Elemental analysis of uncultured magnetotactic bacteria exposed to heavy metals

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Abstract: Natural enrichments of magnetotactic bacteria were used to study the sites where heavy metals accumulate in uncultured bacteria. Most bacteria obtained by magnetic concentration from these enrichments contained, in addition to the magnetosomes, large phosphorus-rich granules in the cytoplasm. Metal (Zn, Mn, Sr, Cd, Al, Cr, and Pb) chlorides were added independently to the enrichments, and after 24 h, the elemental composition of the phosphorus-rich granules, magnetosomes, and "soft parts" (cytoplasm plus cell envelope) of whole bacteria was analyzed by energy-dispersive X-ray analysis on a transmission electron microscope. All bacteria contained Mn and Sr in the phosphorus-rich granules; some of them presented Mn peaks also in the soft parts. Zinc accumulation was variable and was found mainly in the phosphorus-rich granules, but also in the soft part of some bacteria. Some analyzed bacteria presented Zn peaks only in the soft parts, and some of them did not present Zn in any structure. Cadmium and Al were found only in the granules of some bacteria. Chromium was found in the soft parts of some bacteria. Lead was not detected in any bacteria. We concluded that the phosphorus-rich granules are major sites for metal accumulation by these bacteria. No conclusive results for magnetosomes were obtained because of the limitations of the analytical techniques particularly when used for whole cell analysis.

Key words: magnetotactic bacteria, polyphosphate granules, heavy metals, uncultured bacteria, biomineralization, X-ray microanalysis.

Résumé : Des enrichissements naturels pour les bactéries magnétotactiques ont été utilisés pour identifier les sites d'accumulation des métaux lourds chez des bactéries non cultivées. La plupart des bactéries recueillies par concentration magnétique suite à ces enrichissements contenaient, en plus des magnétosomes, des gros granules riches en phosphore dans leur cytoplasme. Des chlorures métalliques (Zn, Mn, Sr, Cd, Al, Cr et Pb) ont été ajoutés indépendamment des produits d'enrichissement et, après 24 h, la composition élémentaire des granules riches en phosphore, des magnétosomes et des «parties éteintes» ou «soft parts» (cytoplasme et enveloppe cellulaire) des bactéries entières a été mesurée par une analyse de dispersion de l'énergie des rayons X en microscopie électronique à transmission. Chez toutes les bactéries, les granules riches en phosphore contenaient du Mn et du Sr et certaines bactéries présentaient des pics de Mn dans les parties éteintes. L'accumulation de zinc était variable et a été retrouvé principalement dans les granules riches en phosphore et aussi dans les parties éteintes de certaines bactéries. Certaines bactéries analysées ont présenté des pics de Zn uniquement dans les parties éteintes et d'autres n'ont pas révélé la présence de Zn dans aucune des structures. Le cadmium et l'aluminium ont été retrouvés seulement dans les granules chez quelques bactéries. Le chrome a été décelé dans les parties éteintes de certaines bactéries. Aucune bactérie n'a démontré la présence de plomb. Nous concluons de cette étude que les granules riches en phosphore sont les principaux sites d'accumulation des métaux chez ces bactéries. Il n'a pas été possible d'obtenir des résultats concluants pour les magnétosomes à cause des limites des méthodes analytiques surtout lorsqu'on utilise des cellules entières.

Mots clés : bactéries magnétotactiques, granules de polyphosphate, métaux lourds, bactéries non cultivées, biominéralisation, microanalyse aux rayons X.

[Traduit par la Rédaction]

Magnetotactic bacteria are motile microorganisms that align themselves along magnetic field lines because they have small membrane-bound magnetic crystals, called magnetosomes, usually arranged in chains inside the cytoplasm (Blakemore 1975). In most bacteria, the magnetic crystals contain magnetite (Fe_3O_4; Frankel et al. 1979). In a few bacteria they are

Received April 30, 2001. Revison received October 4, 2001. Accepted October 15, 2001. Published on the NRC Research Press Web site at http://cjm.nrc.ca on December 17, 2001.

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 DOI: 10.1139/cjm-47-12-1132

composed of iron sulfides (Pósfai et al. 1998); both iron oxides and iron sulfides can be found in different magnetosomes of the same organism (Bazylinski et al. 1993). Magnetotactic bacteria producing magnetite are found mostly in the aerobic–anaerobic transition zone, whereas greigite producers are found below this region, in the anaerobic zone (Bazylinski et al. 1995). It is believed that each magnetotactic bacterium produces crystals with specific morphologies and size ranges (Meldrum et al. 1993; Thornhill et al. 1994), although there are reports of some exceptions (Lins and Farina 1998).

