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Photosynthetic activity and protein overexpression found in Cr(III)-tolerant cells of the green algae *Dictyosphaerium chlorelloides*

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HIGHLIGHTS

- Dc1M$^{Cr(III)R30}$ survives at Cr(III) 18–35 times higher than tolerated by Dc1M^{wt} cells.
- Dc1M$^{Cr(III)R30}$ extracts show protein overexpressions absent in extracts from Dc1M^{wt}.
- Neither *de novo* sequence from higher m.w. band (153 KDa) is consistent with MS-BLAST score.
- The lower m.w. overexpression (23 KDa) is consistent with a GTPase from Rab family.
- This GTPase was identified from genome of the green alga *Chlamydomonas reinhardtii*.

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ABSTRACT

Chromium is an important constituent in effluents obtained from chromium plating industries. Due to the highly toxic nature of Cr(VI), attention has been shifted to less hazardous Cr(III) electroplating processes. This study evaluated aquatic toxicity of Cr(III)-containing laboratory samples representative of effluents from chromium electroplating industries, on the photosynthetic activity exhibited by both Cr(III)-sensitive (Dc1M^{wt}) and tolerant (Dc1M$^{Cr(III)R30}$) *Dictyosphaerium chlorelloides* strains. Additionally, selected *de novo*-determined peptide sequences, obtained from Dc1M$^{Cr(III)R30}$, have been analyzed to evidence the possible Cr(III) toxic mechanism involved in the resistance of these cells to high Cr(III) levels in aquatic environments. Dc1M$^{Cr(III)R30}$ strain exhibited a gross photosynthetic balance of about five times lower than that exhibited by Dc1M^{wt} strain, demonstrating that Dc1M$^{Cr(III)R30}$ has a photosynthetic yield significantly lower than Dc1M^{wt}. SDS–PAGE of Dc1M$^{Cr(III)R30}$ samples showed the presence of at least two protein bands (23.05 and 153.46 KDa, respectively) absent in wild-type strain samples. Although it has achieved a low coincidence between the lower molecular weight band and a GTPase identified from genome of the green alga *Chlamydomonas reinhardtii*, none of *de novo* peptide sequences obtained showed a significant MS-BLAST score, so that further studies will be required.

1. Introduction

Toxic effects of heavy metals on microalgae have often been studied as these organisms occupy the lower most position in the food chain in aquatic ecosystems; thus, stress conditions affecting microalgae may have a great impact on the whole environment. Chromium (Cr) contamination of freshwater and groundwater has become a worldwide problem, due to the important use of Cr in various manufacturing and military industries (Shanker et al., 2005). The Cr concentration in the effluents of leather industries ranges from 2 to 5 g L^{-1}.

The most chemically-stable and environmental common forms of Cr are the trivalent (III) and hexavalent (VI) ones, which differ in their biological toxicity. In photosynthetic organisms, Cr alters several morphological, physiological and biochemical processes including germination, chlorophyll biosynthesis, chloroplast and cell membranes ultrastructure and cell oxidative status (Shanker et al., 2005; Rodríguez et al., 2007).

Laboratory populations of microalgae are widely used as sensitive test object for the evaluation of the phytotoxicity of chemicals and wastewater streams. Cell populations of microalgae are complex systems with resistant and sensitive cells. When pollutants are added to a dense microalgal culture, the cell density will be reduced after a few days due to the death of sensitive cells. However, after further incubations, the culture will sometimes increase in

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density again due to the growth of cell variant, which is resistant to the contaminants (Ipatova et al., 2012). Within limits, organisms may survive in chemically-stressed environments as a result of two different processes: physiological adaptation (acclimation), usually resulting from modifications of gene expression (Sánchez-Fortún et al., 2009b); and, adaptation by natural selection if mutations provide the appropriate genetic variability (Belfiore and Anderson, 2001).

Proteomics studies aim to identify posttranslational modifications of proteins, as well as the organization of proteins in multiprotein complexes and their localization in tissues (de Hoog and Mann, 2004). Technologies enabling proteomic studies have been in development for several decades now. A proteomic experiment involves a number of typical steps, which can be taken using different technological platforms (Molloy and Witzmann, 2001), and the proteins of interest can be excised from a 2-DE gel, digested and identified by mass spectrometry. Peptide mass data obtained for each sample are queried against a peptide database with known peptide masses of unique proteins (Gevaert and Vandekerckhove, 2000).

Proteomics studies in phytoplankton have focused on the freshwater chlorophyte *Chlamydomonas reinhardtii* (Stauber and Hippler, 2004; Kelle et al., 2005; Schmidt et al., 2006). These studies shown that proteomics can be used for the elucidation of certain metabolic pathways. In comparative phytoplankton proteomics, a large fraction or the entire proteome of one strain may be compared to that of another strain, kept under different environmental conditions. Proteins differentially expressed due to the environmental stimuli can be identified, as well as the metabolic pathway they are part of (Jamers et al., 2009). These techniques could be applied to investigate light-induced changes in the proteome and at the same time compare the response of both wild type and mutant strains involved in adaptation processes to aquatic environments highly contaminated.

