Copper association with iron sulfide magnetosomes in a magnetotactic bacterium

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Abstract. Greigite (Fe_3S_4) and pyrite (FeS_2) particles in the magnetosomes of a many-celled, magnetotactic prokaryote (MMP), common in brackish-to-marine, sulfidic, aquatic habitats, contained relatively high concentrations of copper which ranged from about 0.1 to 10 atomic per cent relative to iron. In contrast, the greigite particles in the magnetosomes of a curved magnetotactic bacterium collected from the same sampling site did not contain significant levels of copper. The ability of the MMP to biomineralize copper within its magnetosomes appeared to be limited to that organism and dependent upon the site from which it was collected. Although the chemical mechanism and physiological function of copper accumulation in the magnetosomes of the MMP is unclear, the presence of copper is the first evidence that another transition metal ion could be incorporated in the mineral phase of the magnetosomes of a magnetotactic bacterium.

Key words: Biomineralization – Copper concentration – Greigite – Iron sulfide – Magnetite – Magnetosome – Magnetotactic bacterium – Pyrite – Single magnetic-domain

Magnetotactic bacteria are a diverse group of gram-negative eubacteria whose direction of motility is influenced by the earth's geomagnetic field (Blakemore 1975, 1982). These organisms are ubiquitous in aquatic habitats and cosmopolitan in distribution (Blakemore 1982; Blakemore et al. 1989). All magnetotactic bacteria contain intracellular iron mineral inclusions referred to as magnetosomes (Balkwill et al. 1980). These unique structures confer a permanent magnetic dipole moment to the cell resulting in the magnetotactic response (Frankel 1984; Frankel and Blakemore 1980, 1989). The magnetosome itself consists of a crystalline mineral phase and a surrounding organic phase, (i.e. a membrane; Balkwill et al. 1980; Gorby et al. 1988). The mineral phase consists of ferrimagnetic magnetite (Fe_3O_4; Frankel et al. 1979; Towe and Moench 1981; Matsuda et al. 1983; Mann et al. 1987; Bazylinski et al. 1988), greigite (Heywood et al. 1990, 1991), or greigite and pyrite (Mann et al. 1990). The species- and/or strain-specific morphologies (Blakemore et al. 1989) and the narrow single-magnetic-domain size range (approximately 35 to 120 nm) of the particles indicate that formation of the magnetosomes is highly controlled by the organism (Mann and Frankel 1989). The magnetosome membrane is presumably the structural entity that anchors the magnetosome at a particular location in the cell, as well as the locus of control over the size and morphology of the growing mineral particle (Frankel and Bazylinski 1993).

Towe and Moench (1981) reported the presence of very small amounts of titanium associated with the magnetite particles in the magnetosomes of an uncultured, freshwater, magnetotactic coccus. However, Gorby (1989) reported that no transition metals other than iron were found in the magnetosomes of the freshwater magnetotactic bacterium *Aquaspirillum* ("*Magnetospirillum*"; Schleifer et al. 1991) *magnetotacticum* following incubation of cells with titanium, chromium, cobalt, copper, nickel, mercury, and lead in growth experiments. In this paper, we report that significant amounts of copper are associated with the greigite and pyrite particles of an uncultured, many-celled, magnetotactic prokaryote (MMP) common in brackish-to-marine, sulfidic, aquatic habitats (Farina et al. 1983, 1990; Rodgers et al. 1990a, b; Bazylinski et al. 1990; Mann et al. 1990; DeLong et al. 1993).

