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ORIGINAL PAPER

Subcellular localization of the magnetosome protein MamC in the marine magnetotactic bacterium *Magnetococcus marinus* strain MC-1 using immunoelectron microscopy

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Abstract Magnetotactic bacteria are a diverse group of prokaryotes that biomineralize intracellular magnetosomes, composed of magnetic (Fe_3O_4) crystals each enveloped by a lipid bilayer membrane that contains proteins not found in other parts of the cell. Although partial roles of some of these magnetosome proteins have been determined, the roles of most have not been completely elucidated, particularly in how they regulate the biomineralization process. While studies on the localization of these proteins have been focused solely on *Magnetospirillum* species, the goal of the present study was to determine, for the first time, the localization of the most abundant putative magnetosome membrane protein, MamC, in *Magnetococcus marinus* strain MC-1. MamC was expressed in *Escherichia coli* and purified. Monoclonal antibodies were produced against MamC and immunogold labeling TEM was used to localize MamC in thin sections of cells of *M. marinus*. Results show that MamC is located only in the magnetosome membrane of *Mc. marinus*. Based on our findings and the abundance of this protein, it seems likely that it is important in magnetosome biomineralization and might be used in controlling the characteristics of synthetic nanomagnetite.

Keywords Magnetosome · Magnetotactic bacteria · MamC · *Magnetococcus marinus*

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Introduction

Magnetotactic bacteria are a phylogenetically and morphologically diverse group of prokaryotes that passively align and swim along the Earth's geomagnetic field lines, a behavior referred to as magnetotaxis (Bazylinski and Frankel 2004). Magnetotaxis is thought to be crucial for the survival and growth of magnetotactic bacteria in natural environments and is due to the bacterium's ability to biomineralize magnetosomes, which are intracellular lipid bilayer membrane vesicles containing a magnetic mineral crystal of either magnetite (Fe_3O_4) or greigite (Fe_3S_4) (Bazylinski and Frankel 2004). The vesicle, known as the magnetosome membrane, has been shown to originate from invaginations of the cytoplasmic membrane in some magnetotactic bacteria (Komeili et al. 2006). The formation of magnetosome crystals is an excellent example of a biologically controlled mineralization process, in which the mineral forms in a closed compartment, isolated from the outer environment (Bazylinski and Frankel 2004). Such a mineralization process is exquisitely regulated at the gene level and results in the production of tens-of-nanometer-sized

magnetic particles with specific magnetic and physical properties that have been optimized for the response of the bacteria to the Earth's geomagnetic field (Frankel and Blakemore 1980).

The magnetic minerals produced by magnetotactic bacteria share unique features that make them highly appreciated in nanotechnological applications (Amemiya et al. 2007; Prozorov et al. 2007; Arakaki et al. 2008; Faivre and Schüler 2008), and these include as follows: biomedical imaging, for instance, the use of nanomagnetite as a contrast agent in magnetic resonance imaging (Prozorov et al. 2007); as drug carriers (Arruebo et al. 2007); and for cancer hyperthermia treatment (Thomas et al. 2009). The characteristics that make these microbial nanoparticles unique (such as narrow size distribution, single magnetic domain, perfect stoichiometry and mineral structure and elongation of the particle in one crystallographic direction) (Thomas-Keprta et al. 2000) have thus far not been replicated by inorganic means (Jimenez-Lopez et al. 2010). Furthermore, the fastidious nature of magnetotactic bacteria makes it difficult to produce industrial-scale amounts of nanomagnetite solely by using the bacteria as tiny mineral factories. Thus, there is a great deal of utility in being able to produce high-quality and high-quantity magnetosome-like particles by another strategy. In this context, a number of different studies over the past 10 years have focused on understanding the function of specific magnetosome proteins to determine whether one or more combinations of these proteins may be used for in vitro nanomagnetite synthesis (Komeili 2012; Lower and Bazylinski 2013).

The magnetosome membrane for all magnetotactic species that have been examined to date contains proteins not present in other parts of the cell (Gorby et al. 1988; Matsunaga et al. 2000; Taoka et al. 2006). The gene and amino acid sequences of many magnetosome membrane proteins are reasonably conserved among several groups of magnetite-producing magnetotactic bacteria, for example, in *Magnetospirillum* species including *Ms. magnetotacticum*, *Ms. gryphiswaldense*, *Ms. magneticum* and the marine coccus *Magnetococcus marinus* (Okuda et al. 1996; Grünberg et al. 2001; Matsunaga et al. 2005; Tanaka et al. 2006; Taoka et al. 2006; Schübbe et al. 2009).

The localization of the specific magnetosome proteins is a necessary first step to elucidate their roles in the biomineralization of magnetite magnetosomes. Techniques such as green fluorescent protein (GFP) fusions and fluorescent microscopy, immunogold labeling and transmission electron microscopy (TEM) and atomic force microscopy (AFM) have been used to determine the localization of several magnetosome proteins in cells of *Magnetospirillum* species. For example, Lang and Schüler (2008) constructed GFP fusions with MamC, MamF and MamG of *Ms. gryphiswaldense* and determined that all three proteins localize only to the magnetosome membrane. This method has also been used to localize other magnetosome proteins such as MamJ and MamK (Komeili et al. 2006; Scheffel et al. 2006). Immunogold labeling and AFM have also been used to localize magnetosome proteins. Taoka et al. (2006) determined the localization of Mam12 (equivalent to MamC) and Mam22 (MamA) in *Ms. magnetotacticum* using immunogold labeling of purified magnetosomes and also of ultrathin sections of whole cells using this method. In the cell, MamC was only present in the magnetosome membrane. Yamamoto et al. (2010) using AFM found that MamA localized to the surface of the magnetosome membrane. Moreover, the magnetosome membrane proteins Mms5, Mms6, Mms7 (equivalent to MamD) and Mms13 (MamC) appear to tightly bind to magnetite crystals in the magnetosomes of *Ms. magneticum* (Arakaki et al. 2003) and *Ms. gryphiswaldense* (Grünberg et al. 2001).

