Published in final edited form as:

J Struct Biol. 2013 February ; 181(2): 162–168. doi:10.1016/j.jsb.2012.12.002.

Cryo-electron tomography of the magnetotactic vibrio *Magnetovibrio blakemorei*: insights into the biomineralization of prismatic magnetosomes

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Abstract

We examined the structure and biomineralization of prismatic magnetosomes in the magnetotactic marine vibrio *Magnetovibrio blakemorei* strain MV-1 and a non-magnetotactic mutant derived from it, using a combination of cryo-electron tomography and freeze-fracture. The vesicles enveloping the *Magnetovibrio* magnetosomes were elongated and detached from the cell membrane. Magnetosome crystal formation appeared to be initiated at a nucleation site on the membrane inner surface. Interestingly, while scattered filaments were observed in the surrounding cytoplasm, their association with the magnetosome chains could not be unequivocally established. Our data suggests fundamental differences between prismatic and octahedral magnetosomes in their mechanisms of nucleation and crystal growth as well as in their structural relationships with the cytoplasm and plasma membrane.

Keywords

Magnetosome; Magnetotactic bacteria; Cryo-electron tomography; Biomineralization

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Introduction

Magnetotactic bacteria comprise a diverse group of motile, Gram-negative prokaryotes capable of producing intracellular, membrane-bounded, nano-sized magnetic crystals called magnetosomes (Bazylinski and Frankel, 2004). Each magnetosome consists of a magnetite (Fe_3O_4) or greigite (Fe_3S_4) crystal enveloped by a phospholipid bilayer membrane (Gorby et al., 1988). Specific proteins are expressed by the cell in the magnetosome membrane and modulate the biomineralization of the magnetic crystals within the magnetosome membrane vesicle (Komeili, 2007). As a result of the controlled biomineralization process, magnetosomes contain magnetite and greigite crystals with different morphologies that are dependent on the magnetotactic bacterial species (Bazylinski and Frankel, 2004). For magnetite magnetosomes, crystal habits include cuboctahedral, elongated prismatic and elongated-anisotropic (bullet-shaped crystals).

Magnetite magnetosome formation in the *Alphaproteobacteria* has been extensively characterized in the freshwater magnetotactic spirilla belonging to the genus *Magnetospirillum* (Greene and Komeili, 2012). In *Magnetospirillum gryphiswaldense* MSR-1 magnetosomes consist of cuboctahedral magnetite crystals that are enveloped individually by vesicles of the magnetosome membrane associated with a set of specific proteins (Gorby et al., 1988; Katzman et al., 2010; Schüller, 2004; Scheffel et al., 2006). In *M. magneticum* AMB-1 the magnetosome membrane originates by invagination from the cytoplasmic membrane and remains attached to the cell membrane (Greene and Komeili, 2012; Komeili et al., 2006).

In the genomes of many magnetotactic bacteria, magnetosome genes are located in clusters (Komeili, 2007). These genes encode for proteins that are responsible for controlling the size and morphology of magnetite crystals in magnetotactic bacteria, as well as magnetosome chain organization (Schüler, 2004). In the genus *Magnetospirillum*, these genes comprise the *mamAB*, *mamGFDC*, *mamXY* and *mms6* operons (Jogler & Schüler, 2009) that reside in a genomic region known as the magnetosome island (MAI) (Schüler, 2004). Comparative genomic analysis shows that the gene content and organization in the MAI differ among cultured magnetite-producing magnetotactic bacteria. These differences are thought to be responsible for diversity of magnetosome crystal morphology, size and magnetosome organization within the cell (Jogler et al., 2009a). In uncultured magnetotactic bacteria, *mam* genes have also been detected in both magnetite-producing magnetotactic bacteria belonging to the *Alphaproteobacteria* (Jogler et al., 2009b) and the *Nitrospirae* phylum (Jogler et al., 2011) as well as in greigite-producing magnetotactic bacteria belonging to *Deltaproteobacteria* (Abreu et al., 2011; Lefèvre et al., 2011) suggesting a monophyletic origin for the trait of magnetotaxis.

