Arch Microbiol (1989) 151:223–231

Archives of
Microbiology

Formation of *Metallogenium*-like structures by a manganese-oxiding 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

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Non-standard abbreviations: HEPES, (N-hydroxyethylpiperazine-N′-2-ethane sulfonic acid); DAPI, (4′,6-diamidino-2-phenylindole); PIPES, (piperazine-N,N′-bis[2-ethane sulfonic acid])

also have been recognized by paleontologists for their close resemblance to precambrian microfossils such as *Eoastrion* (Barghoorn 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 Hehn 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 $MnSO_4$-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, Devonian 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 $(NH_4)_2SO_4$, 0.06 g $CaCl_2 \cdot 7\,H_2O$, 0.02 g KH_2PO_4, 0.03 g Na_2HPO_4, 0.06 g $MgSO_4 \cdot 7\,H_2O$ 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 $MnSO_4$ solution was added to all media to the required final concentration after autoclaving.

Agar and liquid cultures. For studies of the effects of Mn^{2+} 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 $MnSO_4$. 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 $MnSO_4$ exclusively. Liquid cultures consisted of 100 ml of PYG or MSVP liquid medium supplemented with 0.1 mM $MnSO_4$ 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 $MnSO_4$ were added on opposite sides of the colony approximately 5 mm from the leading edge of the hyphal growth. Previous tests showed that the $MnSO_4$ 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 $MnSO_4$. 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 Vaspar. Slide cultures were incubated on the microscope stage at 21 – 24° C and observed periodically under the 100× 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× 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.

