Formation of *Metallogenium*-like structures by a manganese-oxidizing fungus

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**Abstract.** Structures resembling *Metallogenium* spp. were observed in agar and in liquid cultures of a Mn-oxidizing basidiomycetous fungus only when Mn$^{2+}$ was oxidized. Fungal viability was necessary for formation of the structures; Mn$^{2+}$ concentration and the presence or absence of agar in the medium were important factors determining their morphology. Slide cultures revealed no identifiable cells in any stage of development. Fluorescent dyes that stained nucleic acids and polysaccharides in the fungal hyphae did not stain the *Metallogenium*-like structures. Likewise, Rhodamine 123, a fluorescent probe for membrane potential, stained fungal mitochondria, but did not stain the structures. Thin sections through the structures showed no biological membranes or other cellular features. Only the characteristic ultrastructure of biological Mn oxides were observed in serial thin sections. In agar, unfixed structures disappeared permanently during reduction of Mn oxides with hydroxylamine. Glutaraldehyde fixation stabilized these structures. Fixed structures lost most of their original phase density during reduction with hydroxylamine, but continuous microscopic observations showed that their phase density could be restored by staining with Coomassie blue. Structures that formed in liquid medium did not require stabilization with glutaraldehyde during reduction of Mn oxides. They, too, lost their original phase density during reduction with hydroxylamine; phase density could be restored by staining with cationic colloidal iron or Coomassie blue. The results suggest that the *Metallogenium*-like structures were formed as a result of Mn oxidation associated with exopolymers produced by the fungus.

**Key words:** *Metallogenium* — Manganese-oxidizing fungus — Mn oxidation — Extracellular polymers

The genus *Metallogenium* was first described by Perfil'ev in the 1930's as a new group of bacteria that he believed played an important role in the formation of ferromanganese ores and in cementing of bottom deposits in lake sediments (Perfil'ev and Gabe 1961). Using ingenious microcapillary methods, Perfil'ev and Gabe (1961) observed characteristic *Metallogenium* structures in aerobic-anaerobic transition zones of lake sediments where seasonal Fe and Mn oxidation occurred. The description of the type species, *Metallogenium personatum*, was based on light microscopic observations of peloscope and flow capillary cultures from these zones (Perfil'ev and Gabe 1961). Although axenic cultures of *M. personatum* were never obtained, the original observations did include a description of its developmental cycle (Perfil'ev and Gabe 1961). Somewhat later, Zavarzin (1961, 1964) described a second species, *M. symbioticum*, which he believed lived in symbiotic association with a fungus and was strictly dependent on the fungal host for its growth. Morphological studies of *M. symbioticum* in fungal cultures led to further speculation that *Metallogenium* spp. may actually be parasitic mycoplasmas that infect fungi (Dubinina 1970; Zavarzin and Hirsch 1974; Zavarzin 1981). Dubinina (1984) expanded on this hypothesis by reporting that it was possible to infect a variety of bacteria and fungi with a putative *Metallogenium* sp. derived from a binary fungal culture. The parasitism was not host-specific, and it apparently resulted in the parasitized organism gaining the ability to oxidize Mn. However, the putative parasite was not isolated or studied in pure culture; only its *Metallogenium*-like morphology was described in detail (Dubinina 1984).

Despite the published description of *Metallogenium* spp., the validity of the genus *Metallogenium* is not universally accepted (for reviews, see Ghiorse 1984a; Marshall 1979). In fact, a number of microbiologists (e.g., Schweisfurth and Hehn 1972; Schmidt and Overbeck 1984) have reported that *Metallogenium*-like structures contained no apparent cellular structures suggesting that their origin is not directly attributable to cellular growth. Nevertheless, the genus has been accepted by geochemists and limnologists. Indeed, *Metallogenium*-like structures similar to those originally described by Perfil'ev and Gabe (1961) have been observed in a variety of aquatic environments, including water treatment filters (Czekalla et al. 1985); rock varnish (Krumbein and Jens 1981; Dorn and Oberlander 1982; Palmer et al. 1986); as well as in the water column and sediments of freshwater lakes (Klaveness 1977; Gregory et al. 1980; Jaquet et al. 1982; Tipping et al. 1985; Maki et al. 1987). Characteristically, their biological properties are poorly defined, but their widespread occurrence and importance in Mn and Fe geochemistry is widely recognized. *Metallogenium*-like forms
also have been recognized by paleontologists for their close resemblance to Precambrian microfossils such as *Eoastrion* (Barthoorn and Knoll 1975). *Metallogenium* spp. are thought to be modern analogs of these fossil structures (Marshall 1979; Zavarzin 1981).

Thus, the *Metallogenium* phenomenon is still an enigma. At present, many believe that the *Metallogenium*-like structures observed in fungal cultures and in natural environments are not themselves living organisms, rather they probably result from the growth or activity of organisms in the environment where they are found (Ghiorse 1984a; Gregory et al. 1980; Klaveness 1977; Maki et al. 1987; Marshall 1979; Schmidt and Overbeck 1984; Schweisfurth and Henn 1972). However, the biological and chemical processes by which they are formed remain unknown. The present work demonstrates unequivocally that *Metallogenium*-like structures that occur in cultures of a Mn-oxidizing fungus do not contain viable cells at any stage of their development. Furthermore, microscopic observations suggest that the structures most likely originate from the activity of extracellular Mn-oxidizing factors associated with exopolymers produced by the fungus.