In freshwater magnetotactic spirilla, the magnetosome vesicles enveloping the magnetite crystals are distributed along a filamentous structural element, formed by the protein MamK (Komeili et al., 2006). MamK, encoded by the *mamK* gene of the *mamAB* cluster in the MAI of several magnetotactic bacteria, is a homolog of the bacterial actin-like protein involved in the determination of cell shape called MreB (Komeili et al., 2006; Jogler et al., 2009a,b). The magnetosome chain is organized by a filamentous network which runs parallel to the cell membrane and extends to the cell poles (Komeili et al., 2006; Scheffel et al., 2006; Pradel et al., 2006). This filamentous network is composed of MamK filaments of about 200-250 nm in length (Komeili, 2007). In *Magnetospirillum magneticum* strain AMB-1, the MamK assemblage is highly dynamic and the protein nucleates at multiple sites resulting in the formation of a new filament or the association with a preexistent one (Pradel et al., 2006). More recently, Katzmman et al. (2011) showed that the function of the magnetosome cytoskeleton elements is also involved in positioning and segregation of the

magnetosome chain. Using cryoelectron tomography and analysis of mutant cells, they also demonstrate that septation in *M. gryphiswaldense* MSR-1 proceeds asymmetrically from one lateral edge of the cell and MamK filaments probably mediate the recruitment of magnetosome chains to the midcell before cell division. Magnetosomes are also organized along a cytoskeletal filamentous structure in the uncultured *Candidatus Magnetobacterium bavaricum* although a *mamK* gene was not found during partial genome sequencing of this organism (Jogler et al., 2011). The filamentous structure observed in this magnetotactic bacterium might be formed from MamK or a MamK-like protein encoded for a gene elsewhere in the genome.

MamJ, another protein that seems to be essential for magnetosome chain organization in *Magnetospirillum* species (Scheffel et al., 2006), is encoded by the *mamJ* gene also present in the *mamAB* cluster (Grünberg et al. 2001). Transmission electron microscopy of *M. gryphiswaldense* mutants lacking a functional *mamJ* gene showed that mature magnetosomes were arranged in clusters within the cell, whereas empty and partially filled vesicles containing immature magnetosomes were scattered throughout the cytoplasm. The filamentous structure, consisting of MamK filaments, was morphologically identical in mutant and wild-type cells. The phenotype generated in the absence of a functional MamJ protein suggests its function involves linking magnetosomes and magnetosome vesicles to the MamK filamentous structure (Scheffel et al., 2006). In *Magnetospirillum gryphiswaldense* MSR-1, it has been recently demonstrated that MamJ and protein LimJ regulate the dynamics behavior of MamK filaments *in vivo* (Draper et al., 2011).

The precise determination of the position of individual magnetosomes and of magnetosome chains and their relation to cytoskeleton elements is pivotal for the understanding of the complex structure underlying magnetosome biomineralization and magnetosome chain formation, as well as magnetotactic behavior of the organism. Cryo-electron tomography (cryo-ET) is the most promising methodological approach (Milne and Subramaniam, 2009) that has revealed the three-dimensional (3D) ultrastructure of the intact magnetotactic bacterial cell in a near-native, frozen hydrated state (Komeili et al., 2006). Most cryo-ET studies on magnetotactic bacteria have focused on *Magnetospirillum* species (Draper, et al., 2011; Katzmam et al., 2010; Katzmam et al., 2011; Komeili et al., 2006; Scheffel et al, 2006.), which biomineralize cuboctahedral crystals of magnetite, and thus little is known regarding the cellular biology of magnetotactic species that produce elongated prismatic magnetosomes.

Here, we applied cryo-ET to characterize cytoplasmic structures of *Magnetovibrio blakemorei* (Bazylini et al., 2012), a marine magnetotactic vibrio which produces elongated prismatic crystals of magnetite. In particular, we determined physical characteristics of magnetosome chain positioning and magnetosome nucleation, as well as established the presence of cytoskeleton elements in this organism.

Materials and Methods

Cell culture

Cells of *Magnetovibrio blakemorei* strain MV-1 were grown at 28°C in heterotrophic medium in sealed flasks purged first with nitrogen and then nitrous oxide (Dean and Bazylini, 1999). A spontaneous non-magnetotactic mutant of *Magnetovibrio blakemorei* (strain NMV-1), unable to produce magnetosomes (Dubbels et al., 2004), was grown similarly and also examined in this study.