Unlike most cultured microorganisms, many bacteria obtained directly from natural environments, including magnetotactic bacteria, present cytoplasmic inclusions rich in phosphorus (Corpe and Jensen 1992; Bazylinski et al. 1992). These inclusions contain phosphorus, magnesium, potassium, and calcium, and sometimes also sulfur, chlorine, iron, and sodium (Jensen and Corpe 1993). In previous works (Spring et al. 1998; Lins and Farina 1999), we have shown that many magnetotactic bacteria from Itaipu Lagoon present phosphorus-rich granules that naturally accumulate Al, Fe, and Zn, even though they were not obtained from a metal-contaminated environment. Because magnetotactic bacteria have an intracellular compartmentalization of biominerals (magnetosomes and phosphorus-rich granules) unique among the bacteria, these bacteria could be good models for the study of metal retention by uncultured microorganisms.

Many uncultured bacteria contain polyphosphate bodies (Corpe and Jensen 1992), capsules, sheaths, and S-layers. These structures are frequently lost after repeated culturing in laboratory medium (Beveridge 1989). These differences make the studies on cultured organisms inappropriate to understand some of the processes that happen in natural environments.

Extensive work has been done on the accumulation of metals in the polyphosphate bodies of cultured bacteria. Titanium was found in the polyphosphate bodies of *Anacystis nidulans* (Crang and Jensen 1975); Ba and Mn in *Plectonema boryanum* (Baxter and Jensen 1980); Cd, Co, Cu, Hg, Ni, Pb, and Zn in *Plectonema boryanum* (Jensen et al. 1982); Cd in *Anabaena flos-aquae* (Rachlin et al. 1984), Al in *Anabaena cilindrica* (Pettersson et al. 1985); Ni in *Staphylococcus aureus* (Gonzalez and Jensen 1998); and Al, Cd, Cu, Pb, and Zn in *Synechococcus leopoliensis* (Goldberg and Jensen 1999). All these experiments were done using high concentrations of metal salts in the culture media. However, some culture media components can complex with the metal ions, changing the metal species present (Hughes and Poole 1991). In this work, we analyzed the sites of metal accumulation of uncultured magnetotactic bacteria from natural enrichments exposed to different metals. We exposed the enrichments to metal (Zn, Mn, Sr, Cd, Al, Cr, and Pb) salts for 24 h and analyzed the elemental composition of the magnetosomes, phosphorus-rich granules, and "soft parts" (cytoplasm plus cell envelope) by energy-dispersive X-ray analysis (EDXA). The metals used are diverse in their concentration in the seawater as well as in their nutritional requirement and toxicity. Manganese and strontium are relatively common metals in sea water (Stein 1973), whereas the others are not. Manganese, zinc, and chromium are essential metals, but are toxic at high concentrations. In contrast, aluminium, cadmium, and lead are toxic, and no biological functions are known for them (Hughes and Poole 1991). Both the essential and nonessential metals may cause serious environmental problems (Rai et al. 1981).

Source of magnetotactic bacteria, enrichment, and assays with metal salts

Samples of sediment and water were collected in the Itaipu Lagoon, a brackish to saline lagoon near Rio de Janeiro, Brazil, and were maintained in glass flasks in the laboratory for a few weeks. The salinity was around 35‰ in all samples. During this period, magnetotactic bacteria grew in some of the flasks. Twice a week, some drops of sediment were taken from each flask and observed through an optical microscope to monitor the presence of magnetotactic bacteria. A magnet was used to direct bacteria to the border of the drop. The flasks with higher numbers of bacteria were chosen for addition of the metal salts.

The metals were added singly to the flasks. To facilitate dissolution, we added the compounds previously dissolved in distilled water to the flasks. We began with 0.1 mM and added increasing (0.2 mM, 0.4 mM, 0.8 mM, 1.6 mM …) concentrations of metals until no magnetotactic bacteria were seen swimming near the border of a drop of sediment within 24 h. For the analytical experiments, the concentrations used were half of those that killed all magnetotactic bacteria. At these concentrations, there were a few bacteria swimming near the edge of the drop of sediment. The compounds and concentrations (mM) used for elemental analysis were $AlCl_3$, 6.4; $CdCl_2$, 0.8; $CrCl_3$, 1.6; $MnCl_2$, 25.6; $ZnCl_2$, 1.6; $SrCl_2$, 12.8; and $PbCl_2$, 12.8. $PbC1_2$ and $SrC1_2$ were saturated at these concentrations. The flasks to which we have added $AlCl_3$ and $CrCl_3$ presented insoluble precipitates near the water–sediment interface after 24 h of exposition. We used metal chlorides because chloride is the most abundant anion present in seawater, and we did not wish to disturb the enrichment environment by adding other anions. Control samples were taken from each flask before addition of metals, with minimal disturbance of the stratification of the sediment.