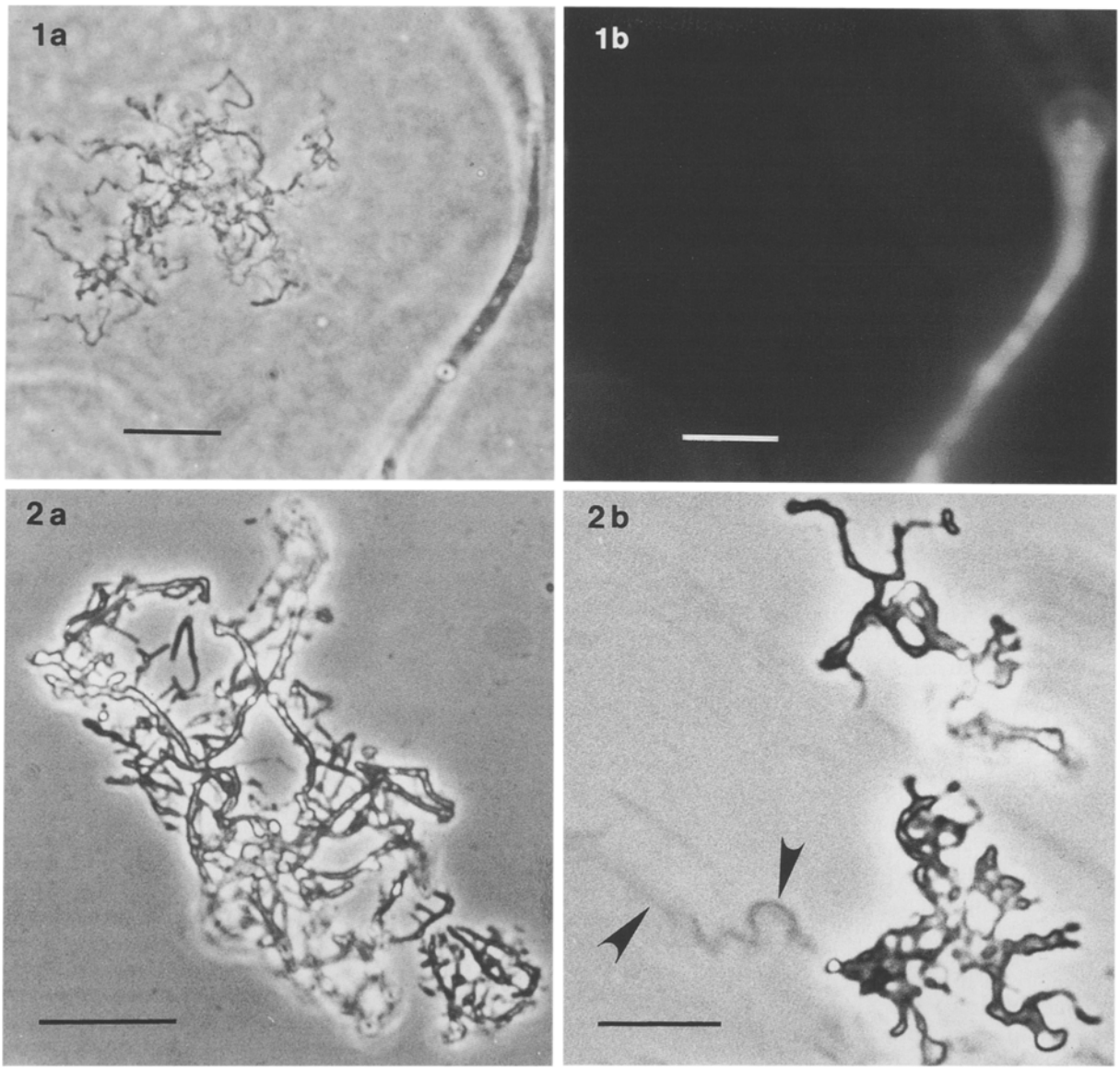

Fig. 1. a Phase-contrast photomicrograph of a *Metallogenium*-like structure (*left*) and fungal hypha (*right*) observed in MSVP agar medium amended with 0.1 mM Mn^{2+}. The sample was stained with 0.01% acridine orange. **b** The same field viewed under epifluorescence. Note absence of fluorescence in the area of the *Metallogenium*-like structure and bright fluorescence of fungal hypha. The sample was taken from a fungus cultures that had been grown for 7 days at room temperature. Bar = 10 μm

Fig. 2a, b. Phase-contrast photomicrographs of *Metallogenium*-like structures observed in stationary phase PYG liquid cultures amended with 0.1 mM Mn^{2+}. **a** Highly refractile, lattice-like free floating structures such as this were common in static cultures. Bar = 10 μm. **b** Similar structures were observed in a surface film that formed at the air-liquid interface. The *arrows* indicate folds in the film. Bar = 5 μm

Other stains were applied to agar blocks after they were fixed in HEPES-buffered 3.0% glutaraldehyde and washed in the buffer. Nile blue A, a phospholipid stain which contains a lipophilic fluorescent component, Nile red, was applied following the procedure of Ostle and Holt (1982). Cellufluor (Polysciences, Warrington, PA, USA) and Sirofluor (gift of Robert P. McGovern, Cornell University), both fluorescent brightening agents that stain structural polysaccharides were applied by treating blocks with a 100 μg/ml aqueous solution of the brightening agent for 10–15 min. A cationic iron-cacodylate stain (Seno et al. 1983) was employed to stain acidic exopolymers. Because of the intense background staining of the agar, this stain could only be used on specimens derived from liquid cultures. These specimens were first attached to microscope slides coated with 0.05% poly L-lysine. Specimens mounted this way were then rinsed with buffer before staining with the iron-cacodylate reagents. Liquid culture specimens were also stained with the other staining reagents described above.

Visualization of underlying structures after removal of Mn oxides. Preliminary trials showed that unfixed structures in agar disappeared permanently when Mn oxides were removed by reduction with 0.1% hydroxylamine hydrochloride. After fixation with glutaraldehyde, the structures also disappeared upon reduction of the oxides, but underlying

structures could be visualized again after staining with Coomassie blue. The following procedure was developed to continuously monitor disappearance and subsequent staining of a structure in the phase contrast microscope: Small blocks of agar containing the structures were fixed in the HEPES-buffered glutaraldehyde solution for 5 to 10 min. After rinsing once in HEPES buffer, the blocks were compressed in a reaction chamber formed under an 11 × 22 mm coverslip which was supported on two sides by coverslips and sealed with Vaspar. Next a drop of 0.1% hydroxylamine was applied to the chamber and a given structure was observed until it disappeared or lost most of its phase density. Then a drop of 0.1% Coomassie blue R-250 was added and the same structure was observed to determine if its phase density was restored.

Light microscopy. A Zeiss standard 18 microscope equipped with phase contrast, bright field, and epifluorescence optics was employed. Photomicrographs were recorded with a Zeiss MC63 35 mm camera system on Kodak Ektachrome T160 color slide film or Kodak Plus-X Pan black and white film. For fluorescence microscopy, the following excitation-barrier filter combinations were employed: DAPI, Rhodamine 123, Sirofluor and Nile red; excitation, 404.7 and 435 nm; barrier, 460 nm. Cellufluor and acridine orange excitation, 440 – 490 nm, barrier, 520 nm.