### Materials and methods

**Fungal culture.** The fungus was isolated as an airborne contaminant on MnSO₄-containing diluted pond water agar medium employed to isolate and enumerate *Leptothrix* spp. (Ghiorse and Chapnick 1983; Ghiorse 1984b). Light microscopic examination showed that in agar cultures *Metallogenium*-like structures occurred near hyphae in brown Mn-oxide zones that formed behind the growing edge of the radially growing fungal mycelium. The fungus has been identified as an arthroconidial anamorph of a basidiomycete similar to those in Group 1 described by Sigler and Carmichael (1976). Although its development and general morphology are similar to *Geotrichum*, it does not fit the modern description of that genus. No genus name has yet been assigned to Group 1 (L. Sigler, personal communication). The culture has been deposited in the Microfungus Collection and Herbarium, Devomian Botanic Garden, University of Alberta, Edmonton, Alberta, Canada T6G2E1 Accession number UAMH 5001.

**Media.** Fungal cultures were maintained initially on the diluted pond water agar medium used for isolation. Two dilute synthetic media also were employed: a complex medium (PYG) consisted of (per liter) 0.25 g each of yeast extract, peptone, and glucose dissolved in 10 mM HEPES buffer, pH 7.0; and a defined medium (MSVP) consisted of (per liter) 0.24 g (NH₄)₂SO₄, 0.06 g CaCl₂, 7 H₂O, 0.02 g KH₂PO₄, 0.03 g Na₂HPO₄, 0.06 g MgSO₄·7 H₂O in the same HEPES buffer. Filter sterilized pyruvate solution (1.0 g/l) and vitamin solution (Staley 1968) (0.025 ml/l) were added to the MSVP medium after autoclaving. Difco agar (15 g/l) was added when solid media were required. Sterile MnSO₄ solution was added to all media to the required final concentration after autoclaving.

**Agar and liquid cultures.** For studies of the effects of Mn²⁺ on fungal growth, Mn oxidation, and morphology of *Metallogenium*-like structures, small blocks of agar containing actively growing mycelium were inoculated centrally on agar media supplemented with 0.1, 1.0, or 10 mM MnSO₄. The plates were incubated at 21–24°C. The growth rate of the fungus was estimated by measuring the rate of increase in the radius of the fungal mycelium. For cytological studies, agar cultures were prepared as described above except that the medium was supplemented with 0.1 mM MnSO₄ exclusively. Liquid cultures consisted of 100 ml of PYG or MSVP liquid medium supplemented with 0.1 mM MnSO₄ in 250 ml Erlenmeyer flasks. The flasks were inoculated with a small piece of mycelium and incubated at 25°C. When increased aeration was required, flasks were shaken on a New Brunswick rotary shaker set at 150 rpm.

**Inhibition experiment.** UV irradiation was used to inhibit growth of the fungus and development of *Metallogenium*-like structures in agar cultures. The fungus was inoculated in triplicate on MSVP agar without added manganese; plates were incubated for 6 days at room temperature. The plates were then irradiated with a shortwave UV germicidal lamp (A. M. Thomas Co., Philadelphia, PA, USA) at a distance of 15 cm for 0, 40, and 80 s. Immediately after irradiation, two drops of 0.1 mM MnSO₄ were added on opposite sides of the colony approximately 5 mm from the leading edge of the hyphal growth. Previous tests showed that the MnSO₄ diffused throughout the agar in a few hours. The plates were subsequently monitored for fungal growth and production of visible Mn oxide deposits.

**Aerobic slide cultures.** A modification of the method of Noller and Durham (1968) was employed. First a glass slide was coated with molten 1.5% Noble agar containing 0.1 mM MnSO₄. Excess fluid was drained away immediately and the agar solidified to form a thin film, which was pared with a razor blade to leave a 10 mm square. Finally, a well was made with forceps in the center of the agar square. The culture was then inoculated by placing in the well, a small block of agar taken from the edge of a fungal colony which was growing in the absence of added Mn. A drop of 10 mM HEPES buffer, pH 7.0, was added to the culture, and then an optically clear teflon membrane (Yellow Springs Instruments, Yellow Springs, OH, USA) was placed over the entire agar square. The edges were sealed with Vaspal. Slide cultures were incubated on the microscope stage at 21–24°C and observed periodically under the 100 x oil immersion phase contrast objective lens on the Zeiss standard 18 microscope (see below).

**Differential stains.** Mn oxides were identified by their reaction with acidified leukoberbelin blue according to the method of Ghiorse and Hirsch (1979). Unfixed samples containing *Metallogenium*-like structures were stained with acridine orange (0.01%) and DAPI (5 μg/ml) (Coleman 1980) by placing small 2 mm square agar blocks in a drop of the stain on a microscope slide, and gently compressing the block under a coverglass. The preparations were observed immediately by epifluorescence microscopy with the 100 x oil immersion bright field objective lens of the Zeiss microscope. Rhodamine 123 (5 μg/ml) (Matsuyama 1984) was applied to unfixed specimens by incubating agar blocks in a Rhodamine solution for 45 min at room temperature. After rinsing several times in distilled water, the agar blocks were mounted in distilled water and viewed by epifluorescence microscopy as described above.