Journal of Nuclear and Radiochemical Sciences, Vol. 6, No.1, pp. 115-118, 2005

Accumulation of Cu and Its Oxidation State in *Tremolecia atrata* (Rusty-Rock Lichen) Mycobiont

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Received: November 15, 2004; In Final Form: April 21, 2005

We studied the association of a Cu tolerant mycobiont, *Tremolecia atrata* (rusty-rock lichen) with Cu. *T. atrata* mycobiont cell aggregates, which grew into a sperical shape, rapidly absorbed Cu into their inner and outer parts. The EDS/SEM study showed that Cu was more highly accumulated in the inner part than the outer part of the aggregates. The XANES study revealed that the Cu absorbed by the *T. atrata* mycobiont was both monovalent and divalent. These results suggested that the *T. atrata* mycobiont's high tolerance to Cu is attributable to its ability to store Cu(I) inside the cytoplasm and to adsorb Cu(II) on the cell wall.

1. Introduction

Lichens, symbiotic organisms combining a fungus (mycobiont) and an alga (photobiont), can be found ubiquitously even in environments extremely unfavorable to other living things. They grow in the polar region, burning deserts, salty seashores, extremely high mountains, and heavy metal mines. Their high adaptability is achieved through their intricate cooperative lifestyle. Algae provide photosynthetic products for fungi, which produce metabolic substances to overcome the stress found in those unfavorable environments. Many researchers[1–3] have reported the accumulation of heavy metals, such as Cu, Cd, and U, in lichens growing in mines and nearby smelters. Brown et al.[4] proposed three principle mechanisms for the metal accumulation by lichens: extracellular uptake through an ion exchange process, intracellular accumulation, and the trapping of particles with metals in their body frame. However, the tolerance mechanisms of lichens at the molecular level have not been clear until now. Their tolerant properties could be used for bioremediation of toxic metals and biomonitoring toxic metal pollutions, once the associations at the molecular level, such as the adsorption sites on cell walls and the transport mechanisms to cytoplasms, are clearly understood.

In order to elucidate the tolerance mechanisms of lichens at the molecular level, the roles of mycobionts and photobionts must be examined separately. Yamamoto et al.[5] succeeded in isolating about 250 mycobionts from their symbionts and found that some mycobionts are highly tolerant to Cu. In this study, the distribution of Cu and its chemical state in the mycobiont-cell aggregates of spherical-shaped *Tremolecia atrata* (rusty-rock lichen) having Cu tolerance were examined using scanning electron microscopy equipped with energy-dispersive spectrometry (EDS/SEM) and X-ray absorption near edge structure (XANES). The effect of Cu on the growth of the *T. atrata* mycobiont was also examined by a batch method.

2. Experimental

Sample preparation. A *T. atrata* mycobiont separated by Yamamoto et al.[5] from natural lichens was used. The cells were cultured in a malt-yeast medium (MY)[6] composed of 20 g/L malt extract and 2 g/L yeast extract. Before inoculation, the medium had a pH of 5.8.

Effect of Cu on the growth of *T. atrata* mycobiont-cell aggregates. About 100 mg of fresh *T. atrata* mycobiont-cell aggregates were inoculated into 100 mL Erlenmeyer flasks containing 25 mL MY medium with 1.2 mM Cu(II)SO_4. The pH of the medium was about 4.7. The cell aggregates were incubated on a rotary shaker at 120 rpm in the dark at 20 ˚C. At 1, 7, and 14 days after inoculation, the culture was filtered with a 125 μm nylon mesh filter, and the mycobiont cell-aggregates on the filter were collected and weighed. The amount of Cu accumulated in the *T. atrata* mycobiont was determined by measuring the Cu concentration in the medium solution filtered through a 0.22 μm membrane filter (ADVANTEC MFS, Inc., DISMIC-25) using induced coupled plasma atomic emission spectroscopy (ICP-AES: Shimadzu, ICP-7000).

SEM/EDS. After 24 hours of incubation with Cu as mentioned above, the cell aggregates were collected, washed 1 time with purified water (pH ~ 6.8), and freeze-dried. The resulting spherical-shaped cell aggregates, with diameters of about 1 mm, were sliced into 1 μm sections. Morphological observations and P, S, K, and Cu contents were determined using scanning electron microscopy (SEM) equipped with energy dispersive spectrometry (EDS) (JEOL JMS-6330 F). The EDS spectra were collected between 0 and 20 keV.