Electron microscopy. MSVP agar blocks containing *Metallogenium*-like structures were fixed for 2 h at room temperature in 2.0% glutaraldehyde in 20 mM PIPES buffer, pH 7.0, containing 10 mM $CaCl_2$. Post fixation was in 1.0% OsO_4, followed by treatment with uranyl acetate, ethanol dehydration and epoxy embedment following the protocol of Adams and Ghiorse (1986). Thin sections were obtained with an LKB ultramicrotome equipped with a diamond knife. The sections were floated on distilled water and picked up on carbon-formvar-coated, copper grids. Slotted grids were employed to retrieve serial sections. When additional specimen contrast was desired, sections were double stained with saturated uranyl acetate and 0.3% lead citrate solutions. Sections were viewed in a Phillips 301 transmission electron microscope operated at an accelerating voltage of 80 kV and instrument magnifications between 5,000 and 50,000 ×. Electron micrographs were recorded on Kodak electron image film.

Results

Formation of Metallogenium-like structures in fungal cultures

Metallogenium-like structures (Fig. 1a) were observed only when the fungus oxidized Mn. In agar media the structures occurred in close proximity to fungal hyphae, sometimes in contact with hyphae, but usually several micrometers away (e.g., Fig. 1a). Typically the *Metallogenium*-like structures possessed fine, pleomorphic filaments that closely resembled the arais of *Metallogenium* spp. (Zavarzin and Hirsch 1974). The filaments ranged in diameter from 0.02 µm to 0.5 µm and they radiated outward to form coenobia-like structures that usually measured 15 – 30 µm in diameter (Fig. 1a), but occasionally reached 50 µm or more in diameter. In liquid cultures, the fungus oxidized Mn under both aerated and stationary conditions; however, the formation of *Metallogenium*-like structures (Fig. 2a, b) was favored by stationary conditions. In liquid cultures they appeared to be more robust, their filaments being more refractile under phase contrast optics than in agar cultures (compare Figs. 1a and 2a). In stationary cultures, the structures were observed floating free in the medium. As the cultures aged, they were often observed in a phase dense film (arrows Fig. 2b) that formed at the air-liquid interface. Budding or motile forms were never observed in agar or liquid cultures.

Table 1. Effect of UV irradiation on fungal growth and formation of *Metallogenium*-like structures. The fungus was inoculated on three Petri plates containing MSVP agar without added Mn^{2+} and allowed to grow for 6 days. At 0 h, two of the plates were exposed to UV light and then $MnSO_4$ was added to all 3 plates. At the first sign of Mn oxidation, agar was examined for presence of *Metallogenium*-like structures

UV dose (s)	Mycelium radius (mm)			Appearance of *Metallogenium*-like structures (hours after addition of $MnSO_4$)
	0 h	36 h	72 h	
0	13.5	17.0	20.5	8
40	14.0	14.0	17.5	34
80	14.5	14.5	15.0	72

Dependence on fungal viability

The dependence of Mn oxidation and formation of *Metallogenium*-like structures on the viability of the fungus was demonstrated by inhibiting fungal growth and formation of the structures with non-lethal doses of UV irradiation. This form of inhibition was preferable to chemical or heat treatments because it offered less chance for simultaneous inhibition of biological Mn oxidation. As shown in Table 1, the radius of unirradiated mycelium continued to increase at the same linear rate (~ 2.0 mm/day) during the entire experiment. *Metallogenium*-like structures were first observed at the same time as Mn oxidation started, approximately 8 h after addition of $MnSO_4$. In cultures irradiated for 40 s, Mn oxidation and *Metallogenium*-like structures were observed 34 h after addition of $MnSO_4$, approximately 16 h before mycelial growth resumed. Cultures irradiated for 80 s did not show Mn oxidation or *Metallogenium*-like structures until 72 h after addition of $MnSO_4$, at approximately the same time as mycelial growth resumed. These data indicate that the formation of *Metallogenium*-like particles was dependent on the growth of the fungus. The longer fungal growth was inhibited, the longer it took for *Metallogenium*-like structures to form.