Abbreviation: MMP, many-celled magnetotactic prokaryote

Material and methods

Organisms

Two morphologically conspicuous magnetotactic bacteria were examined in this study, neither of which has been isolated as a pure

culture. Both cell types could be collected in large numbers as a duotypic cell suspension as described below. Both types are common in reducing water and sediment of brackish-to-marine sulfidic habitats such as salt marsh pools (Bazylinski et al. 1990). The first is referred to as the MMP (Fig. 1a). Analyses of small subunit ribosomal RNA sequences of the MMP (DeLong et al. 1993) as well as consistent ultrastructural and chemical characteristics of its magnetosomes (Mann et al. 1990) suggests that this organism is a single bacterial species. The second is a relatively large, curved bacterium (Fig. 1b), averaging 3.9 by 1.4 μm ($n = 17$), that produces several (usually 2) parallel chains of greigite particles as determined by selected area electron diffraction (SAED). Although the morphology of this second bacterium and its magnetosomes are remarkably consistent, it is not presently known whether it represents a single species. Based on the high hydrogen sulfide concentrations present at the collecting site (up to 5 mM), it is likely both organisms are at least facultatively anaerobic.

Sediment and water, containing large numbers of the microorganisms described above, were collected from brackish to marine sulfidic sites at Morro Bay, Calif. and Woods Hole and Plum Island, Mass., using plastic implements, and transported in plastic containers. Magnetotactic bacteria were separated from the

