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TOXICITY AND ADAPTATION OF *DICTYOSPHAERIUM CHLORELLOIDES* TO EXTREME CHROMIUM CONTAMINATION

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Abstract—Metals are often spilled by industries into inland water environments, with adverse consequences. Numerous papers have reported that heavy metals produce massive destruction of algae. Nevertheless, algal populations seem to become tolerant when they have had previous exposures to heavy metals. Because the mechanisms allowing heavy metal tolerance of algae are not yet known, the present study analyzed the effect of hexavalent chromium on growth and photosynthetic performance of *Dictyosphaerium chlorelloides,* stressing on the adaptation mechanisms to chromium contamination. Growth and photosynthetic performance of algal cells were inhibited by Cr(VI) at 10 mg/L, and the 72-h median inhibition concentration was established as 1.64 and 1.54 mg/L, respectively. However, after further incubation for a three month period in an environment with 25 mg/L of chromium, some rare, chromium-resistant cells occasionally were found. A Luria–Delbrück fluctuation analysis was performed to distinguish between resistant algae arising from rare, spontaneous mutations and resistant algae arising from physiological adaptation and other adaptive mechanisms. Resistant cells arose only by spontaneous mutations before the addition of chromium, with a rate of 1.77×10^{-6} mutants per cell division. From a practical point of view, the use of both chromium-sensitive and chromium-resistant genotypes could make possible a specific algal biosensor for chromium.

Keywords—Adaptation Chromium *Dictyosphaerium chlorelloides* Mutation Toxicity

INTRODUCTION

Today, human activities are altering biosphere-level processes and causing a biodiversity crisis, with global extinction rates 500-fold above background [1,2]. As a result, several million geographically distinct populations and thousands of species go extinct annually [1]. Humans are the world's greatest evolutionary force, and great scientific effort should be made to comprehend the impact of civilization on the natural world [3,4].

The biodiversity crisis is reasonably well understood for terrestrial vertebrates and a few other groups, but little is known about this crisis in organisms as abundant and important as microorganisms. Studies with microorganisms would be interesting, however, because dependable nutrient cycles may become less predictable as essential microorganisms succumb to anthropogenic toxicants [1]. From an ecological point of view, the tolerance of these microorganisms to water pollution is paramount, because they are the majority primary producers of aquatic ecosystems [5]. Furthermore, from a physiological and genetic point of view, the survival and growth of microalgae in extremely contaminated environments is an interesting topic [6].

Heavy metals often are spilled from industrial sources into the environment. These metals are among the main man-made pollutants in aquatic ecosystems and a powerful anthropogenic selection force, with serious environmental implications and evolutionary consequences. Numerous papers have reported that heavy metals have a rapid effect on microalgae, causing massive destruction of sensitive cells [7,8]. These studies usually analyzed the injurious effects of heavy metals on microalgae and, indirectly, its effects on the aquatic ecosystems. Some heavy metal-resistant algal variants, however, may have succeeded by means of physiological or genetic mechanisms. The biological consequences of heavy metal contamination would be very different depending on the ability of microalgae to adapt to chromium. It seems that microalgal populations can be tolerant to the presence of heavy metals if they have had previous exposures [9,10].

In spite of this, little is known about the mechanisms allowing adaptation of microalgae to heavy metals. However, evidence for these mechanisms can be found in previous work that analyzed the effect of a toxic spill of acid wastes rich in heavy metals [11] or the effects of copper used as an algaecide in reservoirs [8,12]. Apparently, microalgae may survive exposure to heavy metals as a result of two different processes: First, physiological adjustment (acclimation or tolerance), usually resulting from modifications of gene expression [13], and second, genetic adaptation by natural selection if mutations provide the appropriate genetic variability, revealing the existence of resistant genotypes [14]. In this sense, a lot of interesting questions arise and require further work to be answered.

Chromium is widely used by industry and is an important water pollutant [15,16]. Untreated effluents from industrial discharges contain approximately 80 to 100 mg/L of Cr(VI)—much higher than the permissible limit of 0.05 to 1 mg/L [17]. The biological effects of chromium, which exists in hexavalent (VI) and trivalent (III) forms, depend on its oxidation state.

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The majority of Cr(VI) found in nature comes from industrial emissions, and Cr(VI) is the most toxic form of chromium to the plant, animal, and microorganism species that inhabit aquatic environments [18,19].