Effect of medium composition

The growth rate of the fungus did not change significantly in the three media employed (Table 2). Also, elevation of the Mn^{2+} concentration from 0.1 to 10 mM did not affect growth rate except in pond water agar where 10 mM Mn^{2+} inhibited growth slightly (Table 2). As expected the intensity of Mn oxidation in each medium generally increased with increasing Mn^{2+} concentration. Also, the onset of Mn oxidation varied between media. However, a more important effect of the medium composition was on the formation of *Metallogenium*-like structures, which formed slightly behind the growing edge of the mycelium in all three of the media

Table 2. Effects of medium composition and manganese concentration on fungal growth rate and formation of *Metallogenium*-like structures. Experiments were done in duplicate. Growth rates were averaged over 5 days; the standard deviation is given in parenthesis. Observations of *Metallogenium*-like structures were made by phase contrast microscopy. The intensity of Mn oxidation was judged by the intensity of the brown color of the oxides

$MnSO_4$ added (mM)	Fungal growth rate (mm/day)	Intensity of manganese oxidation[a]	*Metallogenium*-like structures observed?
Pond water agar			
0.1	1.8 (0.4)	+	yes
1.0	2.2 (0.6)	+++	yes
10	1.4 (0.3)	++++	no
PYG agar			
0.1	2.4 (0.5)	+	yes
1.0	2.4 (0.6)	+++	yes
10	2.2 (0.6)	+++++	no
MSVP agar			
0.1	2.0 (0.3)	+	yes
1.0	2.0 (0.5)	−	no
10	2.1 (0.2)	++	no

[a] + = very light brown or yellow; +++++ = dark brown or black

we tested. In pond water and PYG agar, *Metallogenium*-like structures were observed in cultures containing 0.1 and 1.0 mM $MnSO_4$, but not in those containing 10 mM. In MSVP agar, the structures were formed only at 0.1 mM $MnSO_4$. As the concentration of Mn^{2+} was increased to 1 mM, the structures became progressively less filamentous. Mn oxidation occurred in all media at 10 mM Mn, but no *Metallogenium*-like structures were seen at this concentration (Table 2).

Development in slide cultures

Observation of aerobic slide cultures of the fungus in MSVP agar-containing 0.1 mM Mn^{2+} established a reproducible development pattern for formation of the structures in agar. Initially, small, phase-dense particles appeared (arrows, Fig. 3; 0 h, 8 h), followed after several hours by the appearance of tortuous filaments that radiated outward from the coccoid particles. Ultimately, large, filamentous structures were formed in the agar matrix (Fig. 3; 20 h). This developmental pattern is very similar to that originally reported for *M. personatum* growing in isolated capillary cultures (see Figure 74 in Perfil'ev and Gabe 1961). In our slide cultures, the initial coccoid particles appeared suddenly in many locations near growing hyphae between 30 and 90 min after initiation of the culture, motile cells were never seen. The particles were not observed unless $MnSO_4$ was added to the medium, and they contained significant amounts of Mn oxide as indicated by an intense reaction with leukoberbelin blue, suggesting that they were nucleation centers for the precipitation of Mn oxides. Not all coccoid particles developed into *Metallogenium*-like structures, and in some cases only individual filaments were seen. The structures continued to grow for up to 20 h (Fig. 3).

Differential staining

The *Metallogenium*-like structures reacted intensely with acidified leukoberbelin blue indicating that they contained

