

## Cadmium toxicity and resistance in *Chlorella* sp.

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### Abstract

The Cd<sup>2+</sup> tolerance of *Chlorella* sp. isolated from an urban waste water treatment plant was studied. The growth of this alga was severely inhibited at 10 µM Cd<sup>2+</sup> with an LD<sub>50</sub> of 3 µM. Addition of 100 µM glutathione or 100 µM cysteine reduced the inhibitory effect of Cd<sup>2+</sup>. The amelioration of Cd<sup>2+</sup> inhibition by GSH was not due to direct complexing of the two compounds in the growth medium, indicating that an intracellular mechanism involving GSH or its metabolites is responsible for Cd<sup>2+</sup> detoxification. A Cd<sup>2+</sup> resistant line (CdR-DK), with an LD<sub>50</sub> of 35 µM, was selected by growing the alga on solid medium in the presence of increasing concentrations of the ion. Phytochelatins (PCs), a family of peptides synthesized by plants in response to cadmium, were detected in extracts of the CdR-DK cells. The possible role that these peptides, which are derived from glutathione, may play in cadmium detoxification in *Chlorella* is discussed.

**Keywords:** *Chlorella* sp.; Cd<sup>2+</sup> resistant; Phytochelatins

### 1. Introduction

Heavy metal ions inhibit a variety of metabolic activities and are therefore toxic to most organisms when the concentration of a metal ion in the environment rises above a specific threshold. A number of ecotypes of plants and algae with increased resistance to heavy metals have been isolated from sites that are polluted with metals [1,2]. A variety of mechanisms of metal tolerance have been described in algae. These include sorption of

metal ions to cell wall components [3,4], excretion of organic compounds that form extracellular complexes with metal ions [5,6], and production of intracellular chelators of metal ions [7–11].

A common response of organisms upon exposure to heavy metals is the synthesis of intracellular metal-binding proteins which may function in detoxification of these metals ([12,13] and literature cited therein). The metal-binding proteins formed in *Chlorella* and *Euglena* differ from those found in animals and some fungi. They have a lower molecular weight and are more negatively charged [9,10]. In a number of cases where these

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ligands have been well characterised, induced Cd-binding peptides have been identified as phytochelatins, similar to those found in many higher plants [14,15]. Discussion of the structure and possible function of phytochelatins in plants and algae is presented in a number of recent reviews [16–18].

The ability of microalgae to thrive in environments that are polluted by heavy metals and the mechanisms that enable them to grow under these conditions are of interest. These algae can be utilised in processing and purification of industrial and domestic waste water that is contaminated with heavy metals ([13] and references therein). Isolation or selection of algal strains that are tolerant to the toxic effects of heavy metals, and analysis of the mechanisms that contribute to metal tolerance, may help to improve the efficiency of waste water processing as well as give insight into the processes involved in adaptation to this form of environmental stress.

In the research presented here we describe a mutant of *Chlorella* sp. that displays high resistance to  $\text{Cd}^{2+}$ . This strain was selected from a culture obtained from an urban waste water treatment plant. The growth characteristics of the original unselected culture and the selected Cd-resistant line are described. The role of cysteine, glutathione and phytochelatins in  $\text{Cd}^{2+}$  detoxification is also discussed.

## 2. Materials and methods

The *Chlorella* sp. used in this study was isolated from a stabilization pond of the domestic waste water treatment plant located in Beer-Sheva, Israel.

### 2.1. Growth conditions

Axenic cultures were grown on BG-11 mineral medium [19] without EDTA. All experiments were performed in 250-ml Erlenmeyer flasks containing 100 ml of medium, placed on a gyratory shaker (180 rev./min) at 27°C. Continuous illumination of 150 mmol/m<sup>2</sup>/s<sup>-1</sup>, at the surface of the flasks, was provided by cool white fluorescent lamps.