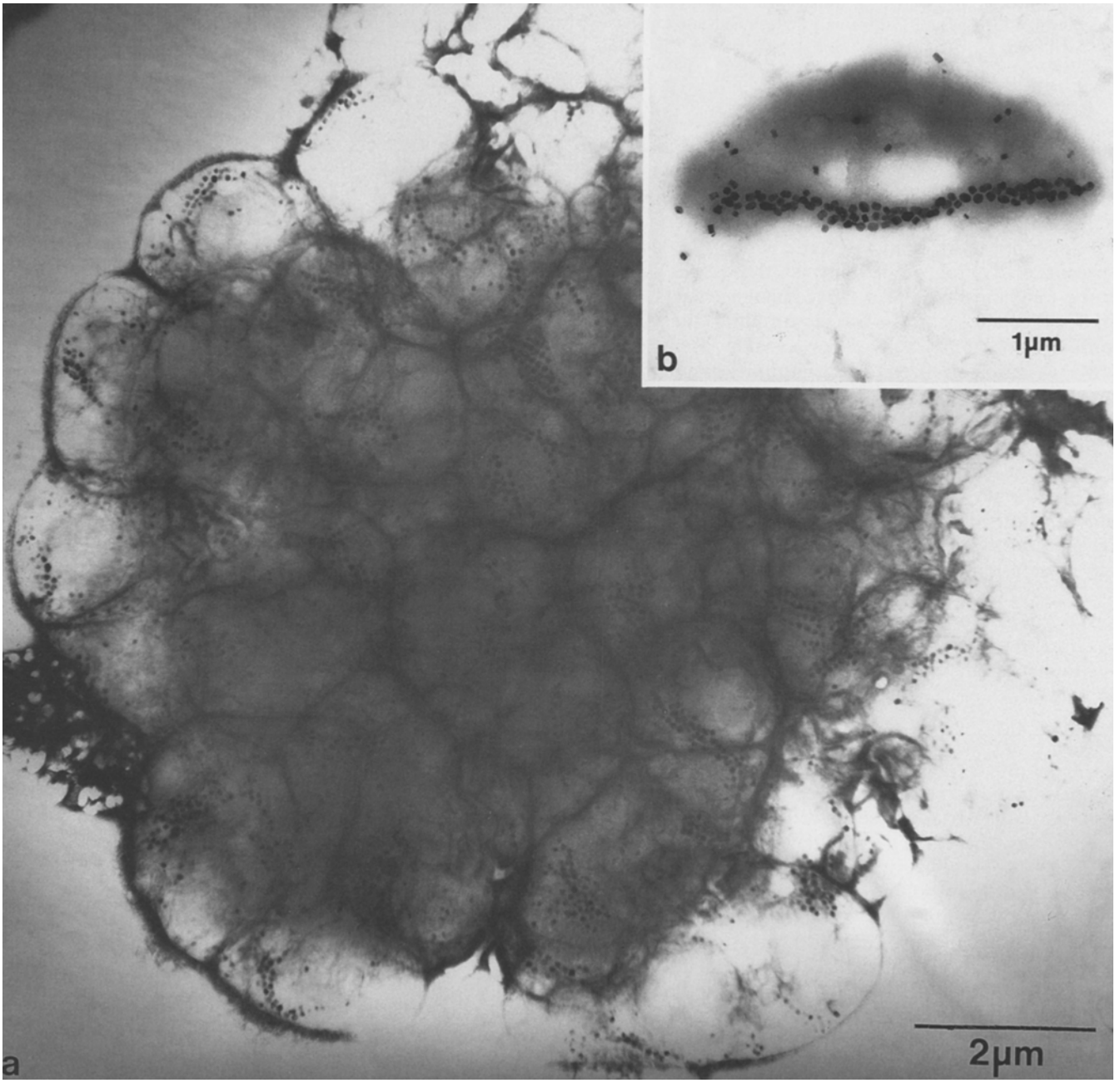

Fig. 1. Transmission electron micrographs (TEM) of magnetotactic bacteria examined in this study. **a** An MMP negatively stained for several seconds with 0.5% uranyl acetate. Note the "many-celled" appearance and the numerous scattered linear arrays of magnetosomes in each cell. **b** An unstained cell of the unidentified curved bacterium. Note that the chain of magnetosomes in this organism is 2 to 3 particles wide

sediments and water and concentrated using a modified magnetic "race-track" technique (Wolfe et al. 1987). In some cases, after concentration, the resultant "cell suspension" was allowed to stand for about 24 h during which time the MMP disaggregated into its constituent cells. Cells of the MMP and the curved bacterium were either deposited directly onto carbon-coated nickel electron microscopy grids, or first fixed with a 3% formalin solution and then deposited on the grids. Grids were allowed to sit for approximately 10 min after which the excess liquid was drawn off with tissue paper. In some cases, cells on grids were washed once with distilled water. At no time during the entire procedure were the cells exposed to exogenous copper salts or metal.

Electron microscopy and energy-dispersive X-ray analysis (EDXA)

Transmission electron microscopy (TEM) observation of whole cells and magnetosomes was performed using various instruments. Simple imaging was carried out either with a JEOL (Tokyo, Japan) Model 200CX microscope operating at 200 kV or a JEOL Model 100X instrument operating at 100 kV.

Analytical electron microscopy was performed on cells and magnetosomes using a VG Microscopes Model HB5 scanning transmission electron microscope (STEM) operating at 100 kV. This instrument was equipped with a field-emission electron gun, a Link LZ-5 X-ray detector, and an AN10000 X-ray analysis system capable of detecting all elements heavier than boron. Energy-dispersive X-ray analyses obtained on this system had an instrumentally limited spatial resolution of about 2 nm (specimen characteristics could degrade this further), and a typical sensitivity (depending on the sample) of about 0.1 weight %. X-ray elemental maps were recorded by rastering the electron beam under computer control and recording the number of X-ray counts in energy windows corresponding to the X-ray energies of the elements of interest. The maps obtained in this study almost all consisted of 128 × 128 pixels, and the dwell time at each pixel was typically 50 m. The total acquisition time was therefore about 15 min. For the major elements the typical number of counts recorded per pixel was 10 to 20. The window for all elements of interest in this work corresponded to the K X-ray lines in the energy range 500 eV to 10 keV.

Selected area electron diffraction (SAED)

Diffraction information on magnetosomes was obtained by setting the selected area diffraction aperture around individual and groups of magnetosomes and switching to the selected area mode using the JEOL Model 200CX TEM operating at 200 kV (described above). The microscope camera length was calibrated by using patterns obtained at the same microscope settings for either gold or thallous chloride samples. This technique provides a calibration precision of 1 to 2% (Hirsch et al. 1977).