XANES. The oxidation state of Cu in the cell aggregates of the *T. atrata* mycobiont and *Saccharomyces cerevisiae* were examined by X-ray absorption near edge structure (XANES) spectroscopy in the fluorescence mode at the BL27B line at the High Energy Accelerator Research Organization, Tsukuba, Japan. The XANES spectra for the Cu Kα1 were collected. About 1 g of fresh *T. atrata* mycobiont-cell aggregates were inoculated into 100 mL Erlenmeyer flasks containing 25 mL of the MY medium with 1.2 mM Cu(II)SO_4. The medium showed a pH of 4.7 before inoculation. The cell aggregates were incubated on a rotary shaker at 120 rpm for 24 hours in the dark at 20 ˚C. The oxidation state of Cu in the yeast *S. cerevisiae* was also analyzed. About 1 g of fresh *S. cerevisiae* cells were incubated in the MY medium with 1.2 mM Cu(II)SO_4 at 120 rpm for 24 hours at 20 ˚C. The medium showed a pH of 4.7 before inoculation. Cells of these two species were collected by centrifugation and the cells were washed with purified water and then with 0.001 M HCl. After being freeze-dried, the cells were sealed threefold in polypropylene bags after exchanging the air in the bags with argon gas. Antioxidants made of zeolite

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Published on Web 07/31/2005

were put in the second bag to prevent the oxidation of Cu(I) in the cells. As standards for mono- and di-valent copper, Cu(I)Cl and Cu(II)SO_4, respectively, mixed with BN (about 10 w/w%) in each sample were used. The oxidation of Cu(I)Cl was also prevented by the antioxidants.

3. Results

Effect of Cu on the growth of *T. atrata* mycobiont cell aggregates. Figure 1 shows the time courses of growth of *T. atrata* mycobiont cell aggregates in the MY media with 1.2 mM Cu and the amounts of Cu accumulated in the cell aggregates. The growth was expressed as the relative growth ratio calculated by dividing the dry weight at 1, 7, and 14 days after contact by the initial dry weight. The cell aggregates grew rapidly in the first 1 day after inoculation. The relative growth ratio at 1 day was approximately 2.3, and then reached about 3.0 and 3.4 at 7 and 14 days, respectively.

The amounts of Cu accumulated in the cell aggregates increased up to 7 days after inoculation; the amounts were about 1.0 and 2.4 mg/g dry weight at 1 and 7 days, respectively. However, the amounts of Cu at 14 days was 2.3, indicating that the Cu accumulation did not increase between 7 and 14 days.

SEM/EDS. Figure 2A shows a photo image of *T. atrata* mycobiont cell aggregates grown in the MY medium with Cu for 1 day and Figure 2B, an SEM image of the rectangular area in Figure 2A. As shown in Figure 2A, the centers of the spherical-shaped cell aggregates were hollow. A morphological difference was observed between the outer and inner parts of the hollow area. The outer part seemed to be dense with cells, while the matrix seemed harsh in the inner part. Figures 3A and B show EDS spectra for the inner and outer areas, respectively, indicated by the squares in Figure 2B. In both areas, peaks corresponding to Kα emissions of P, S, and K were detected at 2.01, 2.31, and 3.31 keV, respectively. The Kα emission of Cu was detected at 8.04 keV in both the inner and outer areas.

Figure 4 shows the sliced cell aggregates grown in the MY medium with 1.2 mM Cu for 14 days. The spherical cell aggregates were broken into small pieces in the process of slicing. Figures 5A and B illustrate the EDS spectra for the

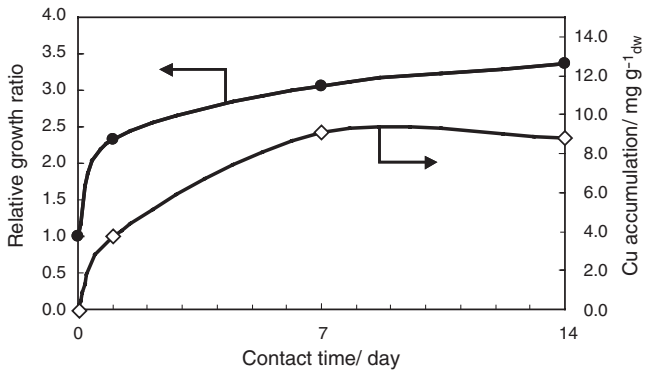

Figure 1. Growth of *T. atrata* mycobiont cell aggregates in the MY media with 1.2 mM Cu (●) and the Cu accumulated in the cell aggregates from the medium (◇). The growth was expressed as a relative growth ratio calculated by dividing the fresh weight at 1, 7, and 14 days after contact by the initial fresh weight. The Cu concentration accumulated in the cell aggregates was calculated by dividing the weight of Cu (mg) by the dry weight of the cell aggregates.