$\text{Cd}^{2+}$  resistant isolates were selected by increasing the  $\text{Cd}^{2+}$  concentration in the growth medium

in a stepwise manner. One hundred thousand cells of the WT culture were plated on solid BG-11 medium supplemented with 10  $\mu\text{M}$   $\text{Cd}^{2+}$ . This concentration of  $\text{Cd}^{2+}$  was toxic to WT cells causing severe inhibition of growth. Surviving colonies were rescued, dispersed in a minimal volume of liquid medium and replated on solid medium containing 20  $\mu\text{M}$   $\text{Cd}^{2+}$ . This procedure was repeated several times and each time the concentration of  $\text{Cd}^{2+}$  was further increased. Colonies capable of growing in the presence of 500  $\mu\text{M}$   $\text{Cd}^{2+}$  on solid medium were finally obtained, and one of them, designated CdR-DK was selected for further studies. This culture is maintained in liquid medium containing 100  $\mu\text{M}$   $\text{Cd}^{2+}$ .

Cultures of WT and CdR-DK at the exponential phase of growth were used for all of the experiments. Cultures of CdR-DK maintained on 100  $\mu\text{M}$   $\text{Cd}^{2+}$  were used unless otherwise stated. Cadmium nitrate, cysteine and glutathione were filter sterilized and added to the medium at the time of inoculation.

### 2.2. Analytical procedures

Cell density was determined at the time of inoculation and each day thereafter for 7 days by measuring turbidity. Growth rate (increase in absorbance at 540 nm/day) was calculated from the change in cell density between the 1st and the 3rd days.

Metal analysis was performed by anodic stripping voltammetry (ASV), in the mode of differential pulse anodic stripping voltammetry (DPASV) which determines the concentration of free  $\text{Cd}^{2+}$  in solutions. Measurements were conducted with a Model EI224 polarographic analyzer developed and built at the department of Electrical and Computer Engineering, Ben-Gurion University (for details see [20,21]). The current peak height observed at a specific potential in a voltammogram is directly proportional to the concentration of free  $\text{Cd}^{2+}$ . Under our experimental conditions the peak of  $\text{Cd}^{2+}$  is at a potential of 511 mV.

For analysis of PCs, cells were harvested by centrifugation (20 000  $\times g$ , 10 min) and frozen at -70°C. Frozen cells were pulverized in liquid nitrogen by grinding with acid-washed sand and extracted in 10 mM Tris-HCl buffer pH 8.0 (2 ml/g

fresh wt.). After removing cell debris by centrifugation, the supernatant was treated with 5-sulfosalicylic acid (5% final concentration) and either assayed immediately or stored at  $-70^{\circ}\text{C}$  for analysis later.  $\text{SH}^-$ -containing compounds were analyzed by HPLC as described by Mendum et al. [22]. This procedure gives a good separation between cysteine, GSH and PCs of various chain length. Thiol compounds were identified based on their retention times relative to the retention times of cysteine and GSH standards and of PCs purified from tomato cells [22].

For gel chromatography, frozen cells were pulverized and ground as above but using 10 mM Tris-HCl buffer, pH 8.5 (1 ml/g fresh wt.). After centrifugation ( $20\,000 \times g$ , 10 min) the protein concentration in the supernatant was assayed ac-

ording to Bradford [23]. Twenty mg of protein were loaded on a Sephadex G-50 column ( $50 \times 2$  cm), equilibrated with 20 mM Tris-HCl, pH 8.0, 0.1 M KCl, and eluted with the same buffer at a flow rate of 0.1 ml/min. Fractions of 2 ml were collected and analyzed for  $\text{SH}^-$  by Ellman's reagent [24], protein concentration by measuring  $A_{280}$ , and  $\text{Cd}^{2+}$  by atomic absorption spectrometry.