The present study has a double aim: First, to study the effect of heavy metals on the growth rate and photosynthetic performance of microalgae under a classic ecotoxicological approach, and second, to analyze the ability of sensitive cells to adapt to heavy metals under an evolutionary ecological procedure. The experimental model used Cr(VI) and *Dictyosphaerium chlorelloides* (Naumann, Komárek, and Perman), an abundant Chlorophyceae with a worldwide distribution that is widely used in studies of evolutionary ecology with algae.

MATERIALS AND METHODS

Experimental organism

Dictyosphaerium chlorelloides, strain Dc, was from the algal culture collection of the Genetics Laboratory, School of Veterinary Sciences, Complutense University. Cells grew axenically in culture flasks (Greiner; Bio-One), with 20 ml of BG-11 medium (Sigma Aldrich Chemie) at 20°C under a continuous light of 60 μmol/m²/s over the waveband of 400 to 700 nm. Cells were maintained in mid-log exponential growth by serial transfers of one-cell inoculums to fresh medium once a fortnight. Before the experiments, the cultures were recloned (by isolating a single cell) to avoid including any previous spontaneous mutants that had accumulated in the cultures.

Effect of chromium on growth rate and photosynthetic performance

Hexavalent chromium oxide (Cr(VI); Sigma-Aldrich Chemical Co.) was used dissolved in distilled water. The toxic effect of Cr(VI) on the growth rate of *D. chlorelloides* was tested previously using 13-ml, polystyrene, sterile tubes (Sarstedt). Those previous studies determined the suitability of using polystyrene, sterile tubes for these toxicity assays, assuring that neither chemicals nor microalgal cells adhered to the tube walls [8,20]. Serial dilutions of Cr(VI) in BG-11 medium were prepared to obtain concentrations of 0, 0.1, 0.5, 1, 2, 5, and 10 mg/L. For each dose, eight replicates were inoculated with 10^4 cells/ml from mid-log, exponentially growing cell cultures.

The effect of Cr(VI) was estimated in both strains (sensitive and resistant to exposure) after 72 h by calculating the acclimated maximal growth rate (m) in mid-log, exponentially growing cells, which derives from the equation $N_t = N_0 e^{mt}$, where t is 3 d, N_t is the cell number at the end of the experiment, and N_0 is the cell number at the start of the experiment. Therefore, m was calculated as $m = \log_e(N_t/N_0)/t$. Acclimated maximal growth rate (m) is the Malthusian parameter of fitness under conditions of r selection [21,22]. Experiments and controls were performed under blind count (i.e., the person counting the test did not know the identity of the tested samples) every 24 h using a hemocytometer in an inverted microscope (Axiovert 35; Zeiss). The number of samples in each case was determined using the progressive mean procedure [22], which assures a counting error of less than 5%.

The photosynthetic response was measured as the effective fluorescence quantum yield (Φ_{PSII}) in triplicates of experiments and controls using a ToxY-PAM fluorometer (Walz) at 72 h. Effective quantum yields were calculated as follows: $\Phi_{PSII} = (F'_m - F_t)/F'_m$, where F'_m and F_t are the maximum and steady-state fluorescence, respectively, of light-adapted cells [23].

The concentration causing 50% inhibition of growth and Φ_{PSII} at 72 h ($IC50_{(72)}$) in algae was evaluated according to the area-under-the-curve method prescribed by the International Organization for Standardization [24]. The $IC50_{(72)}$ values were determined by nonlinear regression analysis. Results are expressed as the mean ± standard deviation, and data are presented as the percentage inhibition of growth rate and Φ_{PSII} with regard to controls (unexposed cells).

Statistical analysis was performed using the computer software package GraphPad Prism (Ver 4.0; GraphPad Software).

Ability of microalgae to adapt to chromium

Massive cultures of *D. chlorelloides* were exposed previously to 25 mg/L of Cr(VI) (~2.5-fold the 100% inhibitory concentration obtained in the toxicity assays) and maintained in culture with chromium for three months to establish if some kind of resistant cells could arise when microalgae are exposed to lethal concentrations of heavy metals. When microalgal cultures are exposed to lethal doses of chromium, the sensitive cells die because of the toxic effect of Cr(VI). After further incubation for three months, however, the growth of some algal variants resistant to the toxic effect of Cr(VI) could be detected. Growth rate and photosynthetic yield of these chromium-resistant microalgae were determined (as described previously) and then compared with those of sensitive microalgae.