Electron microscopy

For cryo-ET, frozen vitreous cells were prepared by pipetting drops of cell culture onto Quantifoil 300-mesh copper EM grids. The grids were then blotted with filter paper and immediately frozen in liquid propane at -175°C using a plunge freezing machine (Leica model KF80, Allendale, NJ). Frozen specimen grids were cryo-transferred into the microscope using a Gatan 626 cryo-holder and cryo transfer stage (Pleasanton, CA). Electron tomography was performed using a Tecnai TF30 transmission electron microscope (FEI Company, Hillsboro, OR) equipped with a Shottky field emission gun and operated at 300 kV. Tomographic tilt series were acquired by tilting the samples from at least -54° to $+54^{\circ}$ at 2° intervals. The defocus value at each image was $-10\text{ }\mu\text{m}$. The total electron dose was $10,000\text{ e nm}^{-2}$. Images at each angular increment were recorded by a 2048×2048 pixel Ultrascan cooled CCD detector (Gatan) after zero-loss filtering by a Tridiem post-column imaging filter (Gatan). Tomograms were reconstructed using the IMOD software package (University of Colorado, Boulder, CO) and surface-rendered in Amira (Visage Imaging, Germany). Most tomograms were post-processed in IMOD by anisotropic diffusion filtering to improve signal-to-noise ratio.

For electron energy-loss spectroscopy, cells were freeze-dried inside the microscope and analyzed with the Tridiem post-column imaging filter and Ultrascan CCD detector. Electron energy loss spectra were obtained with dispersions of 0.2 and 0.3 eV/channel using a $40\text{ }\mu\text{m}$ objective aperture. Data acquired was analyzed with the Digital Micrograph software package.

Freeze fracture

For freeze-fracture experiments, cells were centrifuged at 4°C and washed in phosphate buffered saline and directly placed on gelatin cushions without chemical fixation. The samples were immediately frozen by impact against a liquid-nitrogen cooled sapphire block of a Life Cell CF-100 quick-freezing machine (Research and Manufacturing Co, Tucson, AR). Frozen specimens were then transferred to a Balzers freeze-fracture machine (Balzers/Leica, Allendale, NJ) and fractured at -150°C . After fracturing, the samples were shadowed with platinum at 45°C and carbon at 90°C . The organic material was cleaned from the replicas by hypochlorite bleach for 1 h. Replicas were then washed in distilled water, collected on 400 mesh nickel grids, and examined with a Carl Zeiss model EM922 transmission electron microscope (Carl Zeiss Microscopy LLC, Thornwood, NY), operating at 120 kV.

Results

Filamentous structures occur in the cytoplasm of *M. blakemorei* cells

In cryo-ET images of wild-type *M. blakemorei* cells, the overall appearance of the turgid cell cytoplasm is of a continuous granular matrix containing magnetosomes (Figure 1a) and round inclusion bodies. The cell surface is smooth and a periplasmic layer can be clearly seen. Magnetosomes are absent in the mutant *M. blakemorei*, otherwise the overall morphology appears indistinguishable from the wild-type (Figure 1b).

We performed cryo-ET to characterize the filaments, vesicles and other structures in the cytoplasm of *M. blakemorei*. In particular, we were interested in determining whether filaments and vesicles would be found in association with magnetosome crystals. First, tomographic slices revealed filamentous structures ranging in length from about 100 to 200 nm in length (e.g., see arrows in Figure 2a, c, e). These filaments were scattered throughout the cytoplasm and not necessarily associated with other structures. However, a closer look at Figure 2a also suggested the presence of a filament along the magnetosome chain (Figure

2b). To improve visualization of this presumed filament, we digitally removed the magnetite crystals in the tilt image series, and reconstructed the tomogram from the processed tilt series. With this approach, the strong artifactual white halos around the crystals in the volume reconstruction — attributable to the missing wedge in the tilt series — were minimized, thus allowing the filament to be unambiguously observed (Figure 2c,d). After removing the dense particles and regenerating the tomogram, a bright halo is left around the particles, which are Fresnel fringes due to the defocus of the objective lens. Figure 2f depicts a surface-rendered 3D model for *M. blakemorei*. Interestingly, we also observed in some tomograms numerous unidentified structures that seemed to be formed by pairs of filaments (Figure 4).