Quantitation of copper in magnetosomes

The concentration of copper relative to iron in magnetosomes was quantified by integration of the respective K X-ray emission lines in the EDXA spectra. The bremstrahlung background (Bk) was estimated by integrating a region of each spectrum below the K X-ray line of iron where there were no lines (approximately 5.5 keV), and subtracting this quantity from the copper and iron integrated intensities.

Results

Magnetosome composition

Magnetosomes of both organisms were rather pleomorphic (Fig. 2) as compared to the consistent cubo-octahedral and rectangular prismatic morphologies of greigite observed in other magnetotactic bacteria (Heywood et al. 1990, 1991; Frankel and Bazylinski 1993). However, some of the particles of the MMP and the curved bacterium appeared to be well-ordered cubes and rectangular prisms, respectively (arrows in Fig. 2).
Magnetosomes of both organisms examined contained iron sulfides as demonstrated by EDXA (Figs. 3 and 4.). SAED (data not shown) of particles in the MMP revealed the presence of greigite and pyrite consistent with previous results for this organism (Mann et al. 1990) while only greigite diffraction patterns were obtained from particles of the curved bacterium.

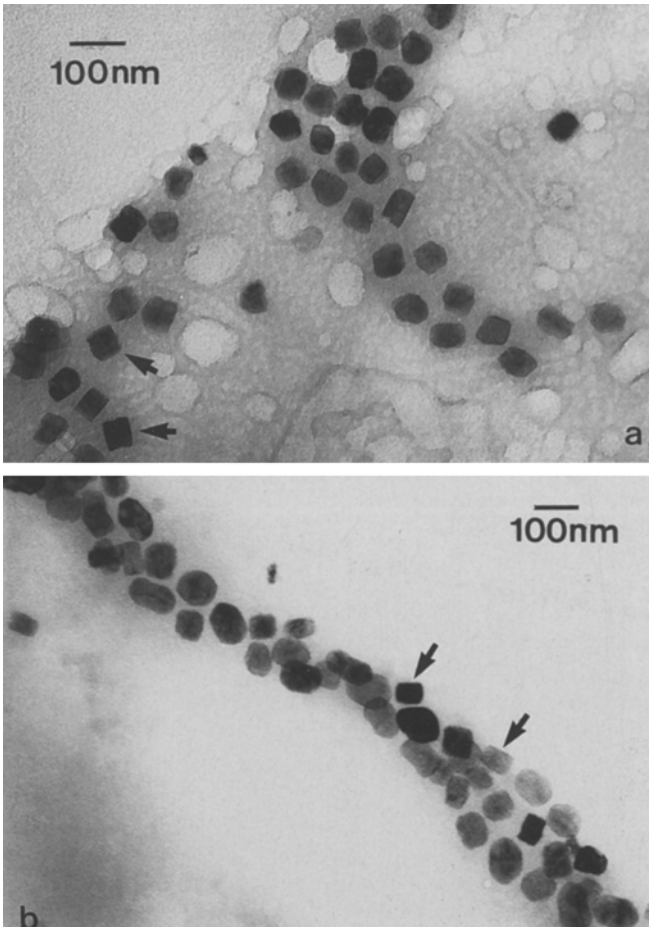

Fig. 2. High magnification transmission electron micrograph of the magnetosomes of the MMP (**a**) and the unidentified curved bacterium (**b**). Note that many of the particles in both organisms appear pleomorphic. *Arrows* indicate well-ordered cubes in **a** and rectangular prisms in **b** within the MMP and the curved bacterium, respectively