The key to understanding adaptation of microalgae so these organisms can endure a chromium-contaminated environment seems to be characterizing the algal variants that appear after the massive destruction of sensitive cells. Heavy metal-resistant cells could arise by two procedures: First, through acquired resistance during the exposure to chromium (physiological adaptation), and second, through spontaneous mutations occurring randomly before chromium exposure (genetic adaptation). A modified fluctuation analysis [25] was performed as described previously by López–Rodas et al. [26] and Costas et al. [20] to distinguish between chromium-resistant cells arising through acquired physiological adaptation during exposure to Cr(VI) and chromium-resistant cells arising as result of rare, spontaneous mutations occurring before Cr(VI) exposure.

The fluctuation value was estimated as follows: Fluctuation $= CV_{set\ 1}/CV_{set\ 2}$, where CV is the variance in the number of cells per culture divided by the mean variance in the number of cells per culture, set 1 is the experimental culture, and set 2 is the control culture. A fluctuation value of approximately one indicates that chromium resistance is a consequence of physiological adaptation, and a fluctuation value of greater than one indicates that chromium resistance is a consequence of genetic mutations. The mutation rate (μ) was calculated as $\mu = -L_e P_0/(N_t - N_0)$, where $\log_e$ is the natural algorithm, P_0 is the proportion of cultures showing no resistant cells, N_t is the cell number at the end of the experiment, and N_0 is the cell number at the start of the experiment.

RESULTS

The Cr(VI) showed acute toxicity to sensitive microalgae, inhibiting both cell growth and $\Phi_{(PSII)}$, with $IC50_{(72)}$ values of 1.64 and 1.54 mg/L, respectively (Table 1). The concentration-

Table 1. Comparison of 72-h median inhibition concentrations ($IC50_{(72)}$) for growth and photosynthetic response (Φ_{PSII}) and associated 95% confidence limits (CL) for sensitive and resistant populations of *Dictyosphaerium chlorelloides* exposed to Cr(IV)[a]

		Growth inhibition (mg/L)		$\Phi_{(PSII)}$ inhibition (mg/L)	
Population	*n*	$IC50_{(72)}$	95% CL	$IC50_{(72)}$	95% CL
Sensitive	8	1.64	1.43–1.98	1.54	1.29–1.89
Resistant	8	20.61*	19.57–21.66	17.26*	15.04–19.48

[a] An asterisk indicates a significant difference ($p < 0.05$) with respect to values obtained for sensitive populations.

response of growth inhibition was similar to the concentration-response of $\Phi_{(PSII)}$ inhibition (Figs. 1 and 2, respectively).

When *D. chlorelloides* cultures were exposed to a 25 mg/L of Cr(VI) (~2.5-fold the 100% inhibitory concentration), these organisms became light in appearance after some days because of destruction of the sensitive cells by the toxic effect of Cr(VI). After further incubation for three months, however, some cultures became colored again because of growth of a rare algal variant that was resistant to the effect of Cr(VI). These resistant cells are on the order of 12-fold more resistant compared with those sensitive cells from Cr(VI)-free cultures. The concentration-response to Cr(VI) growth rate and $\Phi_{(PSII)}$ confirm that the resistant algae obtained from cultures with long-term Cr(VI) exposure developed a higher tolerance to this heavy metal. Comparison of $IC50_{(72)}$ values showed statistically significant differences ($p < 0.05$) between the wild-type, sensitive algae and the rare, resistant algal variants (Table 1).

The key to understanding adaptation of microalgae so that these organisms can persist in a chromium-contaminated environment seems to be characterizing these algal variants that appear after massive destruction of the sensitive cells. The fluctuation analysis has demonstrated that most cells of each experimental culture of set 1 (experimental) and set 2 (control) died because of the toxic effect of chromium. After further

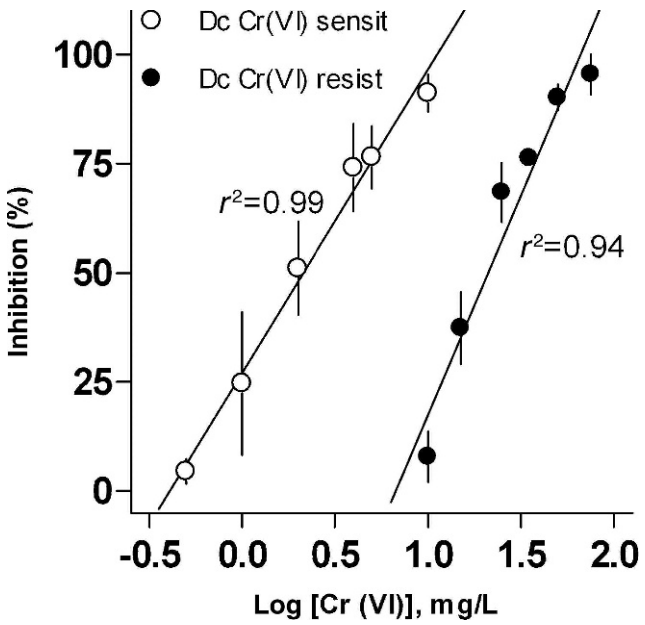

Fig. 1. Growth inhibition response induced by Cr(VI) exposure in sensitive (○) and resistant (●) *Dictyosphaerium chlorelloides* populations. Points represent the mean, with vertical lines showing the standard deviation ($n = 8$).