Magnetic concentration of bacteria, electron microscopy, and elemental analysis

Sediment and water from the enrichments were transferred to a specially designed glass container (Esquivel et al. 1990). This device was placed inside a coil with the capillary end pointing towards the south magnetic direction, so that the magnetotactic bacteria swam to this direction and could be concentrated and collected with a Pasteur pipette.

For description of general morphology of control magnetotactic bacteria, magnetically separated cells were concentrated in the bottom of a 0.5-mL Eppendorf tube, using a magnet, and were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer in seawater overnight, washed in the same buffer, postfixed in a buffered seawater 1% OsO_4 solution for 1 h, washed, dehydrated in acetone series, and embedded in Polybed 812 (Ted Pella) resin. Stained ultrathin sections were examined with a Zeiss 900 electron microscope.

For elemental analysis, a drop of the concentrated sample was placed on a formvar–carbon coated electron microscopy grid. A magnet was placed in such a way to drive the bacteria to one of the limits of the drop, near the center of the grid. The seawater was carefully replaced by distilled water to avoid the formation of salt crystals, and the grid was air-dried. Elemental analyses were done in whole bacteria to avoid elemental redistribution, which might happen in samples processed by standard methods for electron microscopy. EDXA was performed on a Jeol 1200 EX transmission electron microscope equipped with a Noran X-ray detector, with a spot size of about 50 nm, operating at 80 kV for 200 s. When possible, we analyzed also the soft parts. Control spectra were obtained from the formvar–carbon film near each bacterium.

Ultrastructure of magnetotactic bacteria and sites of metal accumulation

Magnetotactic bacteria frequently had one or more kinds of intracellular inclusions, such as large phosphorus-rich granules, sulfur globules, and electron-lucent inclusions. Figure 1 shows an ultrathin section of a bacterium from Itaipu Lagoon where some of the cytoplasmic inclusions are seen, particularly the relatively large phosphorus-rich granules. EDXA of granules (not shown) of control magnetotactic bacteria from the enrichment invariably showed C, O, Mg, P, and occasionally Na, S, Cl, K, Ca, Al, Fe, and Zn, similar to results obtained from several cultured bacteria (Webster et al. 1984) and also from some magnetotactic bacteria (Bazylinski et al. 1992; Spring et al. 1998; Lins and Farina 1999). EDXA spectra of the magnetosomes always showed Fe and O peaks, indicating that all bacteria from the enrichment were of the magnetite-producing type. EDXA spectra of sulfur globules only showed a sulfur peak (not shown). We found no evidence for metal accumulation by this structure. Spectra of the electron-lucent inclusions only showed C and O (not shown), and we believe that they are composed of either poly-hydroxy-alkanoates or lipids. This structure did not accumulate the added metals.

All metals were toxic to magnetotactic bacteria at the concentrations used. The number of bacteria swimming to the border of a drop of sediment diminished substantially when samples were exposed to the heavy metals for 24 h. The same effect was observed in other organisms of the enrichments, such as animals, ciliates, flagellates, and bacteria-sized organisms. Usually the bacteria-sized organisms were the most resistant. Table 1 summarizes the sites of metal accumulation by the magnetotactic bacteria of the enrichment environment. Zinc accumulation in magnetotactic bacteria exposed to $ZnCl_2$ was variable. Some bacteria presented Zn peaks only in the spectra of phosphorus-rich granules (Fig. 2), whereas Zn appeared also in the soft parts of some bacteria. In some cases, no detectable amounts of this metal were observed in any structure. The localization of the accumulation sites for the different metals used in this work followed the same approach as for zinc. All magnetotactic bacteria exposed to $MnCl_2$ or $SrCl_2$ accumulated the respective metals in phosphorus-rich granules; some bacteria presented Mn peaks in smaller amounts in the spectra of the soft parts. Strontium was not detected in the spectra of the soft parts of any bacteria. Cadmium peaks appeared in the spectra of the phosphorus-rich granules in some of the bacteria exposed to $CdCl_2$. No Cd peak was found in the soft parts of any analyzed bacteria. Small amounts of aluminum were detected in the phosphorus-rich granules of some of the analyzed bacteria. Chromium accumulated almost exclusively in the soft parts of some of the analyzed bacteria, and no analyzed bacteria exposed to $PbCl_2$ presented Pb peaks in any structure. No electron-dense deposits, typical of extracellular precipitation, were observed in the electron micrographs of metal-exposed cells. Usually, morphologically similar bacteria presented similar patterns of metal accumulation. We had no conclusive results for metal trapping by the magnetic crystals because the presence of the magnetosome membrane and the soft

