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# Metal metabolism in the red alga *Cyanidium caldarium* and its relationship to metal tolerance

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**Abstract**

The unicellular red alga *Cyanidium caldarium* is tolerant to high levels of various metal ions. Cells of this alga cultured with divalent metal ions at 5 mM contained an elevated concentration of each metal, with the highest level for Zn followed by Mn > Ni > Cu. This order is in fair agreement with the toxicity levels reported previously, with the exception of Mn, which shows a toxicity level comparable to that of Ni. Transmission electron microscopy indicated the presence of electron-dense bodies in the algal cells, and elemental analysis by energy dispersive X-ray spectrometry showed high levels of Fe and P in these bodies. Accumulation of Zn was found in these particles in Zn-treated algal cells, whereas no such deposition was found for Cu, Ni, or Mn in cells treated with the respective metals. Although trapping of Zn in the intracellular bodies may contribute to reduction of metal activity in the cells, this effect can be overcome by high intracellular levels of Zn that result in a high degree of toxicity. The correlation between intracellular concentration and toxic levels of metal ions implies that the reduced incorporation of the metals is a major detoxification mechanism in this alga.

*Abbreviations:* EDX – energy dispersive X-ray; ESR – electron spin resonance

## Introduction

The unicellular red alga *Cyanidium caldarium* is considered one of the most primitive algae (Geitler 1933; Chapman 1974). This alga has a simple cellular structure, with each cell having one mitochondrion, one plastid, and one nucleus plus other organelles, such as Golgi bodies, endoplasmic reticulum, and microbodies (Nagashima and Fukuda 1981; Kuroiwa *et al.* 1994). As expected, based on its distribution in areas where acidic water can leach high levels of metal ions, *C. caldarium* has a high level of tolerance to various metal ions (Wood and Wang 1983; Ahlf 1988; Yoshimura *et al.* 2000). Based on the concentration at which the growth rate is decreased to half that of control cells cultured in the absence of heavy metals, the highest level of tolerance is found for Al, followed by Cu > Cr, Mn, Ni > Zn > Cd.

Observations by electron microscopy indicated the presence of electron-dense bodies in the cells, and these bodies were shown to contain high levels of Fe and P (Nagasaka *et al.* 2002). The ESR spectrum of these bodies represents a clustered structure formed from Fe(III) and phosphate groups, which may be derived from polyphosphates or orthophosphates. The level of Fe-$PO_4$ accumulation depends strongly on the iron status of the culture medium, indicating a role for the electron-dense bodies in iron storage (Nagasaka *et al.* 2003). In addition, accumulation of Al was observed in the electron-dense bodies in *C. caldarium* cells cultured in medium containing Al. This sequestration of Al(III) ions may play a role in increasing tolerance to this metal ion.

Metal ions exert different levels of toxicity on *C. caldarium*. However, it is unclear how the alga deals with the metal ions. Differences in toxicity levels were found between different metal ions. Here, we report the effects of metal concentration in the medium on that within the cells. EDX analysis and ESR spectrometry were used to elucidate the localization and chemical forms of heavy metals in *C. caldarium* cells.

## Materials and methods

### *Culture conditions for* C. caldarium

*C. caldarium* strain R-11 was obtained from the Institute for Molecular and Cellular Biology, University of Tokyo. The alga was cultured autotrophically in Allen's medium (1959) containing: 10 mM $(NH_4)SO_4$, 2 mM $KH_2PO_4$, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 71 $\mu$M $FeCl_3$, 46 $\mu$M $H_3BO_3$, 9.1 $\mu$M $MnCl_2$, 760 nM $ZnSO_4$, 310 nM $CuSO_4$, 200 nM $NH_4VO_3$, and 100 nM $Na_2MoO_4$, and the pH was adjusted to 2.0 with 0.5 M $H_2SO_4$. For metal treatment, $CuSO_4$, $ZnSO_4$, $Ni(NO_3)_2$, or $MnCl_2$ was added to the medium at 5 mM. The algae were cultured in 100-ml shaking flasks at 50 °C under continuous light (36 $\mu$mol photons $m^{-2}s^{-1}$) for three weeks.