While digitally removing the large magnetite crystals enabled the visualization of a filament along situated parallel to the magnetosome chain, this procedure did not help reveal the presence of a magnetosome membrane around mature particles. This could be due to the residual Fresnel fringes after digital removal of the magnetosomes in the tilt series. To search for the presence of a membrane (and proteins) around mature crystals, we therefore examined the cells by the freeze-fracture method. As shown in Figure 3, this approach confirmed that mature crystals are encased by a membrane with embedded proteins. The cell is fractured exposing the cell membrane (Figure 3a) and the cytoplasm (Figure 3b) showing that the chains of magnetosomes associated with the replica and close to the cell surface. In regions of the freeze-fracture replicas, where one crystal was fortuitously removed from the chain during replica cleaning, we could observe the outer or cytoplasmic leaflet of a membranous organelle lined up with the remaining attached magnetosomes.

In a spontaneous mutant that was incapable of producing magnetosomes, the cell contained filamentous structures in the cytoplasm similar to the ones found in the wild-type strain (Figure 5). No empty vesicles or structures were resembling “pairs of filaments” observed in these cells.

Magnetosome crystals nucleate at the periphery of prismatic vesicles

A striking feature of magnetosome formation in *M. blakemorei* revealed by cryo-ET is that nucleation of immature magnetosome magnetite particles occurs juxtaposed to the inner surface of the enveloping membrane vesicles (Figure 6). Furthermore, many of the membrane vesicles containing nucleating or immature magnetite crystals were elongated (Figure 6). Specifically, vesicles containing nucleating crystals were on average 44 ± 5 nm in length and 32 ± 4 nm in width. These measurements were made on selected XY tomographic slices with the largest vesicle cross sections. Mature crystals on the other hand had average dimensions of 61 ± 6 nm in length and 43 ± 3 nm in width. The finding that vesicles containing immature crystals were elongated suggests that the overall magnetosome crystal morphology may be defined by the pre-formed elongated magnetosome vesicles. The difference in aspect ratio of vesicles containing immature and mature crystals (1.38 and 1.42, respectively) is within measurement error, although this difference may be also expected given that vesicles enclosing nucleating crystals may not always be oriented with their long axis lying in the XY plane. We also note that in none of the tomograms analyzed, were any vesicles found permanently attached to the inner surface of the cytoplasmic membrane. Although this observation may lead to conclude that vesicles are not formed by their invagination from the plasma membrane, it is also possible that the frequency of presumed invagination events in MV-1 is very low, thus minimizing the chances that these events would be caught by cryo tomography.

Other cytoplasmic structures are present in the cytoplasm of *M. blakemorei* cells

We observed a periodic structure parallel to the inner membrane of some cells, most likely corresponding to chemoreceptor arrays (supplementary Figure 1). These receptor arrays are known to form hexagonal arrangements (Liu et al., 2012), which we could confirm by Fourier transformation of the images. Similar structures have been described in *Magnetospirillum gryphiswaldense* (Katzman et al., 2010).

We also observed two distinct types of discrete granules in the cytoplasm of the cells, with one type appearing electron-dense and the other electron-lucent (Figure 1a). The electron-lucent bodies, in particular, were much more susceptible to electron beam damage than either the electron-dense inclusions or other random regions in the cell cytoplasm. This damage manifested itself as the typical “bubbling” phenomenon caused by hydrogen gas evolution (see arrowhead, Figure 2a) (Aronova et al., 2011; Leapman and Sun, 1995). It should be emphasized that no damage was visible in other regions of the cells at the maximum dose of 10,000 el nm⁻² used to acquire the tomograms.

Electron energy-loss spectroscopy of the electron-dense granules showed that they contained phosphorus (supplementary Figure 2), whereas this element could not be detected in the electron-lucent bodies. Most likely the electron-lucent inclusions are carbohydrate-rich deposits, and the phosphate-rich granules could be polyphosphate bodies identified by Rashid et al. (2000). Similar types of phosphorus-rich and phosphorus-deficient inclusion bodies were also observed in *Caulobacter crescentus* (Comolli et al., 2006) and in *Magnetospirillum* species (Katzmann et al., 2010). Moreover, the more pronounced sensitivity of the electron-lucent, phosphorus-deficient bodies to electron beam irradiation (Figure 2a) is also in agreement with the previous work by Comolli et al. (2006).