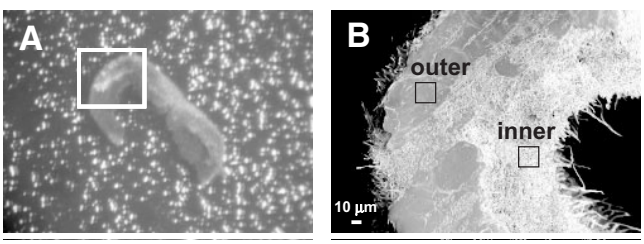

Figure 2. A photo image of *T. atrata* mycobiont cell aggregates grown in MY medium with Cu for 1 day (A) and an SEM image of the area indicated by the rectangle in Figure 2A (B). The bar in Figure 2B corresponds to 10 μm in length.

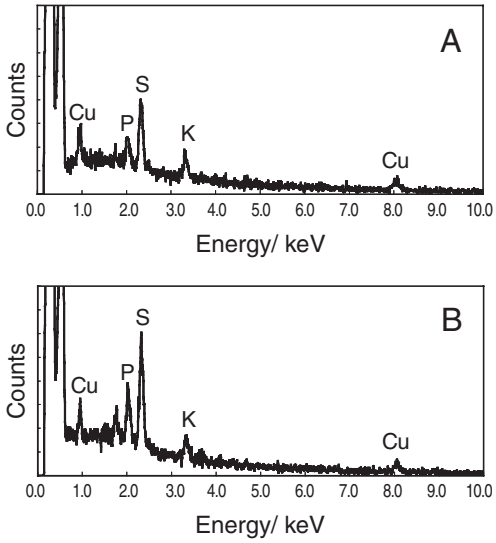

Figure 3. EDS spectra for the inner (A) and outer areas (B), respectively, indicated by the squares in Figure 2B.

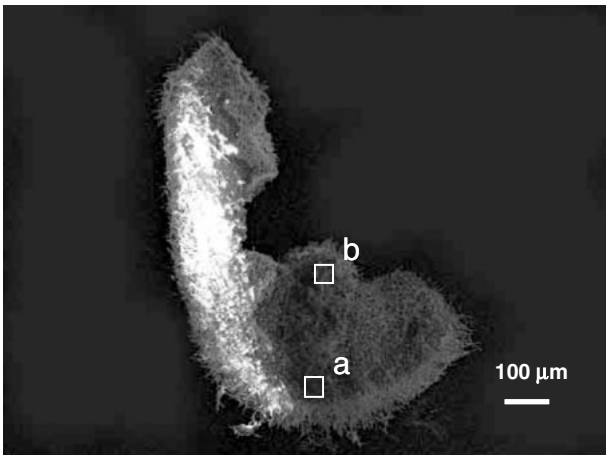

Figure 4. Sliced cell aggregates grown in MY medium with 1.2 mM Cu for 14 days. The bar in the figure corresponds to 100 μm in length.

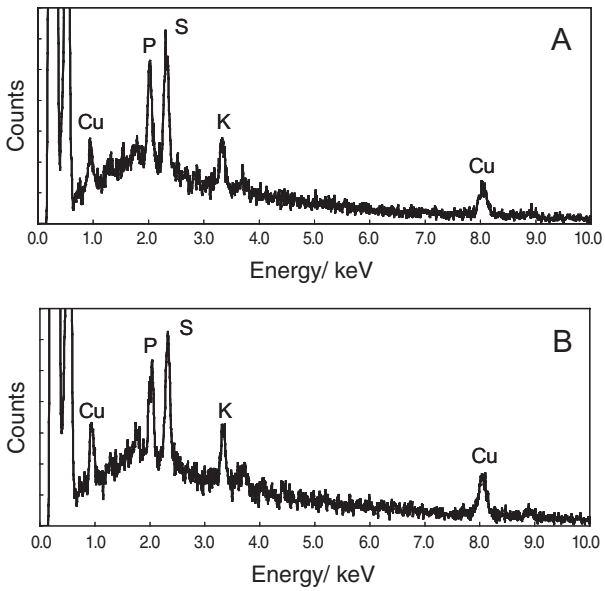

Figure 5. EDS spectra for the areas a (A) and b (B) indicated by the squares in Figure 4.