### *Determination of metal concentration in* C. caldarium *cells*

After cultivation with metal-containing media for three weeks, the cells were collected by centrifugation. To wash out the metals derived from the remaining media, the cells were suspended in dilute sulfuric acid (pH 2.0) and centrifuged again. The washing steps were repeated three times and no metals were detected in the final supernatant. The washed cells were dried, and digested with concentrated $HNO_3$ at 150 °C for 5 h. Metal concentrations were determined by inductive coupled plasma emission spectrometry using a Seiko SPS 1200 VA spectrometer (Tokyo, Japan).

### *EDX analysis*

After cultivation for 3 weeks, the algal cells were frozen using a high pressure freezing apparatus (HPM 010; Bal-Tec, Liechtenstein, Switzerland). The frozen cells were taken up into cryo-substitution vials containing anhydrous acetone at −80 °C. Substitution-fixation was carried out at −80 °C for 2 days, and then the temperature was gradually raised to −20 °C for 2 h, 4 °C for 2 h, and room temperature for 15 min. After several rinses in anhydrous acetone and two rinses in propylene oxide, the samples were embedded in a mixture of Epon 812 and Araldite resins (TAAB, Berkshire, UK). Thin sections were cut using a Leica Ultracut microtome (Leica, Vienna, Austria). Ultrathin sections were mounted on uncoated copper grids (Zn-, Mn-, Ni-treated and control samples) or nickel grids (Cu-treated sample). The elements in the electron-dense bodies were determined using a JEOL 2000FX transmission electron microscope (JEOL, Tokyo, Japan) equipped with an EDX spectrometer. An electron beam with an acceleration voltage of 200 kV and a spot size of 10 nm was focused on the specimens, and X-ray spectra were obtained by collecting X-rays for approximately 100 sec.

### *ESR spectrum study*

ESR spectra were recorded on a JSE-FA 100 spectrometer (JEOL, Tokyo, Japan). The algal cells grown in Allen's media with or without 5 mM $MnCl_2$ were washed three times with dilute $H_2SO_4$ (pH 2.0) and then harvested by centrifugation at 2500 rpm for 5 min. The cell pellet was resuspended in 0.5 ml of dilute $H_2SO_4$. The suspension was put into quartz ESR tubes (5 × 270 mm), frozen in liquid nitrogen, and ESR spectra were measured. The concentration of Mn(II) was determined based on the amplitude of the lowest field Mn(II) signal. The signal was calibrated with 0.36 mM $MnCl_2$ solution. All the spectra were recorded at the following instrument settings: temperature, −196 °C; frequency, 9.13 GHz; modulation frequency, 100 kHz; microwave power, 1 mW; amplitude, 100; time constant, 1 second; scan time, 4 minutes; scan range, 400 mT.

## Results

Table 1 summarizes the metal concentrations in *C. caldarium* cells cultured for 3 weeks in media containing heavy metals. Cultivation in the presence of these metals increased the intracellular concentrations of the respective metals, with the highest level for Zn followed by Mn > Ni > Cu. In the Zn-treated cells, the intracellular concentration of Zn reached 0.23% of dry cell matter, a value corresponding to a concentration of 4 mM on the assumption that wet cells contain 90% water. Thus, Zn and Mn would be incorporated almost freely into the cells. Metal treatment also affected the

*Table 1.* Concentrations of metals in *Cyanidium caldarium* cells cultured in metal-containing media.

| Treatment | Metal concentration ($\mu$g g$^{-1}$ dry weight) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Fe | Mg | Ca | Zn | Mn | Cu | Ni |
| Control | 246 ± 34 | 1670 ± 40 | 102 ± 4 | 14 ± 3 | 21 ± 3 | 1.6 ± 0.2 | – [a] |
| Zn 5 mM | 140 ± 1 | 1220 ± 30 | 77 ± 2 | 2332 ± 26 | 2 ± 4 | 7.7 ± 0.4 | – |
| Mn 5 mM | 422 ± 6 | 1590 ± 10 | 130 ± 10 | 22 ± 1 | 1780 ± 80 | 1.4 ± 0.2 | – |
| Cu 5 mM | 147 ± 5 | 1670 ± 20 | 102 ± 4 | 14 ± 3 | 21 ± 3 | 104 ± 18 | – |
| Ni 5 mM | 304 ± 23 | 890 ± 179 | 53 ± 10 | 15 ± 4 | 17 ± 5 | 1.4 ± 0.5 | 22.8 ± 2.3 |

[a]Concentrations were below the limits of detection by ICP analysis.