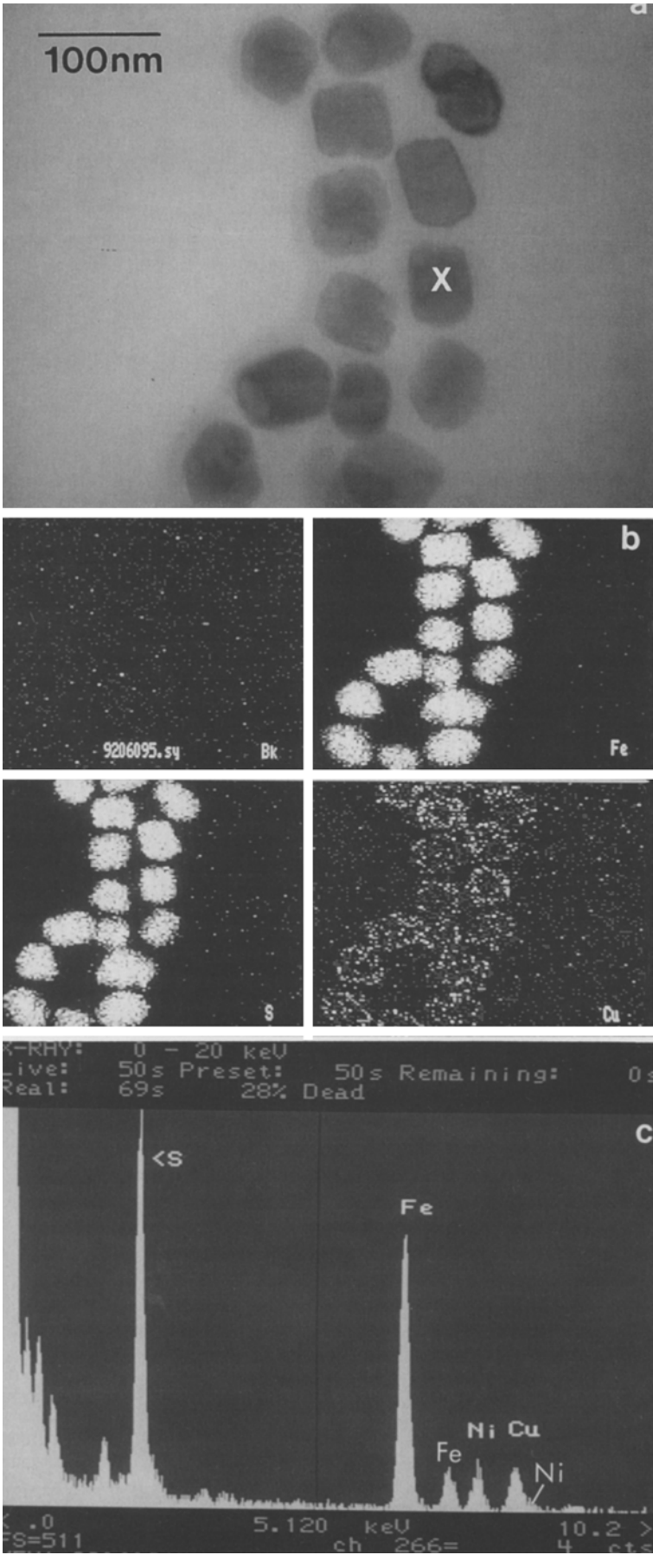

Fig. 3. Copper in the magnetosomes of an MMP collected from Morro Bay, Calif. **a** Scanning-transmission electron micrograph of magnetosomes of the MMP. **b** *Fe*, *S*, and *Cu* elemental X-ray map of the particles shown in **a**. *BK* refers to the bremstrahlung background. Figure 3a and b are slightly displaced relative to one another. **c** Elemental X-ray spectrum obtained with the electron beam focused on the particle labeled "*x*" in **a**