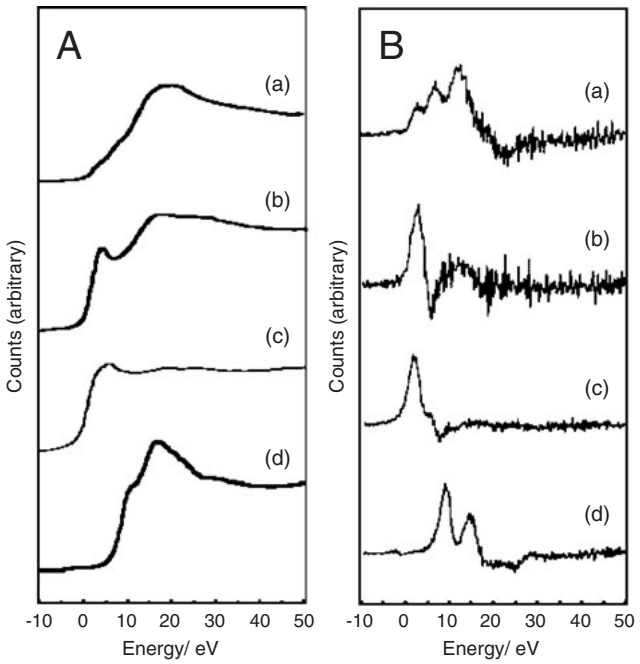

Figure 6. Kα1 edge Cu XANES spectra (A) and their first derivates (B) for Cu in (a) the *T. atrata* mycobiont, (b) *S. cerevisiae*, (c) Cu(I)Cl, and (d) Cu(II)SO_4. The energy calibration was performed to fix the peak maximum of the first derivative of the spectrum for the metal Cu to be 0 eV.

square areas, a and b, in Figure 4. We previously showed that S was distributed in the *T. atrata* mycobiont cell aggregates most homogeneously among these 4 elements.[7] The peak height of S at 2.31 keV in these two spectra was fixed to be the same so that a comparison of the peak strength for P, K, and Cu in these spectra could be easily performed. The peak height of P was almost the same in areas a and b, while that of K in area b was slightly larger than that in area a. The peak height of Cu in area b was larger than that in area a.

XANES. Figures 6A and B show the Kα1 edge Cu XANES spectra and their first derivatives for Cu in (a) *T. atrata* mycobiont, (b) *S. cerevisiae*, (c) Cu(I)Cl, and (d) Cu(II)SO_4. The energy was calibrated by fixing the peak maximum of the first derivative of the spectrum for the metal Cu to be 0 eV (data not shown). As shown in Figure 6A, the spectrum shape of Cu was quite different in each sample. The Cu in the *T. atrata* mycobiont (Figure 6A(a)) and Cu(II)SO_4 (Figure 6A(d)) showed a peak at around 20 eV, while Cu(I)Cl showed a peak at around 5 eV (Figure 6A(c)). Two peaks at around 5 and 15 eV were observed in *S. cerevisiae* (Figure 6A(b)). On the other hand, the first derivative spectra (Figure 6B) showed a couple of outstanding peaks. Three peaks at around 2.5, 7.5, and 12.5 eV were observed in the *T. atrata* mycobiont (Figure 6B(a)), while the spectrum of Cu in *S. cerevisiae* had only one marked peak at around 2.5 eV (Figure 6B(b)). The spectrum of Cu(I)Cl had a characteristic peak of monovalent Cu at around 2.5 eV (Figure 6B(c)). The spectrum of Cu(II)SO_4 showed two large peaks of divalent Cu at around 7.5 and 12.5 eV (Figure 6B(d)).