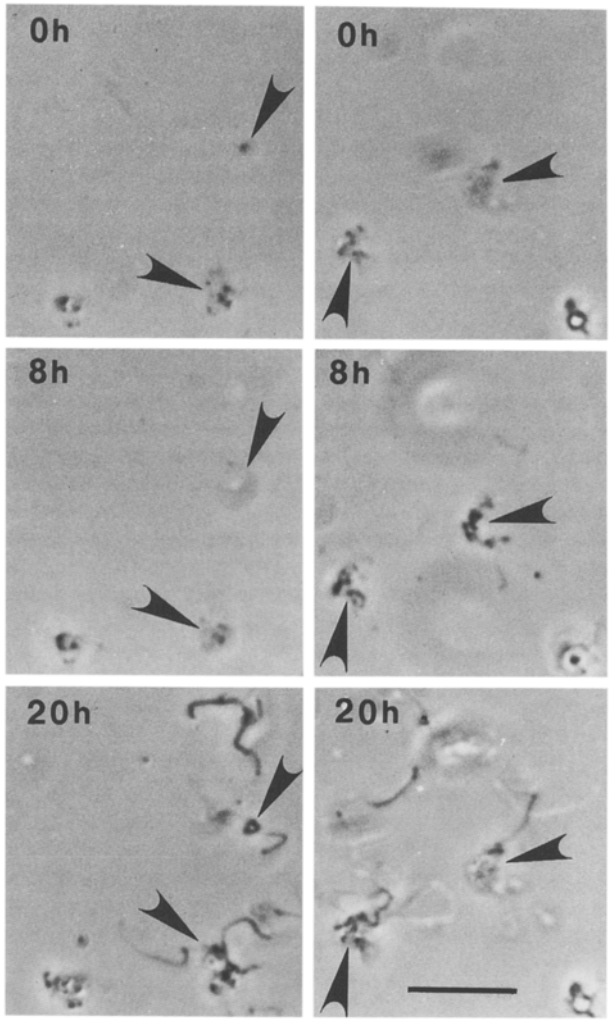

Fig. 3. Two series of phase contrast photomicrographs showing development of *Metallogenium*-like structure in Mn^{2+}-amended MSVP agar slide cultures incubated at 20−22° C. The initial photomicrographs (0 h) were made after approximately 1 h of incubation. Subsequent photomicrographs were made 8 and 20 h later. *Arrows* indicate location of coccoid refractile bodies that formed centers of Mn oxide precipitation. Bar = 10 μm

an abundance of Mn oxide. However, as illustrated in Fig. 1 b, staining with acridine orange resulted in no fluorescence in the *Metallogenium*-like structures while nearby fungal hyphae fluoresced brightly. Similarly, structures stained with DAPI and Rhodamine 123 showed no fluorescence while nuclei and mitochondria in the fungal hyphae fluoresced brightly (not illustrated).

When glutaraldehyde-fixed *Metallogenium*-like structures were treated with the mild reducing agent, 0.1% hydroxylamine to remove the Mn oxides, the structures lost their phase density, in some cases they disappeared almost completely (Fig. 4b). In other cases faint remnants of filaments remained after treatment with hydroxylamine (Fig. 4b′). In most cases, subsequent staining of the fixed structures with Coomassie blue partly restored their phase density (Fig. 4c, c′) suggesting that the structures possessed an underlying matrix that maintained the morphology of the original structures.

It should be emphasized that prior fixation with glutaraldehyde was essential to preserve the delicate underlying

matrix in agar cultures. Structures that were not fixed with glutaraldehyde disappeared completely after reduction with hydroxylamine and their phase density could not be restored by subsequent staining with Commassie blue. Similar structures that formed in liquid cultures (Fig. 2) did not require stabilization with glutaraldehyde. These structures also lost phase density when treated with hydroxylamine, but their density could be restored by subsequent staining with either cationic colloidal iron stain (Fig. 5) or Coomassie blue (not illustrated). Hydroxylamine-treated structures from liquid cultures fluoresced very faintly after staining with Cellufluor suggesting that they may contain a structural polysaccharide. However, the Cellufluor fluorescence was much less intense than that associated with the cell wall of the fungus. Hydroxylamine-treated structures did not fluoresce after staining with Sirofluor or Nile blue A.

Ultrastructure

Fungal hypha encrusted with amorphous Mn-oxide (Fig. 6) were observed frequently in thin sections. Such observations provided evidence that the fixation protocol we employed was adequate to preserve biological structures such as plasma membranes, mitochondria, ribosomes, and the cell wall. These observations also allowed for comparison to the ultrastructure of Mn oxides attached to hyphae with that of the *Metallogenium*-like structures (Fig. 7b). In neither case was there any evidence of biological membranes, ribosomes, cell envelopes, or other ultrastructural features characteristic of cells in the Mn oxide-containing structures. In general, the characteristic leafy and dendritic ultrastructure shown in Figs. 6 and 7b was similar to that of microbial Mn oxides observed in cultures of many different Mn-depositing bacteria (Adams and Ghiorse 1986; Ghiorse 1984a; Ghiorse and Hirsch 1979, 1982) and some abiological Mn oxides produced under laboratory conditions (Stone 1987). Examination of serial thin sections of several different *Metallogenium*-like structures revealed that the structures were composed only of a network of filaments of the amorphous Mn oxides (Fig. 7a). Although it was never possible to reconstruct an entire *Metallogenium*-like structure from serial sections, enough serial sections were examined at random depths in different structures to allow us to conclude that no cellular structures existed in them.

