Biomineralization of Copper by a Fungus Revealed by SEM

T. C. Crusberg, Department of Biology and Biotechnology, Worcester Polytechnic Institute, MA, USA

INTRODUCTION

Many fungi thrive in hostile environments by converting harmful organic toxins to relatively harmless chemical forms. Fungi have a large repertoire of enzymes for this purpose. The response of fungal physiology to challenges by heavy metals is also well documented [1,2]. Heavy metals in the environment have been introduced by a variety of industrial processes, including the mining and electroplating industries, and from the manufacture of a myriad of electronic components. Limits on the discharge of metal-bearing waste waters have been set by governmental agencies around the world. For copper ions, the model used for this study, that maximum contaminant level for industrial discharges is 3.4 mg/l in the US. The fungus *Penicillium ochro-chloron* was found actually growing in an electroplating bath with copper (Cu$^{2+}$) levels in excess of 5000 mg/l [3]. This organism was employed for a study of the potential of such a biotrap for the removal of Cu$^{2+}$ and other toxic metal ions from aqueous solutions and surrogate waste waters. SEM proved vital for the qualitative assessment of the removal of Cu$^{2+}$ from solution by this fungus, through precipitation which entraps insoluble copper phosphate and oxalate salts within the matrix of small fungal mycelia beads.

METHODS

Preparation of fungi

The chemicals used during the course of this work were all of analytical grade whenever available and were obtained from Sigma, Fisher, Difco or Mallinckrodt and the organism was the fungus *Penicillium ochro-chloron* (ATCC 36741). Sterile technique in a laminar flow hood was used for the growth and maintenance of the fungus. Since government-specified maximum contaminant levels (standards) for heavy metals in water are given in mg/l or parts per million (ppm) these units were used here (100 mg/l Cu$^{2+}$ = 1.59 mM).

Corn meal agar (Difco) plates with 400 mg/l Cu$^{2+}$ (as the sulfate) were used to maintain the fungus and to produce spores. After two weeks at 30°C the surface of the plates had acquired a rich lawn of mycelia and spores. Spores were harvested by flooding a plate with 10 ml of sterile dH$_2$O using a Pasteur pipette to direct a stream of water vigorously onto the surface. Spores were decanted and stored for up to two weeks at 4°C. Glucose minimal salts (GMS) (pH 4.0) [4] with Tween 80 (7.5 ml/l) was used to grow *P. ochro-chloron* in liquid culture. Sufficient spore suspension (up to 1 ml) was added to 300 ml of GMS in a baffled Tunair polycarbonate flask (Shelton Scientific IBI, Shelton, CT). In four days, agitating at
300 RPM in an orbital shaking incubator at 30˚C, from 30-50 fungal beads, 2-4 mm diameter, would form. Mycelia beads were washed three times with maintenance medium (GMS minus glucose and Tween 80 and with NaNO₃ at 0.2 g/l), and the (fungal) contents of all flasks combined and used within 24 hours. Twenty to 30 fungal beads were suspended in 100 ml of maintenance medium in a Tunair flask to which Cu²⁺ (as sulfate) was added to 100 mg/l. Over a four day period agitating at 100 rpm the beads took on a deep blue color indicative of the presence of a copper precipitate.

Preparation of copper salts
Insoluble copper (II) phosphate and copper (II) oxalate salts were prepared by adding 5 ml of 10g/l CuSO₄·5H₂O to 5 ml of 1 M K₂HPO₄ or K₂C₂O₄ respectively. In each case the precipitate was collected by centrifugation at 1500 g for 30 minutes in a conical plastic tube using a clinical centrifuge at room temperature, and washed seven times with 10 ml aliquots of dH₂O each wash.

Electron microscopy
To establish how the fungus removed Cu²⁺ ions, scanning electron microscopy of treated beads was performed. Beads challenged with 100 mg/l Cu²⁺ were washed in dH₂O three times to remove any remaining media or metal. Wet beads were individually freeze plunged into liquid hexane at -80˚C, and lyophilized in a Virtis Freezemobile 12 LS freeze dryer. Most of the beads cracked into equal halves during this process (freeze cracking), which allowed examination of the bead interiors or cores. Freeze drying was preferred over critical point drying from CO₂ since results were indistinguishable. Freeze dried beads were mounted on Al stubs with graphite paint and coated with gold/palladium (60:40) in a Fullam sputter coater (Ernest F. Fullam, Inc., Latham, New York), and viewed in secondary electron imaging (SEI) mode using a JEOL JSM-5200 scanning electron microscope at an accelerating voltage of 25 kV. For backscattered electron imaging (BEI) mode using a JEOL JSM-5200 scanning electron microscope at an accelerating voltage of 25 kV. For backscattered electron imaging (BEI) and for energy dispersive X-ray microanalysis (EDX) lyophilized specimens were mounted on graphite stubs, coated with carbon in a Varian vacuum evaporator model PS10E, mounted on graphite stubs and examined using the same instrument with a ThermoNoran LN X-ray detection system (Middleton, WI) with Voyager Software but operated at 15 kV accelerating voltage. For other EDX and X-ray mapping purposes a JEOL 820 scanning electron microscope equipped with a Kevex X-ray analysis system was employed and the images stored in digital form. Copper phosphate and oxalate salts were spread onto graphite stubs, air dried and coated with carbon as described above. Photography was performed using Polaroid Type 53 film.

