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Effect of copper exposure on gene expression profiles in *Chlamydomonas reinhardtii* based on microarray analysis

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Abstract

Copper is a naturally occurring trace metal with toxic properties for man and environment. It is assumed that toxicity is primarily caused by oxidative damage, generated through the production of reactive oxygen species. Copper is, however, also an essential element, which means trace amounts are necessary for biological processes to function properly. Organisms are therefore presented with the challenging problem of maintaining copper concentrations within a well-defined range to avoid stress.

We exposed the green alga *Chlamydomonas reinhardtii* to different copper concentrations and used microarray analysis to investigate the changes in mRNA abundances and to obtain an image of the molecular mechanisms underlying copper homeostasis. The results confirm and extend upon previous findings showing that in the case of lower copper concentrations there is a change in levels of mRNA coding for alternative polypeptides which can take over the function of certain copper containing molecules so as to compensate for the lack of copper. In the case of copper toxicity, there is a strong upregulation of transcripts encoding enzymes involved in oxidative stress defense mechanisms. In both cases, there were significant changes in expression levels of transcripts coding for enzymes involved in several metabolic pathways (photosynthesis, pentose phosphate pathway, glycolysis, gluconeogenesis), in general stress response (heat shock proteins) and in intracellular proteolysis (lysosomal enzymes, proteasome components).

Keywords: Copper homeostasis; *Chlamydomonas reinhardtii*; Stress; Microarray; Gene expression profiles

1. Introduction

Among environmental pollutants, trace metals make up a unique category: contrary to most organic pollutants, they are naturally occurring elements. In addition, they are persistent, have the potential to bio-accumulate and are potentially toxic to biota.

Copper is such a naturally occurring trace metal. Since industrialisation, Cu releases have greatly increased, such that copper contamination and toxicity are occurring more and more in both surface water and groundwater systems (Hong et al., 1996). Copper is known to be toxic to many organisms such as algae, invertebrates and fish (Hughes and Poole, 1989). In addition, a major concern about the effect of copper is its ability to potentially harm humans (Howell and Gawthorne, 1987). The toxic effects of copper are due to the generation of oxidative stress, caused by the production of reactive oxygen species (Pinto et al., 2003). Copper is, however, also an essential element, which means organisms need trace amounts of it for the optimal functioning of certain enzymes.

Given the increasing release of copper into the environment and its potential harmful effects on biota, it is important to expand our knowledge on how organisms deal with copper. This knowledge can be used in the search for efficient and reliable methods for detection and remediation. Moreover, it will aid us in establishing essential concentration ranges of trace elements and help us in determining realistic environmental quality criteria for these compounds.

Copper homeostasis in *Enterococcus hirae* has been extensively studied and reviewed and is probably the best understood

Abbreviations: SSC, saline sodium citrate; SDS, sodium dodecyl sulphate; BSA, bovine serum albumin; EST, expressed sequence tag; RT, room temperature

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prokaryotic copper homeostasis system. It consists of four genes arranged in the *cop* operon. The gene products of *copA* and *copB* are copper transporting ATPases, *copY* encodes a copper-responsive repressor, and *copZ* encodes a chaperone which can catalyze intracellular copper routing (Solioz and Stoyanov, 2003).

In eukaryotic cells, copper transport is best characterized in *Saccharomyces cerevisiae*, where copper is believed to involve a cell-surface cupric reductase activity (Hassett and Kosman, 1995) and separate high-affinity transporter proteins encoded by the *CTR*1 and *CTR*3 genes (Puig and Thiele, 2002; Marjorette et al., 1998).

In plants, relatively little is known about copper acquisition and transport into and within cells. However, recently, rapid progress has been made such that several families of trace metal transporters have now been identified. P-type ATPase trace metal transporters have been identified in a wide range of organisms including plants and are implicated in the transport of a range of essential and potentially toxic metals across cell membranes. The first P-type ATPase reported in plants was PAA1 from *Arabidopsis thaliana* (Tabata et al., 1997). Another widespread family of copper transporters, the COPT proteins, has been identified in plants by functional complementation in yeast. Five members of this family, COPT1–COPT5 have been found in *Arabidopsis thaliana* (Sancenòn et al., 2003). Copper chaperones are involved in the intracellular trafficking of metal ions. In plants, three different members of the copper chaperone family, CCH, COX17 and CCS, have been identified and characterized at different levels of biological organisation (Yruela, 2005).