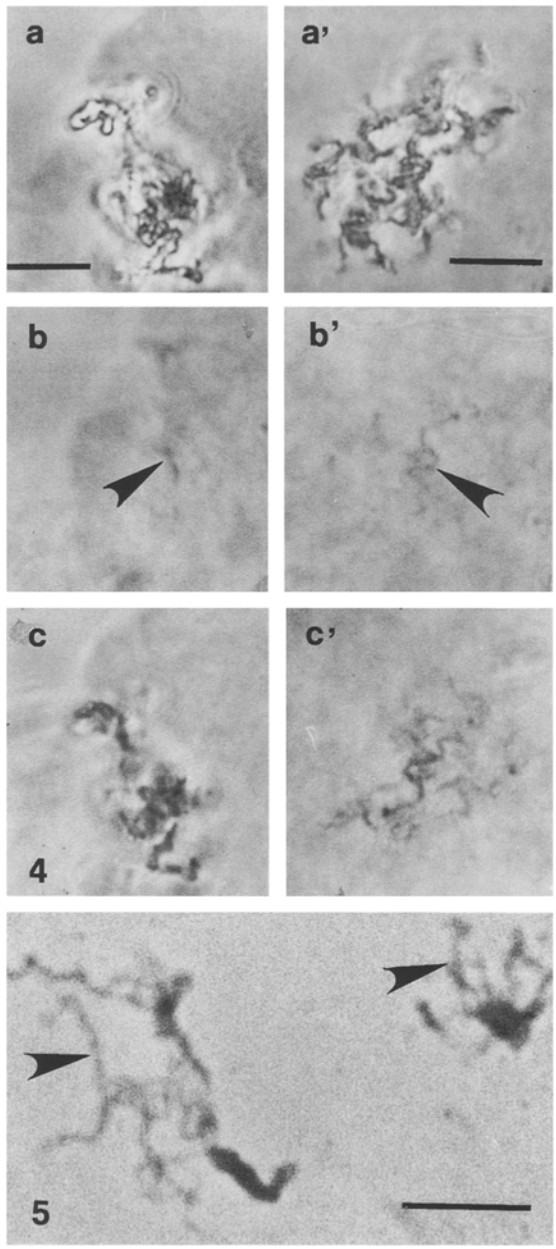

Fig. 4. Two series of phase contrast photomicrographs showing **a, a'** Glutaraldehyde-fixed *Metallogenium*-like structures as they appeared in Mn^{2+}-amended MSVP agar; **b, b'** Same fields after treatment with 0.1% hydroxylamine to remove Mn oxide. *Arrows* indicate faint structural features which had lost most of their phase density after the treatment. **c, c'** Same fields after staining with 0.1% Coomassie blue. Bars = 5 μm

Fig. 5. Phase contrast photomicrograph showing unfixed *Metallogenium*-like structure taken from the surface film of a Mn^{2+}-amended PYG liquid culture and placed on a 0.05% poly L-lysine coated slide. Mn oxides were removed with 0.1% hydroxylamine as in Fig. 4; however, in this case, the structures were stained with cationic colloidal iron instead of Coomassie blue to restore their phase density. Bar = 5.0 μm

Discussion

We have demonstrated that the *Metallogenium*-like structures in our fungal cultures did not contain living cells. Furthermore, we found no evidence that they were formed by cells that had subsequently vacated them. Our results support the suggestions of other workers who have argued that *Metallogenium*-like structures represent morphologically distinctive Mn oxide deposits that result from the growth and activity of viable organisms (Schweisfurth and Hehn 1972; Marshall 1979; Klaveness 1977; Gregory et al. 1980; Schmidt and Overbeck 1984; Palmer et al. 1986; Maki et al. 1987). However, our observations do not support the proposal that *Metallogenium*-like structures in fungal cultures result from the growth or activity of mycoplasmal parasites or bacterial symbionts of any kind. We found no evidence of any cells associated with the structures at any stage of their development. Had cellular forms been present, they would have been revealed by the differential staining, or electron microscopy, or slide culture procedures we employed.