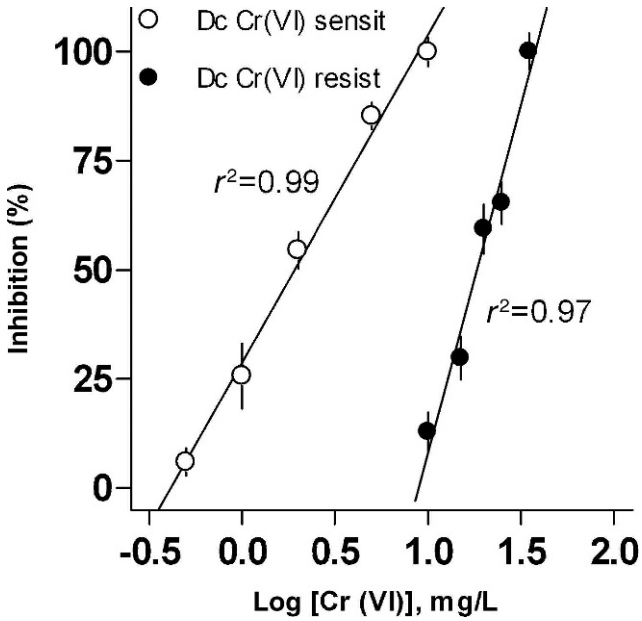

Fig. 2. Photosynthetic inhibition response measured as the effective fluorescence quantum yield (Φ_{PSII}) as induced by Cr(VI) exposure in sensitive (○) and resistant (●) *Dictyosphaerium chlorelloides* populations. Points represent the mean, with vertical lines showing the standard deviation ($n = 8$).

incubation for several weeks, however, some cultures of *D. chlorelloides* had again increased their density, apparently because of growth of a Cr(VI)-resistant algal variant. In set 1, a high fluctuation (from 0 to $>10^5$ resistant cells/culture flask) was observed after 60 d of Cr(VI) exposure. By contrast, nearly 1.5×10^5 Cr(VI)-resistant cells were detected in all cultures from set 2. In addition, a low Cr(VI)-resistant cell fluctuation was observed in set 2 (variance/mean ~0.2), indicating that the high fluctuation found in set 1 cultures was caused by processes other than sampling error. As in set 1 cultures, the variance significantly exceeded the mean (variance/mean ~2.5), so it could be inferred that Cr(VI)-resistant cells have arisen by rare, preselective, spontaneous mutations rather than by physiological adaptation or postselective mutations appearing in response to Cr(IV) (Table 2).

The estimated μ value of Cr(VI)-sensitive organisms to Cr(VI)-resistant organisms in *D. chlorelloides* was calculated as 1.77×10^{-6} mutants per cell division (Table 2). Isolated Cr(VI)-resistant mutants growing in absence of Cr(VI) have shown a small diminution of growth rates with respect to those found in Cr(VI)-sensitive cells. The coefficient of selection (*s*) of Cr(VI)-resistant mutants was 0.125. By using μ and *s* values,

Table 2. Fluctuation analysis of *Dictyosphaerium chlorelloides* exposed to 25 mg/L of Cr(VI)[a]

	Set 1	Set 2
No. of replicate cultures	105	30
No. of cultures containing the following no. of Cr(VI) resistant cells per milliliter		
0	86	0
$<1.5 \times 10^5$	7	0
$>1.5 \times 10^5$	12	30
Fluctuation	13.05	
Mutants per cell division (μ)	1.77×10^{-6}	

[a] Set 1 is the experimental culture, and set 2 is the control culture.

the frequency of Cr(VI)-resistant alleles was estimated as 14 resistant cells per 10^{-6} wild-type cells.

DISCUSSION

The standard toxicological analysis clearly demonstrates that microalgae are very sensitive to the effect of chromium. The $IC50_{(72)}$ obtained with Cr(VI) exposures in *D. chlorelloides* is in agreement with those obtained by other authors using toxicity test assays with marine [27] and freshwater [28] algal species. In spite of this, evidence in the literature demonstrates the existence of some kind of algal interspecies variability for Cr(VI) sensitivity [29,30].