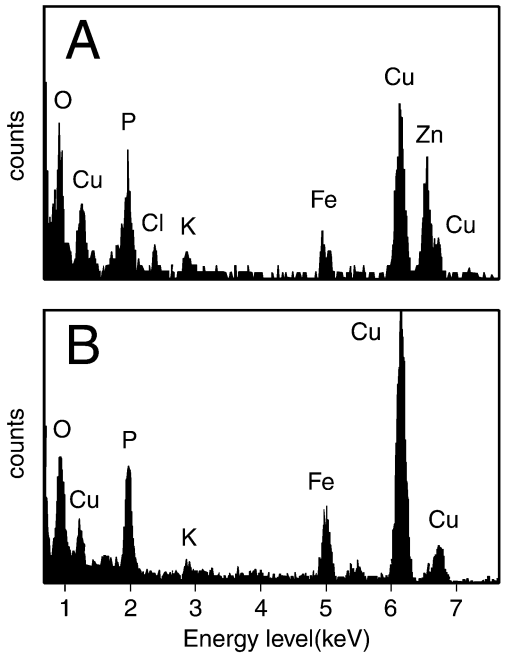


*Figure 1.* EDX spectra of the electron-dense bodies in *C. caldarium* cultured in heavy-metal-containing media. A: Electron-dense bodies in Zn-treated cells. Large Zn peaks were observed with large peaks of Fe, P, and O. B: Electron-dense bodies in Mn-treated cells. Large peaks of Fe, P, and O were observed, although no Mn peaks were detected. The horizontal axis shows energy levels and the vertical axis shows the X-ray counts in each spectrum.

levels of other metals. Zn or Cu treatment reduced the levels of Fe in the treated cells to half that in control cells, while the reverse was true for Mn and Ni treatment. In addition, treatment with Ni reduced the Mg level to half that of control cells.

Figure 1A shows the EDX spectrum of the electron-dense bodies in Zn-treated cells. The X-ray spectrum of the body showed intense signals for Fe, P, O, and Zn (the intense Cu signals were due to the copper grids). No such intense Zn signals were observed for the chloroplast, cytoplasm, or the cell wall of Zn-treated cells (data not shown). The EDX spectrum of the electron-dense bodies in Mn-treated cells is shown in Figure 1B. Whereas intense signals for Fe, P, and O were observed, there was no appreciable Mn signal. No detectable Mn signals were observed in the spectra of the chloroplast, cell wall, or cytoplasm of Mn-treated cells (data not shown). This was also the case for Ni and Cu; these elements were not detected in Ni- and Cu-treated cells (data not shown).

Figure 2A shows the ESR spectrum of *C. caldarium* cells cultured in control medium. The sharp signal at a g-value of 2.00 was due to organic radicals. A broad isotropic signal was observed at a g-value of 2.00, which was assigned to the Fe-$PO_4$ cluster that constitutes the electron-dense body (Nagasaka *et al.* 2003). An increase in recording temperature from −170 to −20 °C depressed the signal intensity without changing line shape (data not shown), indicating paramagnetic nature of the electron-dense body. The six signals that appeared at magnetic field strengths from 301.89 to 349.37 mT (arrowheads) are characteristic of Mn(II) aqua ions. As shown in Figure 2B, signals due to organic radicals and the Fe-$PO_4$ cluster were also observed in the ESR spectrum of Mn-treated cells. In addition, signals for Mn(II) aqua ions were also observed in this spectrum with intensities greater than those in control cells. The Mn(II) aqua ion concentration was estimated to be 30% of total Mn present in the cells, based on the amplitude of the Mn(II) signal at the lowest magnetic field strength.