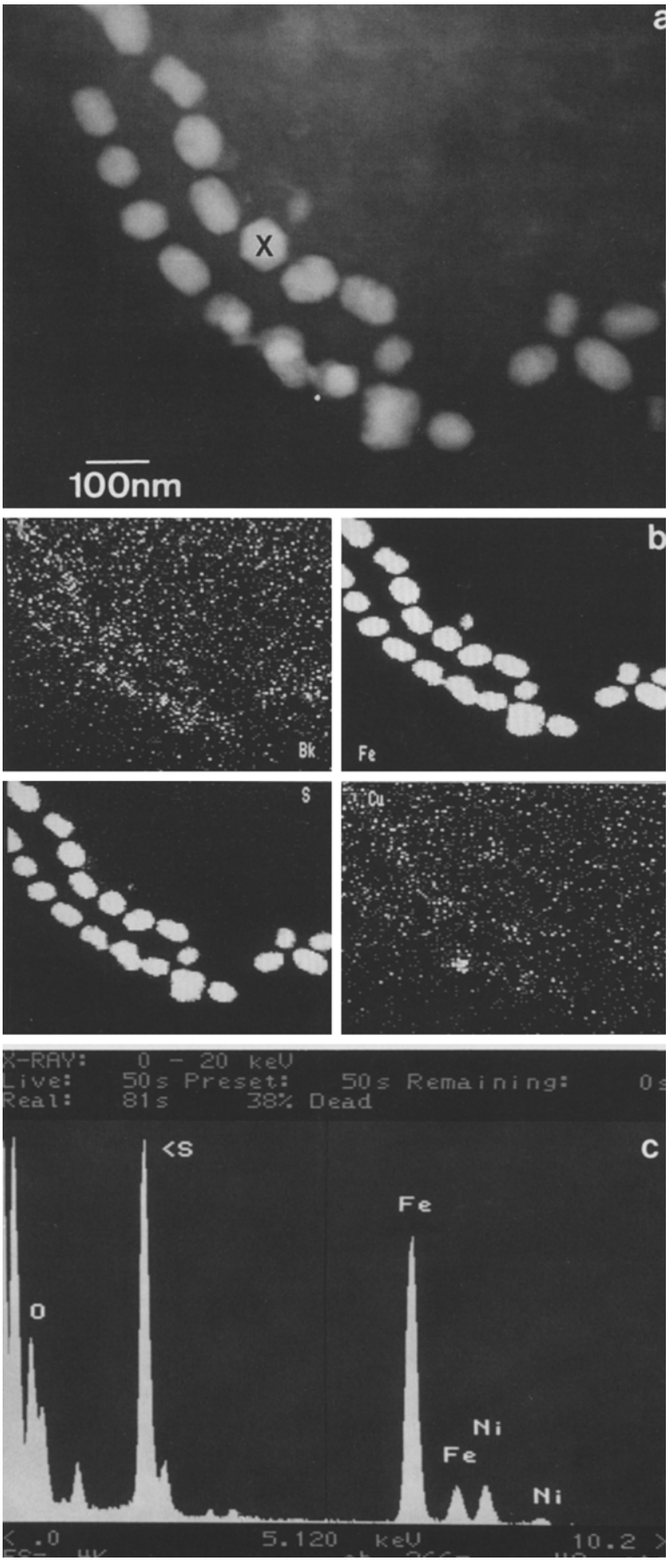

Fig. 4. Absence of copper in the magnetosomes in an unidentified curved magnetotactic bacterium collected from the same site as the MMP analyzed in Fig. 3. **a** Scanning transmission electron micrograph (negative image) of magnetosomes of the curved bacterium. **b** *Fe*, *S*, and *Cu* elemental map of the particles in **a**. Note the correspondence of the weak copper map, the Bk map, and the particle position. This shows that the copper map is essentially due to the bremsstrahlung background. **c** Elemental X-ray spectrum obtained with the electron beam focused on the particle labeled "x" in **a** showing that the copper signal is essentially zero