### 3. Results and discussion

Exposure of *Chlorella* sp. to increasing  $\text{Cd}^{2+}$  concentrations resulted in progressive inhibition of growth (Fig. 1). In the original WT line, growth was inhibited in the presence of  $2 \mu\text{M Cd}^{2+}$ . Growth of the resistant line decreased only slightly in response to  $10 \mu\text{M Cd}^{2+}$ . Higher  $\text{Cd}^{2+}$  concen-

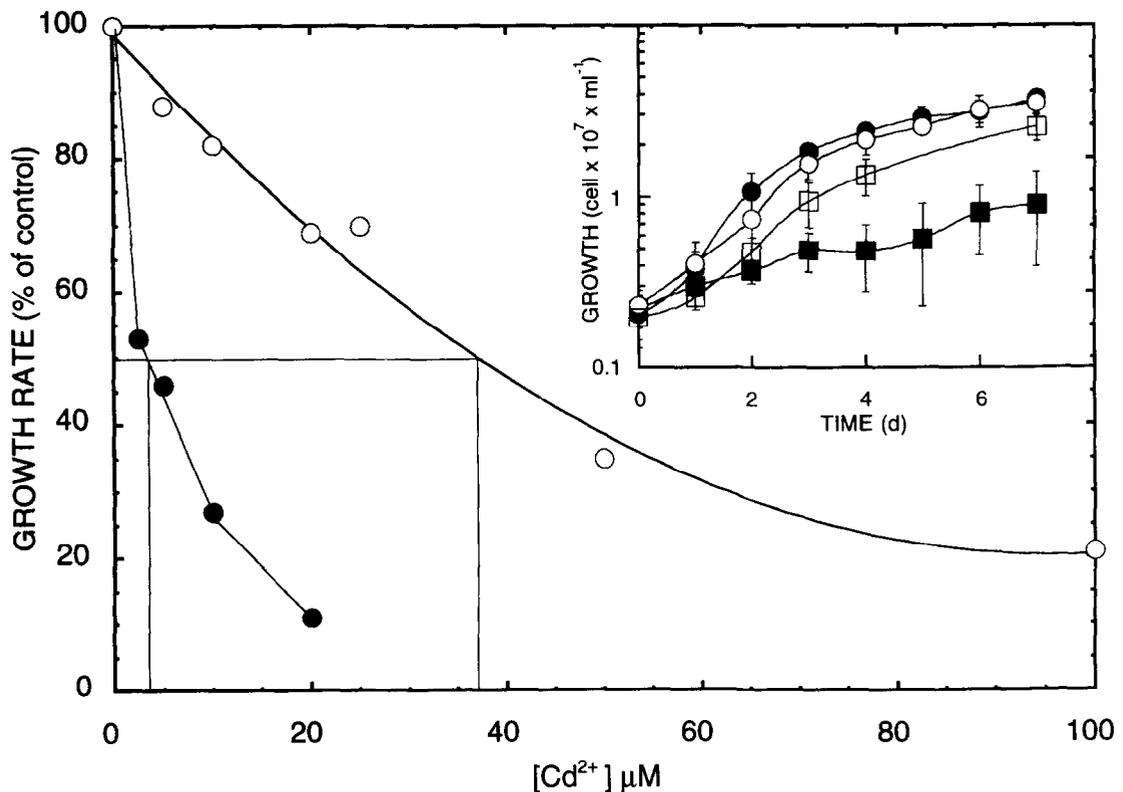


Fig. 1. Growth of WT and CdR-DK *Chlorella* lines as a function of  $\text{Cd}^{2+}$  concentration. The main figure shows the effect of increasing Cd concentrations on growth rate, expressed as % of control. Growth rate was determined between the first and the third day of the culture period. Filled symbols, WT; open symbols, CdR-DK. Results are means of at least 3 experiments at each  $\text{Cd}^{2+}$  concentration. Inset: growth curves of WT (filled symbols) and CdR-DK (open symbols) cell lines in the absence (circles) and presence (squares) of  $20 \mu\text{M Cd}^{2+}$ . Bars indicate S.D.

trations resulted in a gradual decrease in growth (Fig. 1). However, growth of the resistant line was not completely arrested, even upon exposure to 100  $\mu\text{M}$   $\text{Cd}^{2+}$  (data not shown). The growth conditions were identical for WT and CdR-DK lines. Thus, a higher growth rate for the resistant line at a given  $\text{Cd}^{2+}$  concentration, as compared with the WT, indicates a certain degree of tolerance to  $\text{Cd}^{2+}$ .