## Discussion

*C. caldarium* cells possess electron-dense bodies, which contain high levels of Fe and P and can play a role in storage of Fe (Nagasaka *et al.* 2003). Accumulation of Al in these bodies is also found in cells cultured in medium containing Al, indicating a

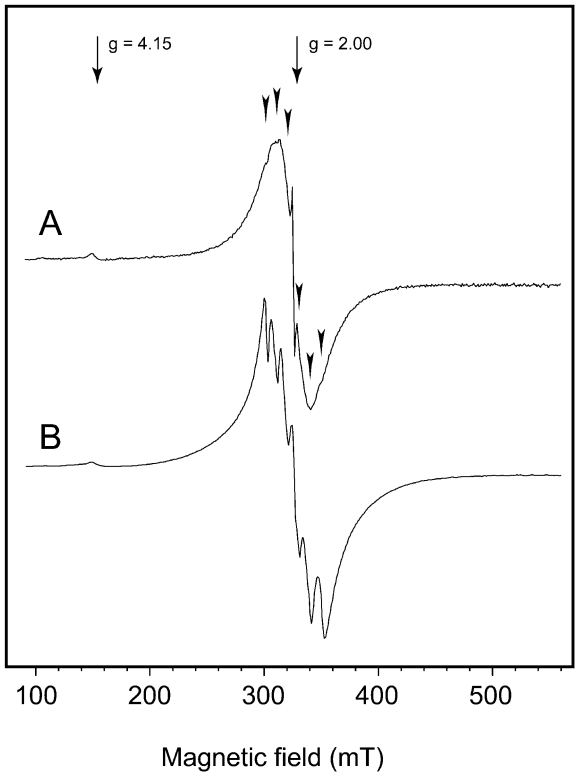


*Figure 2.* ESR spectra of intact *C. caldarium* cells. A: Algal cells cultured in control medium. B: Algal cells cultured in Mn-containing medium. In each ESR spectra, six signals that were characteristic of Mn(II) ions were observed (arrowheads). The spectrometer conditions were as follows: temperature, −196 °C; microwave frequency, 9.13 GHz; modulation frequency, 100 kHz; and microwave power, 1 mW.

role in detoxification of Al (Nagasaka *et al.* 2002). Of the metal ions examined, Zn alone was accumulated in the bodies. It is unlikely that high intracellular metal concentration is a prerequisite for metal deposition in the electron-dense bodies. Algal cells grown in the presence of 5 mM Mn contained a high level of Mn, while no appreciable Mn deposition was observed in the electron-dense bodies. In contrast, deposition of Al was clearly demonstrated in cells treated with 200 mM Al, which had an intracellular Al concentration of 0.025 mg/g of dry matter (unpublished data), a concentration comparable to those of Ni and Cu.

ESR analyses indicated that the phosphate moiety of inorganic phosphate or polyphosphate acts as a ligand for metal ions in the electron-dense bodies (Nagasaka *et al.* 2003), as were also seen in bacterium (Goldberg *et al.* 2001; Schönborn *et al.* 2001). As shown in Table 2, the binding abilities of the trivalent ions Fe(III) and Al(III) are much greater than those

*Table 2.* Solubility of metal ions in the presence of phosphate and deposition in the electron-dense bodies.

| | Metal ions | | | | | |
|---|---|---|---|---|---|---|
| | $Fe^{3+}$ | $Al^{3+}$ | $Mn^{2+}$ | $Ni^{2+}$ | $Cu^{2+}$ | $Zn^{2+}$ |
| pM[a] | 18.9 | 15.2 | – | 8.1 | 10.3 | 8.7 |
| Deposition in the EDB | Yes | Yes | No | No | No | Yes |

[a]Solubility of metal ions in the presence of 1 mM $PO_4^{3-}$ ions. Data are expressed as − log concentrations of metal ions. Formation of metal hydroxides was omitted. Solubility data were taken from Dean (1999).

of the divalent ions Ni(II), Cu(II), and Zn(II), as estimated on the basis of solubility of the metal in the presence of 1 mM $PO_4^{3-}$ ions. Thus, the high affinities of Al(III) and Fe(III) to phosphate ions could facilitate deposition of these ions in the electron-dense bodies. Mn(II) has a much lower affinity to phosphate ions, which is presumably responsible for its failure to accumulate in the electron-dense bodies. High intracellular levels of Zn would facilitate its deposition in the electron-dense bodies in Zn-treated cells, irrespective of its affinity to phosphate.