4. Discussion

Rapid growth was observed in *T. atrata* mycobiont cell aggregates, even though Cu was accumulated in the aggregates, implying that Cu did not seriously discourage growth. The XANES study showed that the peak positions of Cu in the *T. atrata* mycobiont were at the energies for monovalent (2.5 eV for Cu(I)Cl) and divalent (7.5 and 12.5 eV for Cu(II)SO_4) ions, indicating that Cu in the *T. atrata* mycobiont was a mixture of monovalent and divalent Cu. The peak position of Cu in *S. cerevisiae* was at ~ 2.5 eV, indicating that the predominant Cu species in *S. cerevisiae* was monovalent. The requirement of Cu by microorganisms is usually very low. In contrast, the presence of hydrated Cu(II) ions at higher levels is toxic to microbial cells.[8, 9] The toxicity of Cu to microorganisms, such as lichens[10] and yeasts[11] has been widely reported. These microorganisms have detoxification mechanisms for Cu. Copper(II) with a high toxicity is reduced to Cu(I) by glutathione (GSH).[12] Copper induces metallothionein (MT) in *S. cerevisiae* and Cu(I)-MT is formed[13] as well as Cu(I)-GSH.[14] Copper complexed with organic substances shows less toxicity. As the predominant Cu in *S. cerevisiae* was monovalent, Cu(II) incorporated in *S. cerevisiae* cells was rapidly reduced to Cu(I) by biosubstances such as GSH. Monovalent Cu was observed also in the *T. atrata* mycobiont. The photobionts of lichens produce phytochelatin in response to exposure to heavy metals.[15] Mycobionts play a role in producing GSH,[15] which then is converted to MT. MT, therefore, is ubiquitously found in fungi.[16] These facts suggest that the Cu accumulated in the *T. atrata* mycobiont as Cu(I) is also bound to GSH and MT.

In contrast to Cu in *S. cerevisiae*, Cu in the *T. atrata* mycobiont was both monovalent and divalent. Cervantes and Gutierrez-Corona[17] reported that the ability of fungi to accumulate high levels of heavy metals is due to the high capacity of their cell wall components, such as chitin and melanin, to adsorb the metals as well as to the effective storing inside cells, where the metals bind with GSH and MT. Fungal melanin has carboxyl and hydroxyl functional groups that have a high affinity with heavy metals.[18] Purvis et al.[18] showed that the toxic heavy metal U is strongly adsorbed on melanin in the cell walls of lichens.[19] It is known that fungal melanin shows affinities in the order of Cu(II) > Ca(II) > Mg(II) > Zn(II). Moreover, the production of melanin in soil fungus is reported to be accelerated in response to the exposure to Cu(II).[20] The adsorption of Cu on the melanin in cell walls would effectively prevent Cu absorption into the cytoplasm. These facts can explain that the amount of Cu(I) in the *T. atrata* mycobiont was small compared to that in *S. cerevisiae*, because the *T. atrata* mycobiont was in an aggregated form and the cell wall components were well developed. Some fungal species produce a blue-colored mycelium in which Cu is accumulated.[21, 22] However, no blue-colored mycelium was observed in the *T. atrata* mycobiont. These findings suggest that the Cu existing as Cu(II) in the *T. atrata* mycobiont adsorbs to cell wall components such as melanin and chitin, providing the *T. atrata* mycobiont with high tolerance to Cu.

The cell aggregates used had been pre-cultured in a medium without Cu before use. Therefore, the SEM/EDS study signified that Cu reached the inner area of the cell aggregates within 24 hours. The concentration of Cu was higher in the inner part than the outer part. This morphological observation showed that the senescence of cell aggregates proceeded mostly in the center, resulting in production of a hollow there. In the part where the senescence proceeds, cells lyse and only cell wall components remain. These facts indicate that the Cu accumulated in the cell aggregates by adsorption on cell wall components such as melanin and chitin is divalent.

The high tolerance of the *T. atrata* mycobiont to Cu is attributable to two mechanisms. The first is achieved through the reduction of Cu(II) to Cu(I) in the cytoplasm to store Cu(I) as low-toxicity Cu complexes, such as Cu(I)-MT and Cu(I)-GSH. The second is the adsorption of Cu(II) on cell wall components such as melanin. Rizzo et al.[23] reported that the toxic metals accumulated on melanin in fungi protect the fungi from antagonistic microorganisms. This study showed the possibility that one of the strategies of the *T. atrata* symbiont to overcome the difficulty of living in a hostile environment is to protect the symbiont through the accumulation of toxic metals on cell wall components of the *T. atrata* mycobiont, which thereby limits the access of enemies to the symbiont.

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