Fig. 1. Ultrathin section of a magnetotactic bacterium from Itaipu Lagoon. Note the magnetosomes (dark regions), the typical gram-negative structure of the cell wall (arrows), the large phosphorus-rich granules (large asterisks), the sulfur globules (small asterisks) and the glycocalyx extending beyond the cell wall (arrowheads). Scale bar represents 0.5 μm.

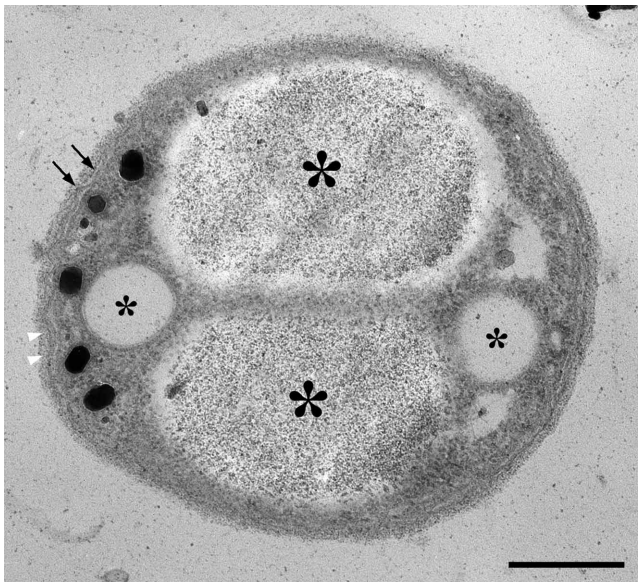


Table 1. Summary of the sites of metal accumulation in magnetotactic bacteria obtained by energy-dispersive X-ray analysis of whole cells.

Metal	Phosphorus-rich granules	"Soft parts"*	Magnetosomes
Zn	+, –	+, –	+, –
Mn	+	+, –	+, –
Sr	+	–	–
Cd	+, –	–	–
Al	+, –	–	–
Cr	–	+, –	–
Pb	–	–	–

Note: (+) The metal was detected in all analyzed bacteria; (–) the metal was not detected in any analyzed bacteria; (+, –) the metal was detected in some of the analyzed bacteria.
*Cytoplasm plus cell envelope.

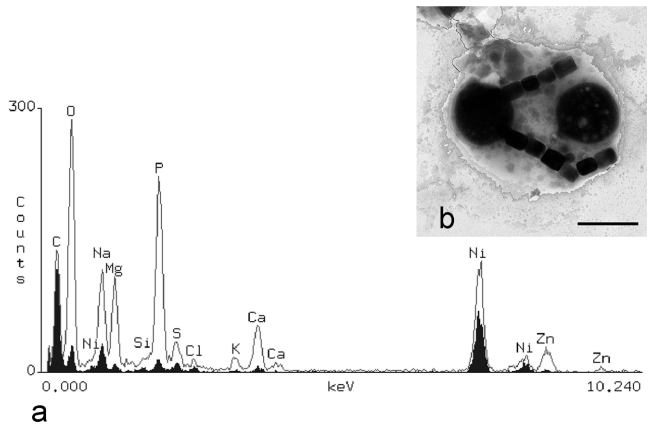
parts that surround them hinders the analysis by EDXA. However, very small peaks of Zn and Mn were obtained from magnetosomes of some bacteria. We are currently working to clarify this point.

The present work is the first systematic study on heavy metal accumulation by uncultured magnetotactic bacteria. We have previously shown that magnetotactic bacteria from Itaipu Lagoon contain phosphorus-rich inclusions (Lins and Farina 1999). Polyphosphate is the phosphorus-containing component in the phosphorus-rich granules of most bacteria (Scharfstein and Keasling 1994; Lawrence et al. 1998). Phosphorus-rich granules of bacteria are assumed to contain polyphosphates, although granules of *Desulfovibrio gigas* are an exception (Hensgens et al. 1996). Because our bacteria were uncultured, we could not unequivocally determine whether polyphosphate or another compound formed their granules. In this case, we can reasonably assume that this compound is present in the bacteria analyzed, and thus, we can compare our results with those found for bacteria containing polyphosphate.