The intracellular concentrations of metal ions increased in the following order: Cu < Ni < Mn < Zn. This is consistent with the levels of toxicity of these metal ions to *C. caldarium* cells, except for Mn, which shows toxicity comparable to that of Ni (Yoshimura *et al.* 1999). It is, therefore, likely that the intracellular concentration of metals roughly determines the toxicity level, although the chemical nature of the metal ions is also a factor. Mn-treated cells contained very high levels of Mn (Table 1), while this ion shows only moderate toxicity. Approximately 30% of Mn is found as Mn(II) aqua ions in cells cultured with 5 mM Mn. This indicates that 70% of Mn is transformed into unobservable forms, such as Mn(III) ions (Sakurai and Nishida 1982). These Mn ions could interact weakly with molecules with crucial roles in the cells, as Mn(II) shows little toxicity irrespective of the intracellular Mn concentration. Zn is deposited in the electron-dense bodies, while it is highly toxic to this alga. High levels of intracellular Zn could outweigh the deposition of Zn, resulting in high toxicity. Al has high affinity to phosphate, which may facilitate its deposition in the electron-dense bodies and thus ameliorate its toxicity. However, it is unlikely that the electron-dense bodies play a critical role in detoxification of divalent metals. Prevention of the entry of metal ions into the cell would be the most important

factor in the tolerance of this alga to high levels of metal ions.

## References


Ahlf W. 1988 Recovery of metals from acid waste water by *Cyanidium caldarium. Appl Microbiol Biotechnol* **28**, 512–513.

Allen MB. 1959 Studies with *Cyanidium caldarium*, an anomalously pigmented chlorophyte. *Archiv Mikrobiol* **32**, 270–277.

Chapman DJ. 1974 Taxonomic status of *Cyanidium caldarium.* The porphyridiales and goniotrichales. *Nova Hedwigia* **25**, 673–682.

Dean JA. 1999 Lange's Handbook of Chemistry. 15th ed. *Electrolytes, Electromotive Force and Chemical Equilibrium.* New York: McGraw-Hill; 8.6–8.17

Geitler L. 1933 Diagnosen neuer Blaualgen von den Sunda-Inseln. *Arch Hydrobiol Suppl* **12**, 622–634.

Goldberg J, Gonzalez H, Jensen TE, Corpe WA. 2001 Quantitative analysis of the elemental composition and the mass of bacterial polyphosphate bodies using STEM EDX. *Microbios* **106**, 177–188.

Kuroiwa T, Kawazu T, Takahashi H, Suzuki K, Ohta N, Kuroiwa H. 1994 Comparison of ultrastructures between the ultra-small eukaryote *Cyanidioschyzon merolae* and *Cyanidium caldarium. Cytologia* **59**, 149–158.

Nagashima H, Fukuda I. 1981 Morphological properties of *Cyanidium caldarium* and related algae in Japan. *Jap J Phycol (Sôrui)* **29**, 237–242.

Nagasaka S, Nishizawa NK, Negishi T, Satake K, Mori S, Yoshimura E. 2002 Novel iron storage particles may play a role in aluminum tolerance of *Cyanidium caldarium. Planta* **215**, 399–404.

Nagasaka S, Nishizawa NK, Watanabe T, Mori S, Yoshimura E. 2003 Evidence that electron-dense bodies in *Cyanidium caldarium* have an iron-storage role. *Biometals*, **16**, 465–470.

Schönborn C, Bauer HD, Röske I. 2001 Stability of enhanced biological phosphorus removal and composition of polyphosphate granules. *Water Res* **35**, 3190–3196.

Sakurai H, Nishida M. 1982 EPR detection and characterization of manganous(II) ion in subcellular fractions of the liver and kidney and thyroid homogenate of mice treated with manganese chloride. *Inorganica Chimica Acta* **66**, L17–L20.

Wood JM, Wang H-K. 1983 Microbial resistance to heavy metals. *Environ Sci Technol* **17**, 582–590.

Yoshimura E, Nagasaka S, Sato Y, Satake K, Mori S. 1999 Extraordinarily high aluminium tolerance of the acidophilic thermophilic alga, *Cyanidium caldarium. Soil Sci Plant Nutr* **45**, 721–724.

Yoshimura E, Nagasaka S, Satake K, Mori S. 2000 Mechanism of aluminium tolerance in *Cyanidium caldarium. Hydrobiol* **433**, 57–60.