watanabe et al., 2003; Rocchetta et al., 2007).

Metal toxicity depends on the organism, the nature and concentration of the metal, and culture conditions (Küpper and Kroneck, 2005). In many cases metal resistance results from a

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very efficient defense/detoxification system, usually the system producing chelating compounds that reduce or inactivate the metal (Cobbett and Goldsbrough, 2000; Küpper and Kroneck, 2005), or metal exclusion and efflux mechanisms to prevent their accumulation inside the cell (von Hoof et al., 2001).

Euglena gracilis is a useful model to study cell damage caused by toxic compounds such as heavy metals. It has been used as a test organism to determine pollutant tolerance in urban aquatic ecosystems (Johnstone et al., 2006; Ahmed and Häder, 2010). This microorganism can grow in heterotrophic or auxotrophic conditions (with light, vitamins and exogenous organic matter or carbon source) depending on the culture medium and light conditions (Barsanti et al., 2000a), making them an interesting model to study different metabolic pathways. *E. gracilis* cells grown under constant darkness can lose their chloroplast, but this condition can be reversed (Schiff and Schwartzbach, 1982). Streptomycin (Sm) is an antibiotic that does not inhibit cell division or viability of *E. gracilis*, but 'bleaches' the cells by causing the permanent loss of plastids and plastid DNA in dividing photosynthetic cells and by blocking the development of chloroplasts in nondividing cells (Schwartzbach and Schiff, 1974).

Previous work revealed that the cytotoxic effect of hexavalent chromium in photosynthetic *E. gracilis* cells produces changes in cellular growth, proteins, and lipids (Rocchetta et al., 2006a; Rodríguez-Zavala et al., 2007; García-García et al., 2009). Recently, some studies have analyzed the effect of this metal on *E. gracilis* grown in heterotrophic conditions (Jasso-Chávez et al., 2010; Lira-Silva et al., 2011). Ultra-structural studies showed damages in the nucleus, mitochondria, and chloroplasts, the last being the most affected (Rocchetta et al., 2007). Changes of fatty acids related with thylakoids and chloroplast membranes were also observed (Rocchetta et al., 2006b). These previous results could be related with oxidative stress processes produced by chromium reacting with molecular oxygen (O_2), producing ROS via non-enzymatic or enzymatic reactions (Bagchi et al., 2002; Rai et al., 2004).

In this study, we propose to analyze oxidative stress parameters and antioxidant defense compounds in relation to different chromium concentrations in *E. gracilis*, and the modulation of their toxicity in auxotrophic and obligated heterotrophic cultures. Two strains have been used for this purpose, one isolated from a highly polluted river and the other obtained from a culture collection. Previous work demonstrated a natural resistance to Cr(VI) by the strain isolated from the polluted river (Rocchetta et al., 2003). We want to compare the effect and response of these strains to Cr exposure. For that, cell density, chlorophyll, carbohydrate and lipid contents were measured as proxies for metabolic damage. Fatty acid composition, lipid peroxidation and intracellular Cr content were analyzed and antioxidant responses as superoxide dismutase (SOD) activity and reduced glutathione (GSH) were measured.

2. Materials and methods

2.1. Micro-organism, culture conditions and metal toxicity assays

We used two auxotrophic strains of *E. gracilis*; one from the Culture Collection of Algae of Texas University, USA (UTEX 753), and the other isolated from a highly polluted river from Matanza River, Buenos Aires, Argentina (MAT) (Ruiz et al., 2004). This type of growth, auxotrophic, leads to live using the energy by the photosynthetic activity and the organic matter incorporation. Their obligated heterotrophic variants (MAT and UTEX) were obtained by bleaching with streptomycin (100 μg/ml during 7 day; Ruiz et al., 2004).

Master cultures were grown on static conditions in 500 ml glass flasks, with manual mixing twice per day, containing 250 ml of *E. gracilis* medium, characterized by being rich in organic matter (EGM; CCAP, 1988), pH 7, at 24 ± 1 °C with cool-white fluorescent continuous light at 150 $\mu E\, m^{-2}\, s^{-1}$ irradiance. On this hand, *E. gracilis* have a good growth condition in both photosynthetic and heterotrophic way. Axenicity was monitored plating cultures in bacteria medium. A new culture was initiated six days before each experiment in order to obtain an inoculum in the exponential growth phase. For the toxicity assays, aliquots of the master cultures were used to inoculate each flask to a final concentration of 1×10^5 cells ml^{-1}. The assays conditions are the same as outlined above. $K_2Cr_2O_7$ was added from a 0.1 M stock solution until reaching total Cr concentrations. Several concentrations were used (0, 5, 10, 20, 40, 100 μM) to find out the 50% growth concentration ($IC_{50,96h}$, assays lasted 96 h; U.S. Environmental Protection Agency (1985)) using the Probit Algae Program (Walsh et al., 1987). Once we obtained the $IC_{50,\ 96h}$ values, which are presented in Results (Section 3.1), we used Cr concentrations (0, 10, 20, 40 μM) above and below the obtained $IC_{50,96h}$ for the biochemical determinations. Cellular density was determined with a Neubauer chamber, with less than 10% error, α 0.05 (Venrick, 1978). The percentages of growth inhibition were calculated according to Wong et al. (1983) on the basis of cell density, which was determined every day with a Neubauer chamber, with less than 10% error, α 0.05 (Venrick, 1978).