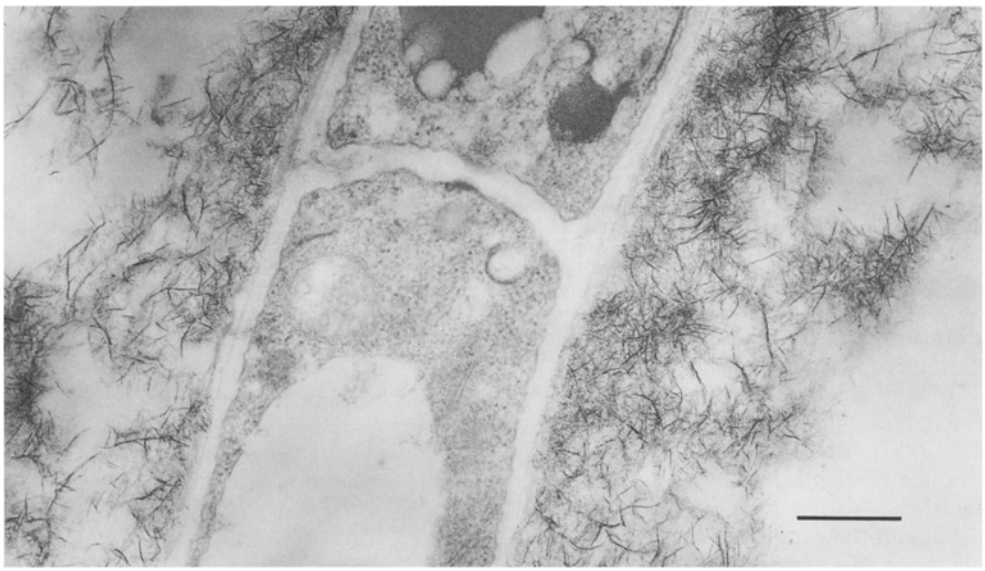

Fig. 6
Transmission electron micrograph of a thin section through a portion of a fungal hypha encrusted with Mn oxides. Note preservation of the fungal cell wall, plasma membrane, and other cellular structures, also note morphology of the Mn oxides deposited by this fungus on the surface of the cell wall. The fungus was grown in Mn-amended MSVP agar. The section was stained with lead citrate and uranyl acetate to enhance contrast. Bar = 0.4 μm

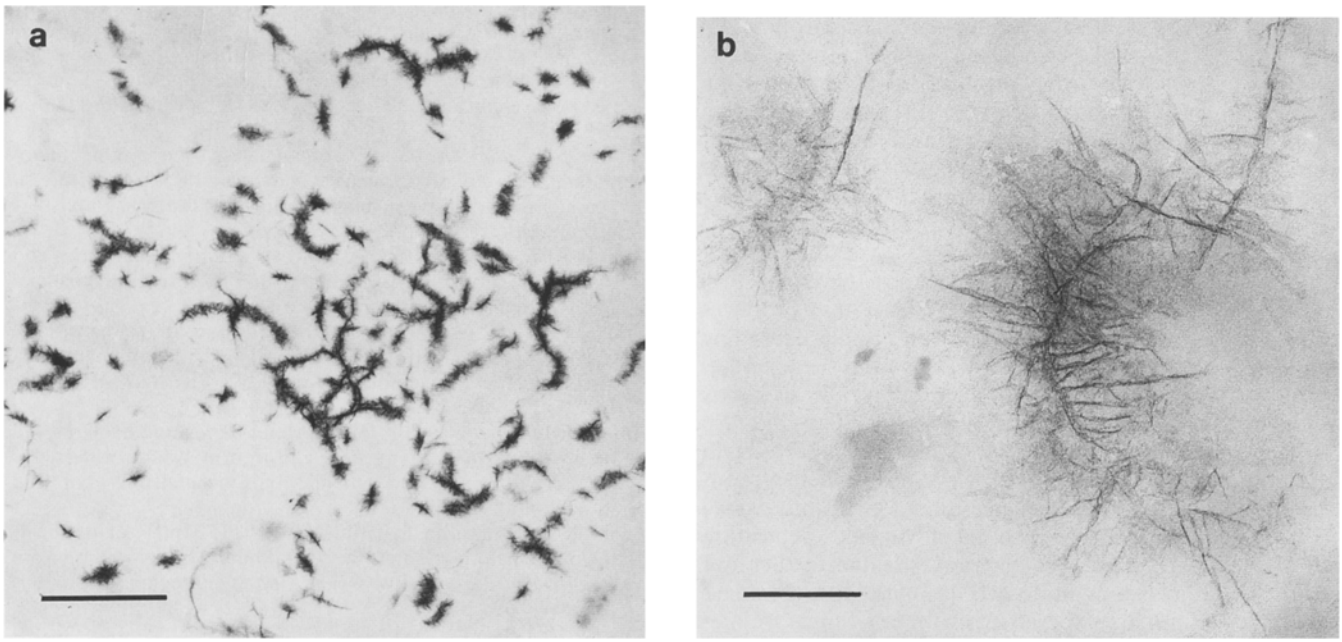

Fig. 7a, b. Transmission electron micrographs of thin sections through *Metallogenium*-like structures that formed in Mn-amended MSVP agar. **a** Low magnification view of a section through a *Metallogenium*-like structure. This was one of a set of serial sections that revealed no membranes or other cellular fine structures. It is unstained. Bar = 1.0 μm. **b** High magnification view showing Mn oxide structure similar to that on surface of hyphae (compare with Fig. 6). Section stained with lead-citrate and uranyl acetate. Bar = 0.2 μm