Presence of copper

Copper association with the magnetosomes in an MMP collected from Morro Bay, Calif. is displayed in Fig. 3. The image of a number of particles is shown in Fig. 3a, with "x" denoting the particle for which the spectrum shown in Fig. 3c was obtained. A substantial copper X-ray peak can be seen in the spectrum. Fig. 3b shows the elemental X-ray maps of iron, sulfur, and copper as well as the background (Bk) of the particles shown in Fig. 3a. All three elements clearly map with particle position while Bk does not. Copper appears to most concentrated on the surface of several of the particles. Copper-association was found in a large number of particles in least 10 MMPs from the collecting site. The amount of copper observed in the magnetosomes of the MMP was extremely variable and ranged from about 0.1 to 10 atomic per cent relative to iron. Representative data from the particles from two MMPs and a curved bacterium are shown in Table 1. Copper was never observed in the magnetosomes of the MMP collected from other brackish sites in Woods Hole and Plum Island, Mass., for a period of approximately three years of periodic collection of the MMP. Representative elemental X-ray spectra from these particles have been previously published (Bazylinski 1991; Bazylinski et al. 1993).

Figure 4 presents the image (4a), elemental X-ray map (4b), and X-ray elemental spectrum (4c) of the particle marked "x" in Fig. 4a, for a magnetotactic curved bacterium collected from the same site and deposited on the same electron microscopy grid as the MMP shown in Fig. 3. In contrast to the MMP, the X-ray spectrum and the elemental map show no copper association with the magnetosomes in the curved bacterium. At least 5 particles in at least 20 cells of the curved bacterium also showed no association with copper.

Representative ratios of copper to iron (reported as percent) in the magnetosomes of 2 MMPs and a curved bacterium are presented in Table 1. MMP-1 and MMP-2 were collected from different sites and a different times. The curved bacterium was collected from the same site and and time as MMP-1. As can be seen from Table 1, the relative copper concentrations varied greatly from particle to particle within the same cell and from MMPs collected from different sites. Consistent with the copper map shown in Fig. 3b, copper appeared to be concentrated on surface of some particles because the relative copper intensity was higher when the beam was focused on the edge of the particle than when it was focused on the center of the particle.

Table 1. Representative copper concentrations relative to iron in two MMPs and a curved magnetotactic bacterium collected from the same site at Morro Bay, Calif., and analyzed on the same nickel electron microscopy grid

Organism	Particle	Cu/Fe (%)
MMP-1	1	4.8[a]
	2	4.2
	3	1.4
	4	0.7
	5	0.6
	6 (edge)[b]	8.6
	6 (center)[b]	1.4
MMP-2	1	10.0
	2	7.2
	3	5.7
	4	2.4
	5	1.1
Curved bacterium	1–5	0.0

[a] Standard error of all measurements is ± 0.2
[b] Position on particle at which electron beam was focused

Discussion

The results presented herein show that under certain conditions, significant concentrations of copper can be incorporated into iron sulfide magnetosomes of a magnetotactic bacterium. Presently, this copper association appears to be limited to the MMP and to be collection site dependent having only been observed in MMPs collected from Morro Bay, Calif., and not in those from two other sites.

The physiological function, if one exists, of the presence of copper in the magnetosomes of the MMP is unclear although the ability of microorganisms to concentrate metal ions against marked concentration gradients is well known (Jones et al. 1976). The concentration of copper within cells is often linked to the toxicity of copper to bacteria which also has long been recognized, its toxicity presumably due to its binding to sulfhydral groups in and the subsequent inactivation of crucial enzymes (Rheinheimer 1992). In fact, copper and copper-containing formulations have been used as antibacterial agents (Bender and Cooksey 1986, 1987). Mechanisms of copper resistance in bacteria have not been well characterized but they are generally recognized to be plasmid-mediated (Bender and Cooksey 1986, 1987; Silver and Misra 1988) and, in at least two bacterial species, intracellular accumulation of copper appears to be important (Erardi et al. 1987; Silver and Misra 1988). In one of these, *Mycobacterium scrofulaceum*, intracellular precipitation of copper sulfide occurs as an apparent result of detoxification by this organism (Erardi et al. 1987). Thus, a function or consequence of the copper association in the magnetosomes of the MMP could be the sequestration and resultant detoxification of free copper within the cell to a non-toxic sulfidic form in the magnetosome. The MMP has not been examined for the presence of plasmids.