RESULTS
Composition of biomineralized deposits
The mycelia of fungal beads consists of cells linked together with considerable branching, forming a vast network of fibers (Figs 1 and 2). In the presence of Tween 80 in liquid culture, these fibers condense to form spheres (Fig 2a) which grow in size at the rate of about 1 mm in diameter per day in culture. Freeze cracking allowed the hollow interior of beads to be examined (Fig 1a). Surprisingly, small microspheres 20-40 µm in diameter were seen throughout the core (Fig 2b-d) with a dense array of those objects appearing at around half radius (Fig 2b). Two forms of beads were actually observed, one rather porous and numerous, and another exhibiting a smooth surface but less numerous. Using backscattered electron imaging or BEI mode, and adjusting contrast and brightness of the image appropriately, the structures rich in one or more high Z (atomic number) atoms stood out magnificently (Fig 2b). X-ray mapping of one section (Fig 2d, IM) showed that the brightest regions were especially rich in Cu and P with a strong EDX signal for Mg, Ca, Cl and K appearing as well (Fig 2c,d). Beads with smooth sur-

Figure 2: Backscattered electron images (15 kV) of a fungal bead showing the surface of tangled mycelia (a) and the core of a freeze-cracked bead with its accumulation of microspheres at about half radius (b). X-ray digitized maps (c,d) showing the backscattered image (IM) and 6 elements as indicated. In these images only porous beads were observed.

Figure 3: Backscattered electron image (20 kV) of a smooth microsphere entrapped within the core of a fungal bead.
faces were rich in Cu but no other major element, suggesting that the counter anion is organic since the detector was rather insensitive for C and O (Fig 3). Since some *Penicillium* species are known to produce oxalate [5,6] it might be that copper (cupric) oxalate was also formed during the four days of incubation. In fact EDX spectra of copper phosphate and oxalate salts prepared in this laboratory presented very similar results with those of porous and smooth surface microspheres respectively.

**DISCUSSION**

Scanning electron microscopy coupled with EDX analysis showed that under suitable conditions in culture copper precipitates extracellularly and is held within the mycelial matrix of *Penicillium ochro-chloron* fungal beads. Below pH 5 copper hydroxide does not precipitate when copper is at 100 mg/l [7]. The porous spherical complexes are most likely copper phosphate since X-ray mapping (Fig 2) shows that Cu, P and O are the predominant elements. Depending on how long the beads have been challenged in copper containing media, the number of these microspheres decreased over time. The most abundant number of spheres was found in samples taken after approximately 20 to 25 hours of copper challenge. The reason for the reduction in the number of porous intermycelial spheres is not known but some condition in the medium after 24 hours is sufficiently different that the solubility product for copper(II) phosphate must favor dissolution. Since pH of the bulk phase remains between 4.0 +/-0.2 during the uptake experiments it is not believed that more precise control of this variable is needed to prevent dissolution of the copper salts. Several fungi are known to produce oxalic acid as a secondary metabolite and since copper oxalate is quite insoluble ($K_{sp} = 4.43 \times 10^{-10}$) [8] it is also not surprising to find this salt deposited within fungal mycelia as smooth microspheres. Copper phosphate is not soluble in the bulk solution at pH 4 either ($K_{sp} = 1.40 \times 10^{-37}$) [8] at the concentrations used in our media suggesting that the conditions for its precipitation within the mycelial core differ from those of the bulk phase. It is indeed possible that an increase in phosphate anion concentration exists for a period of time in the core of the mycelial bead. Such a condition could be brought about by loss of cell viability due to anoxia and release of organophosphates from the cytoplasm into the core by dying or physiologically compromised cells. Then, complexes of copper phosphate in the periplasm of the cells [6, 9] could hydrolyze the organophosphates presenting the inward diffusing copper ions with an increase in phosphate anion and a condition that favors precipitation. A similar argument can be made for copper oxalate precipitation [6]. EDX analysis was also performed on carbon coated preparations of cupric phosphate and cupric oxalate prepared in our laboratory. The similarities of these EDX spectra with those obtained from the spherical precipitates also confirms that the smooth microspheres are probably copper oxalate and the porous precipitate is copper phosphate. Indeed, smooth copper phosphate spheres of sub-micrometer dimensions have been reported to form during non-biological mediated precipitation [10] and lead phosphate precipitates have been observed when fungi were challenged with that metal [11]. Copper oxalate is most likely produced by viable cells as a detoxification process carried out in *P. ochro-chloron* through the action of the enzyme oxaloacetase. Oxalate is released from the cells and forms a precipitate with copper when the $K_{sp}$ is exceeded.

**CONCLUSION**

The use of electron microscopy proved invaluable in understanding what conditions need to be attained for the bioremediation of copper and hopefully other heavy metals in industrial waste waters which to date has proved elusive [12].

**REFERENCES**