If the structures did not form around cells, then what was responsible for their formation? A clue to the answer lies in the finding that the structures lost much of their phase density after treatment with hydroxylamine. Also, subsequent staining with Coomassie blue or colloidal iron restored some of the density to an underlying matrix of polymers that maintained the original morphology of the structure. Fixation with glutaraldehyde was essential to stabilize the underlying polymeric structures in an agar medium, suggesting that protein cross-linking may be important to maintain the integrity of the structures after Mn oxide is removed. These results suggest that the structures were composed of Mn oxides deposited on a matrix of anionic polymers, probably proteins and acidic polysaccharides. Presumably, the fungus excreted the polymers along with Mn oxidizing factor(s) which would be necessary to catalyze Mn oxidation. Such a mechanism of extracellular polymer-associated Mn oxidation has been proposed to account for Mn oxide encrustations on the surfaces of budding bacteria (Ghiorse and Hirsch 1979, 1982) and sheathed bacteria (Adams and Ghiorse 1986, 1987). The formation of such metal-encrusted structures appears to be very common among iron and manganese depositing microorganisms of many different types (Ghiorse 1984a, 1988).

Thus, it appears that the fungus-related *Metallogenium* phenomenon is a special case of exopolymer-associated ferromangenese deposition by microorganisms. Similar to results reported earlier by Schweisfurth (1969, 1971), we have observed that many laboratory cultures of manganese-depositing fungi are capable of producing *Metallogenium*-like structures under the proper laboratory conditions. Schweisfurth was able to reproduce the structures by adding gum arabica and Mn^{2+} to an aqueous suspension of a Mn-oxidizing fungus (R. Schweisfurth, personal communication). In addition, we observed *Metallogenium*-like structures in Mn-oxidation agar diffusion plates several millimeters away from wells containing cell suspensions of *Leptothrix discophora* strain SS-1 (see Fig. 8 in Ghiorse 1984a) indicating that extracellular Mn-oxidizing components (Adams and Ghiorse 1987; Boogerd and DeVrind 1987) were capable of producing *Metallogenium*-like structures as they diffused through agar. Also, we recently isolated a ferromanganese-depositing fluorescent pseudomonad that produces copious quantities of extracellular polymer that form *Metallogenium*-like structures in the presence of Mn^{2+} during the stationary phase of growth (D. Emerson, unpublished observations). In an environmental context, it is noteworthy that in our liquid cultures, static conditions encouraged the production of free-floating *Metallogenium*-like structures (Fig. 2). This observation might help to explain the frequent occurrence of *Metallogenium*-like particles in aerobic-anaerobic transition zones of lakes when mixing is minimal (Klaveness 1977; Gregory et al. 1980; Jaquet et al. 1982; Tipping et al. 1985; Maki et al. 1987).

Finally, it is important to point out a possible connection between our basidiomycetous fungal isolate that produces *Metallogenium*-like structures, and the lignin-degrading white-rot fungus, *Phanerochaete chrysoporium* that excretes a Mn-oxidizing peroxidase which normally utilizes redox cycling of Mn(II) and Mn(III) for lignin oxidation, but also has been reported to produce Mn(IV) oxide under certain conditions (Glenn et al. 1986). *P. chrysosporium* also produces extracellular mucopolysaccharides (Kirk and Farrell 1987). To our knowledge the process of Mn oxide deposition by *P. chrysosporium* has not been investigated further. We were not able to demonstrate extracellular Mn peroxidase activity in preliminary experiments with our fungal isolate; however, we have not yet attempted to isolate or purify the Mn oxidizing activity from spent medium components which could interfere with Mn oxidation (Bromfield 1979). Future research on fungal Mn oxidation will focus on how the *Metallogenium* phenomenon may be related to lignin oxidation and exopolymer production.

Acknowledgements. We thank Jeff Lafavre and Lynn Sigler for help with identification of the fungus. Professor R. Schweisfurth kindly provided unpublished results from his laboratory. Robert P. McGovern supplied us with Sirofluor. Research in our laboratory is supported by Department of Energy, Office of Health and Environmental Research, and the National Science Foundation. We are indebted to Patti Lisk for expert secretarial assistance.

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Received May 2, 1988/Accepted October 27, 1988