The aim of this study was to analyze the impact of chromium trivalent on the photosynthetic activity in both Cr(III)-sensitive and tolerant strains of the unicellular green algae *Dictyosphaerium chlorelloides* as well as interpret, based on *de novo*-determined peptide sequences obtained from $Dc1M^{Cr(III)R30}$, the possible toxic mechanism involved in the resistance of this Cr(III)-tolerant *D. chlorelloides* strain to high Cr(III) concentrations in aquatic environments.

2. Materials and methods

2.1. Chemicals

Chromium(III)–chloride hexahydrate ($CrCl_3{\cdot}6H_2O$) was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). This compound was dissolved in distilled water. In all assays, the working solutions were freshly prepared each time.

2.2. Experimental organisms

Trivalent chromium sensitive ($Dc1M^{wt}$) and tolerant [$Dc1M^{Cr(III)R30}$] strains of *D. chlorelloides*, from the algal culture collection of the Environmental Toxicology Laboratory (Toxicology and Pharmacology Department, UCM, Madrid, Spain), were used in these assays. $Dc1M^{Cr(III)R30}$ strain was obtained in the laboratory from cultures of the wild strain in culture medium supplemented with 30 mg L^{-1} $CrCl_3{\cdot}6H_2O$.

Wild-type Dc1M were grown axenically in culture flasks (Greiner, Bio-One, Longwood, NJ, USA) with 20 mL of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany), while $Dc1M^{Cr(III)R30}$ strain was cultured in BG-11 medium supplied with 30 ppm $CrCl_3{\cdot}6H_2O$.

Both strains were maintained at 20 °C under continuous light of 60 μmol m^{-2} s^{-1} over the 400–700 nm waveband. Cells were maintained in mid-log exponential growth by serial transfers of one-cell inoculums to fresh medium once a fortnight. Prior to the experiments, the cultures were re-cloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the cultures.

2.3. Toxicity tests

The toxic effect induced by chromium has been valued on effective quantum yield (Φ_{PSII}) and gross photosynthesis P_g rate in both strains. These parameters (Φ_{PSII} activity and P_g rate) were used as toxic endpoints and expressed as 72-h median inhibitory concentration [$IC_{50(72)}$].

To determine Φ_{PSII} inhibition and P_g rate, toxicity tests were performed in 5 mL polystyrene sterile culture tubes (Sarstedt Co., Nümbrecht, Germany) filled with BG-11 medium. Previous studies determined the suitability of polystyrene sterile culture tubes for these toxicity assays, assuring that chemicals and cells do not adhere to the wall tubes (Costas et al., 2001; García-Villada et al. 2004). The water used for media preparation was of ultrapure quality, distilled by means of Milli-Q device (Millipore, Bedford, MA, USA).

Preliminary toxicity tests were performed to define the range of concentrations that included 0% and 100% inhibition. The results obtained established a concentration range of 20–50 μM and 750–3000 μM to $Dc1M^{wt}$ and $Dc1M^{Cr(III)R30}$, respectively.

Each assay was repeated eight times ($n = 8$). Both control and test tubes were inoculated with 10^4 cells mL^{-1} as initial concentration. All the cultures (control and treatments) were incubated for 72 h at 20 °C and at 60 μmol m^{-2} s^{-1} irradiance in a thermostatically controlled chamber (Velp Scientifica, Usmate, Italy) to ensure exponential algal growth. Every 24 h the algal density was quantified under the light microscope.

The quantity causing 50% inhibition of photosynthetic yield (Φ_{PSII}) was obtained by means of the dual-channel PAM chlorophyll fluorometer (ToxY-PAM, Heinz Walz GmbH, Germany). The ToxY-PAM dual-channel yield analyser obtains highly sensitive measurements of effective quantum yield of the algae pigment system II centres via assessment of the chlorophyll fluorescence yield and the saturation pulse method (Schreiber et al., 2002).

Gross photosynthesis rate (P_g) was estimated from de formula:

$$P_g = P_n + R$$

where P_g corresponding to the oxygen production rate of photosystem II, R (respiration) corresponding to the process by which in the presence of light microalgal cells consume oxygen and releases carbon dioxide, and P_n (net photosynthesis rate) is defined as the difference between P_g and R. The oxygen values were obtained employing a Clark-type electrode. Dissolved O_2 was measured in a 1 mL reaction chamber from a Chlorolab 2 System (Hansatech, Norfolk, UK). Chlorolab 2 provides a system for the study of respiration and photosynthesis from liquid samples under automated illumination from red (660 nm) LED light. In the toxicity assays, measurements were taken at 20 °C and 975 μmol m^{-2} s^{-1} irradiance.

2.4. Proteomic study

2.4.1. Cell disruption

For both *D. chlorelloides* strains, 100-mL algae culture was washed twice with distilled water and then centrifuged at 7*g* for

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