The effect of increasing  $\text{Cd}^{2+}$  concentrations on growth of the two lines is presented in Fig. 1. The growth rate at any given  $\text{Cd}^{2+}$  concentration was determined from the increase in cell density between day 1 and day 3 since this was the period of logarithmic growth (Fig. 1 insert). Inhibition of 50% in growth rate was observed at concentrations of 3  $\mu\text{M}$  and 35  $\mu\text{M}$   $\text{Cd}^{2+}$  in the WT and resistant lines, respectively.

As shown in Table 1, the inhibitory effect of  $\text{Cd}^{2+}$  on growth rate of both the sensitive and resistant lines was alleviated when either cysteine or GSH (100  $\mu\text{M}$  each) was added to the growth medium. This is in agreement with previous reports showing that GSH attenuates the inhibitory effect

Table 1  
Alleviation of cadmium toxicity to *Chlorella* WT and CdR-DK lines by cysteine and glutathione

Cadmium $\mu\text{M}$	Test compound $\mu\text{M}$	Growth rate Increase $A_{540}/\text{day}$
A. Sensitive cell line		
0	—	0.678
0	100 Cysteine	0.657
0	100 Glutathione	0.620
100	—	0.067
100	100 Cysteine	0.511
100	100 Glutathione	0.650
B. Resistant cell line		
0	—	0.619
0	100 Cysteine	0.600
0	100 Glutathione	0.625
100	—	0.236
100	100 Cysteine	0.442
100	100 Glutathione	0.660

Cells in the exponential phase of growth were subcultured into fresh medium containing the indicated compounds. (The results are of a representative experiment).

of  $\text{Cd}^{2+}$  in tomato cell cultures [22]. Compared to GSH, cysteine was not as effective in restoring the growth of WT and CdR-DK lines in the presence of 100  $\mu\text{M}$   $\text{Cd}^{2+}$ . Differences between GSH and cysteine in restoring growth in the presence of  $\text{Cd}^{2+}$  may reflect different rates of uptake for cysteine and GSH, availability of cysteine for GSH synthesis (the outcome of degradation or utilization by other metabolic processes) or the rate of GSH synthesis.

Several possible mechanisms could be proposed to account for the effects of cysteine or GSH on growth in the presence of  $\text{Cd}^{2+}$ . Being thiol compounds, cysteine or GSH could form organic complexes with  $\text{Cd}^{2+}$  in the medium, thereby lowering the availability of free ions in the medium and reducing uptake of  $\text{Cd}^{2+}$  into the cells. We used ASV to determine the concentration of  $\text{Cd}^{2+}$  in the presence of added GSH or cysteine. Either freshly made medium, or clarified medium obtained after removal of cells by low velocity centrifugation (2000  $\times g$  for 10 min), were tested. The results in Fig. 2, show that the current peak height at 511 mV, representing free Cd ions, was unchanged by the addition of glutathione to the medium. This indicates that formation of  $\text{Cd}^{2+}$ :GSH complexes is unlikely to account for the positive effect of GSH on growth of *Chlorella* sp. in the presence of  $\text{Cd}^{2+}$ . Similar results were obtained when cysteine was added to the medium (data not shown).