Discussion

In the current model of magnetite magnetosomes biomineralization in the freshwater spirilla, *Magnetospirillum*, the magnetosome is an invagination of the cell membrane that contains a specific set of proteins capable of guiding the synthesis of the nanoscale magnetite crystals (Komeili, 2007). To understand the dynamics of the magnetosome formation, cryo-ET in combination with genetic techniques has been used to investigate the molecular architecture of the magnetosomes in *M. magneticum* (Komeili, 2007; Schüler, 2008). Because of the lack of an established genetic system for *M. blakemorei* very little can be said about the molecular dynamics of magnetosome formation, but some conclusions can be drawn from the structural data combined with the presence/absence of genes involved in magnetosome formation.

In *M. gryphiswaldense* the magnetosome vesicle is associated with a filamentous structure extending from one cell pole to the other, and lying adjacent to the cytoplasmic membrane (Scheffel et al., 2006). We observed filamentous structures in the cytoplasm of *M. blakemorei*, but they appear to differ from the wild-type strain of *Magnetospirillum* in that they are neither strictly associated with the magnetosome chain nor are the filaments attached to the cell poles. The role of these filaments is unknown. It is possible that they may not constitute mamK filaments, although *mamK* is present in the genome of *M. blakemorei* (unpublished data; Jogler et al., 2009), and in this case the gene may not be expressed in the same way as in *Magnetospirillum*. In fact, the filaments in *M. blakemorei* resemble those observed in the *mamJ*-deficient *Magnetospirillum* mutants since they are scattered in the cytoplasm and show no conclusive association with the magnetosome vesicles (Scheffel and Schüler, 2007). In addition, *mamJ* is not present in the magnetosome gene cluster of *M. blakemorei* (Jogler et al., 2009a), which may indicate that there is no connection of the magnetosome vesicle to the putative cytoskeletal filament. The presence

of filaments in the *M. blakemorei* mutant suggests that the filament formation is independent of the magnetosome vesicle formation. The absence of a permanent connection of the filaments to the cell pole may indicate that they do not exert the same role as in *Magnetospirillum*, i.e., in assembling the magnetosome chain, although the attachment of the filaments to the cell poles observed in magnetospirilla was not unequivocally determined (Komeili et al., 2006; Scheffel et al., 2006; Katzmann et al., 2010). It is interesting that filamentous structures were observed in magnetotactic bacteria from the *Nitrospira* group (Jogler et al., 2011). In this group of microorganisms, *mamK* is present but the relationship between the filaments and the MamK protein has not been determined. In the absence of cytoskeletal structures associated with the chain of magnetosomes, it is unclear how the torque is transmitted to the cell when the cell's orientation changes relative to the magnetic field. In uncultivated cocci, two chains of magnetosomes seem to be the common configuration, and it has been proposed that the magnetic repulsion between the chains is the mechanism responsible for the torque transfer (Shcherbakov et al., 1997). Again, the presence of the *mamK* gene in *Magnetococcus marinus* (Jogler et al., 2009) and its corresponding protein's involvement with cytoskeleton filaments in these microorganisms are unknown.