Bacteria, other than those mentioned above, also concentrate metal ions from growth medium for reasons apparently unrelated to toxicity. Dissimilatory sulfate-reducing bacteria, including *Desulfovibrio* and *Desulfotomaculum* species, are particularly efficient in this regard extracting most of the copper in growth medium and apparently "fixing copper in the cell wall-membrane region" (Jones et al. 1976). Interestingly, analyses of small subunit ribosomal RNA sequences of the MMP revealed it has a phylogenetic affiliation with the

dissimilatory sulfate-reducing bacteria in the delta subgroup of the Proteobacteria (DeLong et al. 1993).

The chemical mechanism of copper deposition in the magnetosomes of the MMP is unknown at present. However, sedimentary iron sulfides, the production of which is often microbially mediated in an indirect biologically-induced mineralization process (Lowenstam 1981) by sulfate-reducing bacteria (Morse et al. 1987; Bazylinski 1991), often contain significant amounts of copper. These include mackinawite (tetragonal iron sulfide, FeS_{1-x}; Morse et al. 1987), pyrite (Kluckhohn 1990), and others. Sedimentary greigite can also be expected to contain at least small amounts of copper (Morse et al. 1987).

Kluckhohn (1990) found much higher levels of pyritic copper in Chesapeake Bay sediments in which greigite appeared to be an intermediate of pyritization than in those in which it did not appear to be. This might suggest that the transformation of greigite to pyrite is a reaction in which the mineral is susceptible to the incorporation of copper. It is also thought that greigite transforms to pyrite in the MMP (Bazylinski et al. 1991). This might explain why copper appeared to be associated with the surface of the particles after they had formed. It is likely that the surface would pyritize before the core. Small particles of magnetite are known to pyritize in this manner (Kobayashi and Nomura 1972; Canfield and Berner 1987). Alternatively, this might suggest that the deposition of copper in the magnetosomes of the MMP is episodic, that is, it occurs during episodes of copper exposure to the MMP.

Copper, as well as cobalt and nickel, can replace iron in the inverse spinel structure of greigite and in the cubic structure of pyrite, forming compounds such as $CuFe_2S_4$ or chalcopyrite ($CuFeS_2$) (Kostov and Minceva-Stefanova 1981; Morse et al. 1987). It is difficult to determine whether copper is in true solid solution in mineral structures or is an impurity in a discrete mineral (Morse et al. 1987). However, the relatively low but significant levels of copper present in the magnetosomes of the MMP and the SAED patterns of greigite and pyrite obtained from these particles suggest that copper is present as the latter case. It would be interesting to determine if, under controlled experiments, the MMP can take up high levels of copper, cobalt, and nickel and incorporate them into the mineral structure of greigite and/or pyrite to form inverse spinel-type minerals such as linnaeite (Co_3S_4), polydymite (Ni_3S_4), carrollite ($CuCo_2S_4$), violarite ($FeNi_2S_4$), siegenite ($CoNi_2S_4$), and intermediate mineral species or cubic minerals such as chalcopyrite (Kostov and Minceva-Stefanova 1981).

The presence of pleomorphic particles of greigite and pyrite rather than well-ordered crystals of greigite alone present in other iron sulfide-type magnetotactic bacteria and the fact that the magnetosomes of the MMP don't always contain copper suggests that copper deposition in the magnetosomes of this organism is a non-specific process. These data also seem to indicate that the biomineralization process (Mann 1986) involved in magnetosome production in the MMP is not as controlled as in other magnetotactic bacteria (Bazylinski et al. 1993) Regardless, the presence of significant concentrations of copper in the magnetosomes of a magnetotactic bacterium is an important finding. Controlled experiments with pure cultures of the MMP, once available, will address and hopefully answer many of the questions presented in this paper.

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