Another possibility is that the combination of  $\text{Cd}^{2+}$  and GSH in the medium induced the release of organic compounds from the cells into the medium. These compounds could form complexes with  $\text{Cd}^{2+}$  thereby lowering the concentration of free cadmium ions. Cells of WT and CdR-DK lines were grown for 1 week in medium containing  $\text{Cd}^{2+}$  and GSH. Cells and debris were then removed by low velocity centrifugation leaving low molecular weight organic compounds in solution. The concentration of  $\text{Cd}^{2+}$  in the clarified used medium was determined before and after acid digestion. No change in concentration of free cadmium ions was observed after acid digestion (data not shown). Thus, it was concluded that soluble extracellular organic complexes of  $\text{Cd}^{2+}$  which could contribute to the effectiveness of GSH in in-

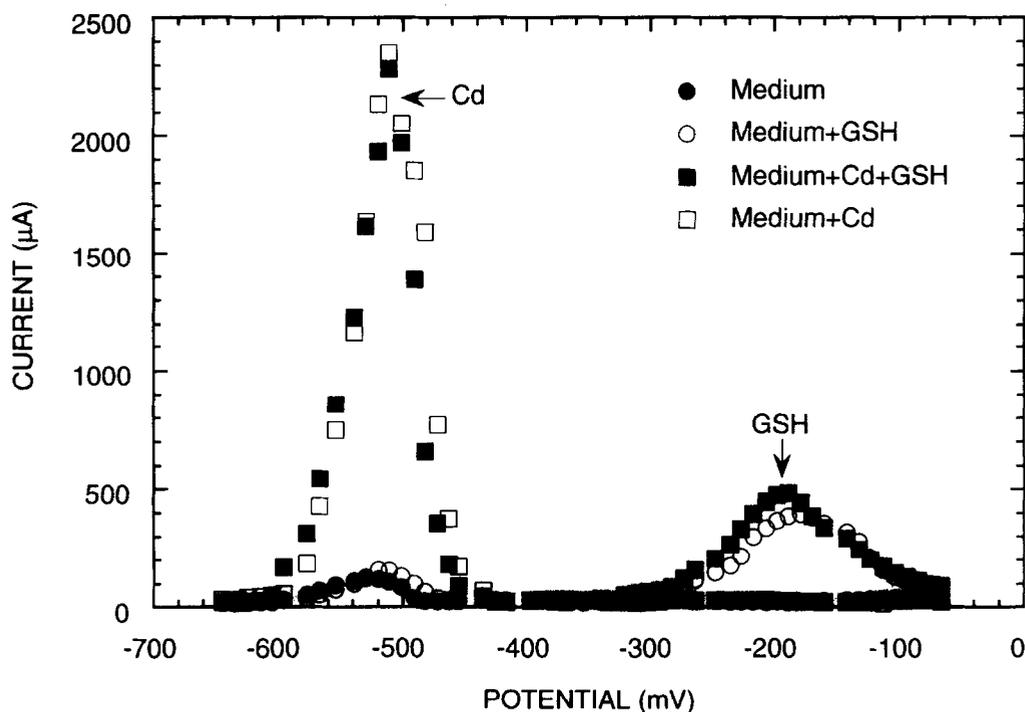


Fig. 2. Effect of GSH on availability of free Cd ions in medium. Voltammogram of: clarified medium (M), clarified medium containing, 20  $\mu\text{M}$   $\text{Cd}^{2+}$  (M +  $\text{Cd}^{2+}$ ), clarified medium containing, 20  $\mu\text{M}$  GSH (M + GSH) and clarified medium containing 20  $\mu\text{M}$   $\text{Cd}^{2+}$  plus 20  $\mu\text{M}$  GSH (M +  $\text{Cd}^{2+}$  + GSH). The tested compounds were added to the clarified medium 30 min before measurement. The results are of 1 experiment performed on medium obtained in 2 other experiments.

creasing tolerance to cadmium were not formed under these conditions.

The results presented above indicate that the protection provided by cysteine or glutathione against the toxic effects of  $\text{Cd}^{2+}$  is due, at least in part, to intracellular mechanism(s) involving these compounds or their metabolites.

The results summarized in Fig. 3 support the involvement of thiol compounds in intracellular binding of  $\text{Cd}^{2+}$  in CdR-DK cells. An extract prepared from these cells grown on 100  $\mu\text{M}$   $\text{Cd}^{2+}$ , was subjected to gel filtration on Sephadex G-50. Most of the cadmium in the extract was associated with low molecular weight, thiol compounds. The low molecular weight compounds could be PCs, which are considered to function as metal detoxification agents in plants by binding the toxic metal ions [16,17]. Similar gel filtration chroma-

tography profiles were obtained with extracts from plant cells exposed to  $\text{Cd}^{2+}$  [12].