For another important primary producer, (micro)algae, much less is known on the mechanisms of copper toxicity. Yet, as this trophic level forms the basis of many aquatic food webs, it is crucial to gain more in depth understanding on the mechanisms underlying copper homeostasis and toxicity. In *Chlamydomonas reinhardtii*, a green freshwater alga, copper homeostasis mechanisms have been investigated but have not been fully elucidated yet. Hill et al. (1996) found that copper uptake in *C. reinhardtii* is regulated in response to copper availability. The same uptake system appears to operate in both copper-replete and copper-deficient cells, but its expression or activity must be induced under copper-deficient conditions. In the case of copper deficiency, *C. reinhardtii* can still survive because of the existence of a well characterized metal-responsive pathway which consists of the reciprocal accumulation of plastocyanin and cytochrome *c*6 (cyt c6) in response to the amount of copper supplied in the growth medium (Quinn et al., 1999). Several regulatory and target genes in nutritional copper signaling have been identified (Eriksson et al., 2004).

In order to further investigate the fate of copper in organisms, changes in gene expression are highly informative. A toxicogenomic approach, combining the fields of genomics and toxicology, presents important opportunities to improve our understanding of the molecular mechanisms underlying stress responses to copper (Bradley and Theodorakis, 2002; Nuwaysir et al., 1999). Microarrays provide a means to simultaneously assess the expression of thousands of genes on the mRNA level and therefore present a sophisticated, high throughput screening tool for the identification of molecular mechanisms of copper toxicity and of novel biomarkers for copper exposure (Neumann and Galvez, 2002; Vrana et al., 2003).

In order to better understand gene expression patterns in response to copper, we have used *C. reinhardtii*, a unicellular green alga, as a model organism. With the completion of the *Chlamydomonas* genome project (http://www.chlamy.org), several genes that are involved in the response to copper may be identified. *C. reinhardtii* has several advantages as a model organism for stress response. Growth is rapid with cells attaining logarithmic growth phase in 2–3 days. It is also sensitive to environmental contaminants and responds to small changes in the environment by regulating transcription by the activation or repression of genes (Hanikenne, 2003). Genes identified in *C. reinhardtii* may also be transformable into common green algae ubiquitous to the environment.

In this study, we have characterized both the effects of low and high copper levels on the gene expression activity in this micro alga using microarray analysis. Clear distinct effects, reflecting "deficient" conditions as well as toxic stress could be discriminated in the exposed algal population.

2. Materials and methods

2.1. Chlamydomonas strain and culture conditions

C. reinhardtii (11-32a, Culture Collection of Algae (SAG) at the University of Göttingen) were maintained in Tris–Acetate–Phosphate (TAP) liquid medium (Harris, 1989) at $25 \pm 1\,^{\circ}C$ under alternating light:dark cycles of 14-h light:10-h dark with a light intensity of 200 $\mu E/(m^2\,s)$. To prevent cultures from being contaminated with metals, all glassware was soaked in 2.5N HCl overnight before use and all solutions were prepared with MQ water.

A growth inhibition test for *C. reinhardtii* was set up with a range of $CuSO_4$ concentrations to determine the appropriate exposure conditions for the microarray experiment. Four test media were prepared with concentrations of 5, 50, 100 and 150 μM $CuSO_4$. These test media together with one control medium (no $CuSO_4$ added) were inoculated with 3×10^4 algal cells. At time points $t = 0$, 24, 48 and 72 h cells were counted using a Multisizer 3 Coulter Counter (Beckman Coulter, USA). The percentage growth inhibition due to copper exposure was calculated from which the EC_{50} was derived.

Based on the growth inhibition test, it was concluded to use the following total exposure concentrations in triplicate for the microarray experiment: 8, 25, 55 and 125 μM, together with a control culture (no $CuSO_4$ added). The metal content of the media was assessed at the start and during the experiment using graphite furnace atomic absorption spectroscopy (AAS, SpectrAA 800, Varian, USA). The media were inoculated with 3×10^4 algal cells and during 72 h, growth was determined by counting cell numbers. Using the chemical speciation model VisMinteq, the free copper ion activity in the medium was calculated. Furthermore, RNA was extracted at fixed time points and microarray hybridizations were carried out. Microarray results