Subsequently, it was verified that after destruction of most algae following a chromium exposure, some rare, resistant algal variants are able to grow in an environment with chromium levels approximately 26-fold higher than those that destroy the sensitive, wild-type algae. Apparently, resistance of algae to chromium seems to be higher than that found in other aquatic organisms [9]. Because only algae previously exposed to chromium seem to be resistant to treatments with this metal, it has been proposed that the reduction of toxic effects of chromium results from interaction among chromium, algal-excreted organic molecules, and the algal cell surface [31]. Microalgae and related eukaryotic photosynthetic organisms have preferentially developed the production of peptides capable of binding heavy metals [32–34]. This complex interaction between chromium and algal excretions takes place only after the algae have been stressed by a previous contact with the metal.

The present results unequivocally demonstrate that chromium-resistant algal variants also occur within clonal cultures that have not been exposed previously to chromium. Consequently, chromium resistance may be explained in terms of physiological adaptation, mainly as a consequence of changes in gene expression [13] or genetic adaptation as a result of rare mutations that occurred within the clonal cultures before chromium exposure [14]. The key to understanding the adaptation of unexposed algae to the extremely adverse effects of chromium is to analyze the rare algal variants that occur after the massive destruction of sensitive cells by Cr(VI). Fluctuation analysis is the appropriate procedure to discriminate between chromium-resistant algae arising by rare, spontaneous mutations occurring randomly during replication of organisms before exposure to this selective agent and chromium-resistant algae arising through specifically acquired adaptation induced by chromium exposure. The large fluctuation observed in the number of chromium-resistant cells in set 1, in contrast to the insignificant fluctuation observed in set 2, unequivocally demonstrates that resistant algal variants arose by a rare, spontaneous, single mutation that occurred before exposure to geothermal waters and not through physiological adaptation in response to Cr(VI). Exposure did not stimulate the appearance of resistant cells at all. Accordingly, algae can rapidly adapt to chromium contamination by single mutations. Recent evidence suggests that mutation at one loci can achieve adaptation of algae to other very hostile, heavy metal-enriched, natural environments [35–37].

The mutation rate from Cr(VI)-sensitive organisms to Cr(VI)-resistant organisms in *D. chlorelloides* (1.77×10^{-6} mutants/cell division) was in the middle range of the mutation rates (from 2.1×10^{-5} to 2.7×10^{-7} mutants/cell/generation) described in algae for resistance to complex mixtures of heavy metals from several extreme, natural environments [11,35–37]. The presence of Cr(VI)-resistant cells in populations of *D. chlorelloides* is caused by a rare, spontaneous mutation that occurs before Cr(VI) exposure. New resistant mutants arise in each generation. However, most of these mutants are eliminated, because Cr(VI)-resistant mutants are detrimental to species fitness in the absence of chromium contamination. At any one time, the balance between the continuous appearance of mutants and their selective elimination determines the number of remaining, chromium-resistant mutants in algal populations growing in the absence of chromium (i.e., this may be the case for wild-type populations developing in nonpolluted waters). Therefore, a natural population would be constituted predominantly by wild-type, chromium-sensitive genotype cells and, at the same time, in a very small fraction by a clone line of chromium-resistant mutant cells (i.e., in an experimental population at any given time are ~14 resistant mutants per 10^6 sensitive, wild-type cells).

Finally, the present results tentatively indicate that the different response in photosynthetic activity observed between sensitive and resistant cells of *D. chlorelloides* in the presence of Cr(VI) could be used to obtain a chromium-specific microalgal biosensor. Hexavalent chromium and other heavy metals in water usually are analyzed with time-consuming techniques that require laboratory hardware and that are not suitable for in situ, continuous monitoring [38]. As a result, many efforts have been made to develop biosensors for continuous monitoring of Cr(IV) [39]. Unfortunately, microalgal biosensors are not specific. Altamirano et al. [40], however, propose a new genetic approach for increasing the specificity of microalgal biosensors based on the use of a sensitive genotype (sensitivity) and a resistant mutant (specificity). In this sense, it should be possible to use the differential response in photosynthetic activity of sensitive and resistant cells of *D. chlorelloides* in the presence of Cr(VI) for continuous monitoring of this specific pollutant in water. The biosensor could be based on the joint use of two different genotypes to detect chromium: The sensitive Cr^s to obtain sensitivity, and the resistant Cr^r mutant to obtain specificity.

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