To determine if *Chlorella* sp. synthesize PCs in response to  $\text{Cd}^{2+}$ , acid-soluble extracts were prepared from WT and CdR-DK cells grown under a variety of conditions. These extracts were analyzed by HPLC for the presence of PCs (Table 2). Two criteria were used to identify PCs in extracts from *Chlorella*: reactivity with Ellman's reagent indicating the presence of  $\text{SH}^-$  groups; and elution times that were identical with those of authentic PCs purified from tomato cells. In several cases, purified PCs were added to extracts from *Chlorella* cells and analyzed together to further support the identification of these compounds. The observations that these compounds increase in abundance upon exposure of cells to  $\text{Cd}^{2+}$ , and that this response is stimulated by ad-

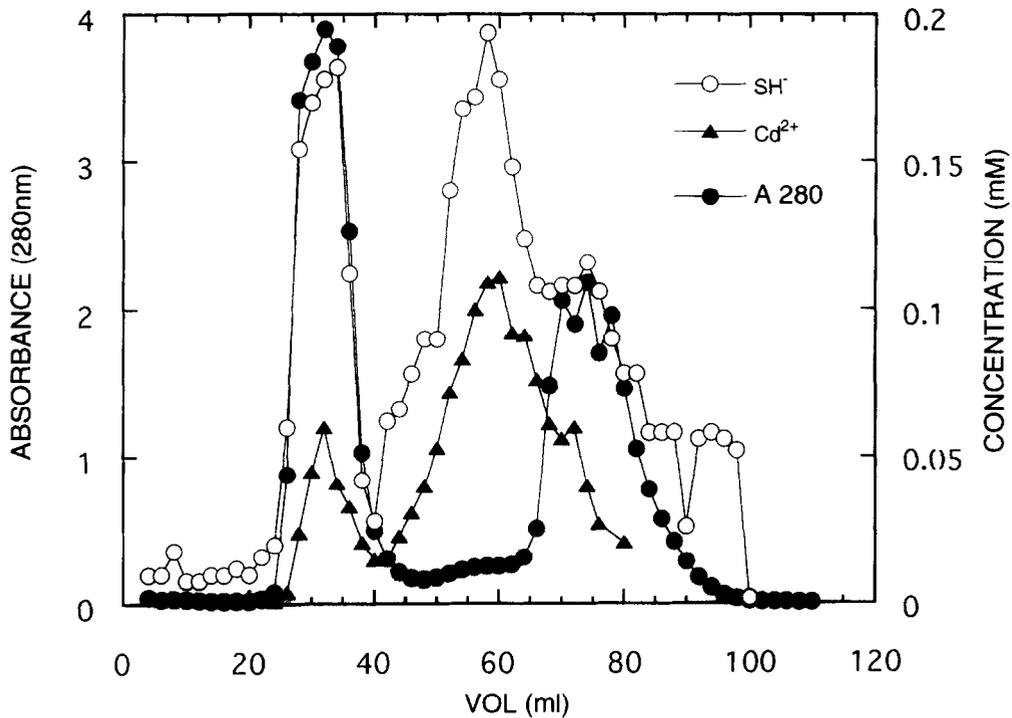


Fig. 3. Chromatogram of gel filtration on Sephadex G-50 of crude extract from CdR-DK cells grown in the presence of 100  $\mu\text{M}$   $\text{Cd}^{2+}$ .

dition of GSH, provide additional support that these are indeed PCs.