Zinc, Mn, Sr, Cd, and Al appeared mostly in the phosphorus-rich granules. These results agree with Jensen et al. (1982), who found Cd, Co, Cu, Hg, Ni, Pb, and Zn mainly in the polyphosphate bodies of the cyanobacterium *Plectonema boryanum*. In *Lactobacillus plantarum*, large amounts of Mn, needed for defense against free radicals, are stored mostly inside the polyphosphate granules (Archibald and Fridovich 1982). However, our results are in partial agreement with Baxter and Jensen (1980), who found Ba and Mn but not Sr in the polyphosphate bodies of *Plectonema boryanum*. Furthermore, our results for Pb-exposed bacteria are different from Jensen et al. (1982), who found this metal both in the polyphosphate bodies and in the soft parts of *Plectonema boryanum*. Different morphological types of magnetotactic bacteria exposed to Zn, Cd, and Al had diverse responses, in the same experiment, probably because they have different membrane permeability and (or) phosphorus-rich granule binding properties. From the above results, it is apparent that different organisms have different metal-accumulation behaviors.

Chromium was the only metal found uniquely in the soft parts of the magnetotactic bacteria. We could not indicate the specific bacterial structures where chromium binds, but our results are consistent with Kong et al. (1992, 1994). Their fractionation studies point to the soluble and membrane fractions as the main sites for chromium binding in *Pseudomonas stutzeri* and also, to a less extent, to the cell wall fraction. Although a growing volume of work points to the cell wall, S-layers, and extracellular polymers as the main sites for metal accumulation by bacteria (Douglas and Beveridge 1998), only Mn, Zn, and Cr were found in the soft parts of some of the analyzed bacteria. We found no evidence for precipitation of these metals in the cell wall or extracellular polymers. Because Mn and Zn amounts in phosphorus-rich granules were always higher than in the soft parts and because we did not find Sr, Cd, Al, or Pb in the soft parts of any bacteria, we conclude that the phosphorus-rich granules are the most important metal-accumulation site in the magnetotactic bacteria of Itaipu Lagoon, under enriched conditions.

Fig. 2. Representative spectra and image of a whole magnetotactic bacterium after 24 h of zinc exposure. (*a*) Energy-dispersive X-ray analysis of one phosphorus-rich granule (line) and "soft parts" (darkened area) of the corresponding bacterium in (*b*). Ni peaks come from the supporting grid. (*b*) Transmission electron micrograph of a whole magnetotactic bacterium placed over a formvar–carbon coated grid. Scale bar represents 0.5 μm.



The functions of polyphosphate bodies are not well defined. As phosphate–phosphate linkages have high amounts of energy, it seems likely that this polymer is an energy store (Wood and Clark 1988; Kornberg 1995). Some authors suggest that polyphosphates are involved in the regulation of the intracellular concentration of cations (Jensen et al. 1982; Wood and Clark 1988; Kornberg 1995) and in metal detoxification (Sicko-Goad and Stoermer 1979; Jensen et al. 1982; Rai et al. 1990). Our results are consistent with the metal-detoxification hypothesis because the metals accumulated mostly in the phosphorus-rich granules, despite the absence of physical barriers between granules and the cytoplasm. Because Zn, Mn, Sr, Cd, and Al are present in higher amounts in the phosphorus-rich granules than in the soft parts, the selective distribution of the metals in the granules can relatively lower the concentration of these metals in the cytoplasm. This fact could be an indication of a nonspecific heavy metals detoxification mechanism in these bacteria.

The imaging and analytical procedures used in this work allowed the discrimination among different morphological types of bacteria in such a way that differences in metal accumulation could be studied. In addition, the EDXA device attached to the transmission electron microscope could show which compartment of the bacteria accumulated the metals. We believe that the site of and amount of metal accumulation by uncultured magnetotactic bacteria may give information about environmental conditions of the microaerophilic microbial community when metal pollution occurs in a saline aquatic environment.

Acknowledgements

We thank Flávia F. Freitas for help in collecting samples and Laboratório de Ultraestrutura Celular Hertha Meyer for electron microscopy facilities. PRONEX, CNPq, and CAPES Brazilian grants supported this research.

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