2.2. Chromium determination

Cultures were centrifuged (20 ml), the pellet was washed three times with distilled water, and the cells were digested with concentrated nitric acid. Total chromium concentration was measured in the cells and in the culture medium in the controls and treated cultures, using a SHIMADZU 6800 atomic absorption spectrophotometer (Kyoto, Japan) equipped with an autosampler ASC 6100. A Hamamatsu hollow cathose lamp was employed as radiation source at 357.9 nm with a slit width of 0.2 nm and 6 mA lamp current. Working solutions of chromium were prepared by appropriate dilution of a stock standard solution of 1000 $mg.ml^{-1}$ Cr (trace to SRM from NIST) from Merck Chemical. These solutions were used as standards for obtaining the calibration curve. Appropriate blank controls were conducted during all analytical methodology. The detection limit of the blank, based on the mean of three times the standard deviation, was estimated to be 0.020 $mg.ml^{-1}$.

2.3. Antioxidants determination

Cells were harvested by centrifugation for 15 min at $7400 \times g$, and washed three times with 0.154 M phosphate buffer, pH 7.0. The pellet was sonicated with 3% 5-sulfosalicylic acid and centrifuged 15 min at $7000 \times g$. The resulting supernatant was used for the assays. Reduced glutathione (GSH) levels were measured following the Anderson (1985) procedure. Absorbance at 412 nm was measured after 30 min incubation at room temperature. Results were expressed as nmol mg $proteins^{-1}$.

Superoxide dismutase (SOD) activity was determined by measuring the inhibition of the reduction of nitroblue tetrazolium (NBT) by the light activated generation of O_2^- by riboflavin in the presence of methionine. The rate of reduction of NBT was measured at 560 nm (Fridovich, 1997). Cells were harvested by centrifugation for 15 min at $7400 \times g$, and washed three times with 0.154 M phosphate buffer (pH 7). The pellet was sonicated and the resulting supernatant was used for the assays. Protein concentration was determined by the Bradford (1976) method using bovine serum albumin as standard.

2.4. Chlorophyll determination

Chlorophyll content was determined by following Wellburn (1994) procedure. Cells were harvested by filtering 5 ml sample using Whatman GF/C filter papers. The pigments (chlorophyll *a* and *b*) were extracted with 80% acetone solution (v/v) for 24 h at 4 °C, and measured spectrophotometrically. Results are expressed as Chlorophyll *a* and *b* ratio (Chl *a*/Chl *b*)

2.5. Carbohydrate determination

Total sugars were measured spectrophotometrically using the Dubois et al. (1956) procedure standardized with glucose. Cells were harvested by centrifugation of 5 ml culture for 15 min at $7400 \times g$. Dubois reagents were used and optical densities were analyzed at 490 nm.

Paramylon (β-1,3-glucans, storage carbohydrates in Euglenida) was extracted and purified following Barsanti et al. (2000b) specifications. To verify effectiveness of the procedure, paramylon granules in the supernatant were observed and measured with an Olympus BX 50 optical microscope. Before extraction, cells were washed three times. Then, 100 ml of the culture medium was centrifuged at $3700 \times g$. Pellets were frozen at −21 °C overnight and then resuspended in 1% SDS (w/v) and 5% Na_2 EDTA (w/v), shaken for mixing, and incubated for 60 min at 37 °C. Paramylon granules were recovered by centrifugation for 60 min at $3700 \times g$. The SDS-Na_2 EDTA treatment was repeated until a translucent supernatant was obtained. Paramylon granules were then washed twice with hot glass-distilled water (70 °C). After the second wash, granules were located in glass fiber filters (APFC type, Millipore) and dried overnight at 90 °C for weight determination. Results were expressed as μg paramylon 10^6 $cells^{-1}$. The quality of the grains