Cells were grown in the presence or absence of added cadmium, with or without GSH. The concentrations of cadmium used were 10 and 100  $\mu\text{M}$  for WT and CdR-DK lines, respectively, causing 80% growth inhibition in both lines. In the WT control (without added  $\text{Cd}^{2+}$ ), a low level of  $\text{PC}_2$  and  $\text{PC}_3$  could be detected (Table 2). In the presence of 10  $\mu\text{M}$   $\text{Cd}^{2+}$  the level of total PCs increased more than 2-fold. Accumulation of PCs in WT cells was further stimulated by addition of GSH to the medium. A larger peptide ( $\text{PC}_4$ ) was also detected when GSH was supplied. It should be noted that the addition of GSH to the growth medium in the absence of  $\text{Cd}^{2+}$  did not have any substantial effect on PC content (data not shown). In CdR-DK cultures grown continuously in the presence of 100  $\mu\text{M}$   $\text{Cd}^{2+}$  the levels of  $\text{PC}_2$  and  $\text{PC}_3$  were 30 and 140 times higher, respectively,

than in WT control cultures. Significant quantities of  $\text{PC}_4$  and  $\text{PC}_5$  were also detected in the resistant line. The levels of  $\text{PC}_2$  and  $\text{PC}_3$  in these cells were 10 and 50-fold higher than observed in WT cells exposed to 10  $\mu\text{M}$   $\text{Cd}^{2+}$ . Addition of GSH to CdR-DK cells along with 100  $\mu\text{M}$   $\text{Cd}^{2+}$  resulted in a further increase in PCs, mainly  $\text{PC}_3$  and  $\text{PC}_4$ .

In CdR-DK cells transferred from 100  $\mu\text{M}$   $\text{Cd}^{2+}$  to  $\text{Cd}^{2+}$  free medium, the content of PCs gradually decreased. After 5 generations (one transfer), only  $\text{PC}_2$  and  $\text{PC}_3$  were detected (Table 2, CdR-DK control). The total PC content in these cells was about 50% of that of the cells maintained on 100  $\mu\text{M}$   $\text{Cd}^{2+}$ . As the CdR-DK cells were grown on  $\text{Cd}^{2+}$ -free medium for a longer period of time, the level of PCs continued to decline. However, even after 50 generations (10 sequential transfers) under these conditions the concentration of PCs was still 2–3-fold higher than in the WT control. When subsequently exposed to in-

Table 2  
Effect of cadmium alone, or cadmium and GSH on the levels of PCs in WT and CdR-DK cells

Treatment	PC <sub>2</sub> mmoles	PC <sub>3</sub> GSH	PC <sub>4</sub> eqv./g fresh wt.	PC <sub>5</sub>	Total
<b>WT</b>					
control	0.016	0.015	<0.01	<0.01	0.031
+10 $\mu\text{M}$ Cd <sup>2+</sup>	0.044	0.038	<0.01	<0.01	0.082
+10 $\mu\text{M}$ Cd <sup>2+</sup> + 100 $\mu\text{M}$ GSH	0.074	0.308	0.079	<0.01	0.461
<b>CdR-DK</b>					
control	0.380	1.785	<0.01	<0.01	2.165
+100 $\mu\text{M}$ Cd <sup>2+</sup>	0.481	2.068	1.004	0.220	3.773
+100 $\mu\text{M}$ Cd <sup>2+</sup> + 100 $\mu\text{M}$ GSH	0.517	3.813	1.390	0.175	5.900
<b>CdR-DK (off Cd<sup>2+</sup> for 10 transfers)</b>					
control	0.030	0.057	0.023	<0.01	0.110
+10 $\mu\text{M}$ Cd <sup>2+</sup>	0.088	0.082	0.024	<0.01	0.194
+25 $\mu\text{M}$ Cd <sup>2+</sup>	0.097	0.157	0.053	<0.01	0.307
+100 $\mu\text{M}$ Cd <sup>2+</sup>	0.170	0.675	0.197	<0.01	1.042

Cells at exponential phase of growth were transferred to fresh medium including the additives mentioned above. After 4 days cultures were harvested and PCs were analyzed by HPLC. Note: control had no added Cd<sup>2+</sup> in the growth medium. (The results are of a representative experiment).