In several bacteria producing bullet-shaped magnetosomes, the crystals start as isotropic particles and later elongate as the bullet shaped crystals grow (Mann et al., 1987a,b). The bullet-shaped magnetite crystals display a broader range of morphological variation and defects than cuboctahedral and elongated prismatic crystals, suggesting that there is less control over biomineralization of bullet-shaped crystals. Thus, it is expected that there are fundamental differences in the control mechanisms for biomineralization between octahedral and prismatic magnetosomes. Our data suggest that the biomineralization of prismatic magnetosomes may be different from octahedral particles. Magnetosome vesicles, including immature magnetosomes, were not found connected to the cell membrane, which suggests that they form and detach rapidly and therefore exist only transiently as reported for *M. gryphiswaldense* (Katzmann et al. 2010 and Katzmann et al., 2011). Also, the nucleating point of the biomineralization seems to be associated with the inner surface of the magnetosome membrane, which contains numerous trans-membrane proteins, as revealed by freeze-fracture. This site of nucleation may play an important role in orientation of the nucleation and growth of the magnetosomes. Because vesicles are elongated in *M. blakemorei* and round in *Magnetospirillum*, it is possible that the template generated by the membrane is responsible for the overall shape of the mature particle. The direction of the biomineralization of the particles has been demonstrated to be [111] for magnetite in most magnetotactic bacteria, except for some bullet-shaped magnetosomes (Lins et al., 2007, Lefèvre et al., 2011). It is reasonable to suppose that membrane proteins may be responsible for the growth in the direction of crystal formation. In fact it has already been proposed that the protein encoded by *mms6* gene modulates the shape of the magnetosome particle in *Magnetospirillum* (Tanaka et al., 2011). It is possible that other proteins might be responsible for specific orientations of magnetosomes relative to adjacent magnetosomes in the chain in uncultured magnetotactic cocci (Lins et al., 2005; Abraçado et al., 2010).

The formation of the magnetite crystal in magnetosomes involves several steps such as nucleation, growth, and morphological/size control. Based on our data, we propose a set of events for the formation of prismatic magnetosomes in magnetotactic bacteria. At least four steps are necessary for the formation of a mature particle: 1) the magnetosome forming vesicle is internalized from the cell membrane; 2) the vesicle becomes elongated to form a template (predefining the size and shape) for the mature magnetosome; 3) the magnetosome crystal nucleation is initiated at a nucleation site on the membrane inner surface. This lateral nucleation site may be an important step in defining the long axis of the magnetosome ([111] direction for magnetite); 4) the crystal grows by molding its shape to that of the

vesicle. It would be interesting to develop a genetic system to study the dynamics of the magnetosome formation in *M. blakemorei*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

UL and FA acknowledge partial financial support from CNPq, FAPERJ and CAPES Brazilian agencies. DAB is supported by US National Science Foundation grant EAR-0920718.

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Figure 1. Cryoelectron microscopy image of (a) wild type *M. blakemorei* and (b) mutant lacking magnetosomes. The electron-dense granule (arrow) is a phosphorus-rich inclusion body, whereas electron-lucent granule (arrowhead) is presumably a sugar body (see also Supplementary Figure 2).

Figure 2.

Cryo electron tomography of *M. blakemorei*. (a) Tomographic slice of a cell showing a chain of magnetosomes. Arrowhead marks a putative sugar inclusion body where hydrogen bubbling has occurred (see text for details). (b) Expanded view of region near magnetosomes. The artifactual bright contrast arising from the missing wedge effect precludes a clear visualization of structures in close proximity to the magnetite crystals. (c) Tomographic slice after crystals were digitally removed in the tilt series and the tomogram reconstructed. Arrows mark filaments in the cytoplasm. (d) Expanded view of region near magnetosomes. A filament can be now seen running along the magnetosome chain. (e) Tomographic slice highlighting the presence of another filament (arrow) in the cytoplasm as well as small vesicles (arrowheads). (f) Surface-rendered model of tomogram showing: plasma membrane (yellow); magnetosome crystals (pink); filaments scattered throughout the cytoplasm and along the magnetosome chain (blue); vesicles (green), one of which contains a nucleating magnetite particle.

Figure 3.

Freeze-fracture images of the cell cytoplasm showing chains of magnetosomes associated with the replica. In each of the two selected images, a crystal had been removed from the replica to reveal the inner leaflet of a membranous organelle (arrow), which is situated co-linearly with the remaining attached magnetosomes.

Figure 4.
Tomographic slices showing unidentified filamentous structures (arrows) in the cytoplasm of *M. blakemorei*, which appear to have a “double membrane”.

Figure 5.

Cryo-electron tomography of *M. blakemorei* mutant cell. Tomographic slices from three different cells show the presence of filaments in the cytoplasm (arrows). In addition to the absence of magnetosomes in this mutant, no empty vesicles or “tubular” structures were found in the tomograms.

Figure 6.

Gallery of images showing magnetosome vesicles with immature, developing magnetosomes. Magnetite crystals can be seen to nucleate at the periphery of each vesicle. Magnetosome vesicles in the first two images have a prismatic, non-circular profile similar to the shape of a mature magnetosome.