creasing concentrations of Cd<sup>2+</sup>, the CdR-DK line retained both its ability to grow in the presence of 100  $\mu\text{M}$  Cd<sup>2+</sup> and the capacity to synthesize PCs (Table 2). The alteration which imparts Cd<sup>2+</sup> tolerance to CdR-DK cells is relatively stable since the cells did not lose the capacity to grow in the presence of higher concentrations of Cd<sup>2+</sup> after extended growth under non-selective conditions. We can conclude from the data presented above that PCs are indeed present in a relatively low level in cells of the wild type *Chlorella* sp. and that these peptides are more abundant in the resistant line CdR-DK.

It is clear that *Chlorella* synthesizes PCs in response to Cd<sup>2+</sup>. A number of observations indicate that PCs are involved in Cd<sup>2+</sup> detoxification in this species. Intracellular Cd<sup>2+</sup> is associated with thiol compounds that have gel filtration chromatography properties similar to Cd-PC complexes isolated from plants and fungi. Exposure to Cd<sup>2+</sup> stimulates production of PCs in

both WT and CdR-DK cells. This response is further stimulated by addition of GSH to the growth medium. GSH also restores growth of *Chlorella* in the presence of Cd<sup>2+</sup>, and this is not the result of Cd<sup>2+</sup> forming extracellular complexes with GSH or other organic compounds. Similar results have been obtained with tomato cells [22].

Resistant cells accumulate large amounts of PCs when maintained on 100  $\mu\text{M}$  Cd<sup>2+</sup>. Similar observations have been made with selected Cd-resistant plant cells [25,26]. Is the accumulation of PCs responsible for Cd<sup>2+</sup> tolerance in CdR-DK cells? The results presented here cannot directly answer this question, but do raise a number of points of interest. The PC content of CdR-DK cells grown continuously in the presence of 100  $\mu\text{M}$  Cd<sup>2+</sup> is 3.5 times higher than that of resistant cells grown in Cd-free medium and then transferred to medium containing 100  $\mu\text{M}$  Cd<sup>2+</sup>. Despite the lower level of PCs in these cells, their growth rate is similar to that of cells maintained in the presence of Cd<sup>2+</sup>. This suggests that there is a superfluous, non-functional overproduction of PCs in CdR-DK cells.

CdR-DK cells maintain their resistance to Cd<sup>2+</sup> in the absence of Cd<sup>2+</sup> selection. When grown for many generations in medium without added Cd<sup>2+</sup>, CdR-DK cells contain more PCs than WT cells. Again, similar results have been reported in Cd-tolerant tomato cells [25,27]. The level of PCs in WT cells exposed to 10  $\mu\text{M}$  Cd<sup>2+</sup> is less than half that in CdR-DK cells under similar conditions. This relatively small difference in PC accumulation could still account for the contrasting growth of WT and CdR-DK cells in the presence of 10  $\mu\text{M}$  Cd<sup>2+</sup>. The low level of PCs in WT cells may be insufficient to form complexes with all of the intracellular Cd<sup>2+</sup>. Addition of GSH both stimulates PC synthesis and restores growth to WT cells.

CdR-DK cells accumulate large amounts of PCs when they are grown in medium containing 100  $\mu\text{M}$  Cd<sup>2+</sup>. Gel filtration chromatography suggests that these PCs form complexes with Cd<sup>2+</sup>. The results presented here do not prove that increased production of PCs is responsible for the resistance of CdR-DK cells, although that is indeed one possible mechanism. It has been shown that PCs are

unlikely to be involved in increased metal tolerance of some plants isolated from metal contaminated environments [28–31]. Other mechanisms that may lead to metal tolerance include the rate of formation and altered composition of Cd-PC complexes [32,33], the subcellular localization of these complexes [34], the rate of cadmium uptake or exclusion of cadmium. Further physiological and genetic studies are needed to establish the mechanisms involved in increasing Cd tolerance in these *Chlorella* strains.

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