Among magnetosome membrane proteins, MamC is of particular interest because (1) it is present in all magnetotactic bacteria studied thus far (e.g., Grünberg et al. 2001, 2004; Tanaka et al. 2006; Schübbe et al. 2009); (2) its gene does not have any obvious homologs in non-magnetotactic bacteria (Grünberg et al. 2001, 2004; Tanaka et al. 2006); (3) it is the most abundant magnetosome membrane protein in several magnetotactic bacteria (Grünberg et al. 2001); and (4) due to the presence of acidic domains, some authors (Scheffel et al. 2008) have attributed a putative important role on the control the size and shape of magnetite crystals. However, to our knowledge, studies on the localization of MamC in magnetotactic bacteria have focused solely on *Magnetospirillum* species. It is thus important to determine where this protein is located in other magnetotactic bacteria before general conclusions regarding these proteins can be made.

Therefore, the present study is focused on determining the subcellular localization of MamC in a different, unrelated magnetotactic bacterium, *Mc. marinus* strain MC-1 (Bazylinski et al. 2013). This bacterium was chosen because MamC from MC-1 differs from the homologous protein in *Magnetospirillum* species (Fig. 1). In particular, differ in the nucleotide sequence and also in the amino acid sequence and in the organization within the magnetosome island (Matsunaga et al. 2005; Schübbe et al. 2009). For instance, while *mamC* is located in the cluster *mamGFDC* in the species of the genus *Magnetospirillum*, it belongs to the cluster *mamCEIH* in *Mc. marinus* strain MC-1 (Schübbe et al. 2009). Interestingly, magnetosome magnetites from MC-1 are also different than those from *Magnetospirillum* species [Magnetospirillum species usually produce cubooctahedral crystals (both equidimensional (*Magnetospirillum magneticum* AMB-1; Mann et al. 1984) and elongated (*Magnetospirilo marino* MMS-1; Meldrum et al. 1993b) or truncated hexaoctahedral (*Magnetovibrio blakemorei* strain MV-1 and MV-2; Meldrum et al. 1993a)]; however, MC-1 produces magnetites having a pseudo-hexagonal prismatic crystal

Fig. 1 Sequence alignment of different MamC proteins from *Mc. marinus* (YP866172), *Ms. gryphiswaldense* strain MSR-1 (AAL10004), *Ms. magneticum* strain AMB-1(YP420314), *Magnetospirillum* sp.SO-1 (WP008620745) and *Magnetovibrio blakemorei* strain MV-1 (CAV30776). Similar amino acids are shown in *bold* and are *shaded*

habit, elongated along the <111> crystallographic direction and truncated in {111}, {100} and {110} faces (Meldrum et al. 1993b). Determining the localization of MamC in MC-1 may provide insight in elucidating whether this protein plays a significant role in the biomineralization of magnetosomes by *Mc. marinus* strain MC-1.

Materials and methods

Bacteria, growth conditions and DNA extraction

Magnetococcus marinus (ATCC BAA-1437) is a marine coccus originally isolated from water collected from the oxic–anoxic interface (OAI) of the Pettaquamscutt Estuary (Narragansett, RI) (Bazylinski et al. 2013). Cells were grown microaerobically under chemolithoautotrophic conditions with thiosulfate as the electron donor in liquid cultures as described by Williams et al. (2006). Genomic DNA was isolated from *Mc. marinus* following the method of Martín-Platero et al. (2007).

Purification of magnetosomes and preparation of cell soluble and membrane fractions

Cells of *Mc. marinus* were harvested by centrifugation at 10,000×*g* for 20 min at 4 °C. The supernate was removed, and the resulting cell pellet was resuspended in artificial seawater [ASW; Bazylinski and Frankel (2004)] buffered with 20 mM TrisHCl, pH 7.1 and recentrifuged. Again the supernate was removed and the cell pellet resuspended in 20 mM TrisHCl, pH 7.1 containing 1 μg mL^{-1} of the serine protease inhibitor, 4-amidinophenylmethanesulfonyl fluorideHCl (Bazylinski et al. 1994). Cells were lysed by passing the cell suspension through a French pressure cell at 18,000 lb/in^2 three times in succession.

Magnetosomes were purified from the crude cell extract at 4 °C using a variable gap magnet (PASCO Scientific, Roseville, CA) as described by Bazylinski et al. (1994). After magnetosomes accumulated at the poles of the gap magnet, the crude cell extract minus the magnetosomes was removed and centrifuged at 10,000×*g* for 20 min at 4 °C to remove unbroken cells and cell debris. Soluble (containing both soluble cytoplasmic and periplasmic proteins) and general membrane (containing both outer and cytoplasmic membranes) fractions were separated by ultracentrifuging the supernate at 200,000×*g* for 3 h at 4 °C. The supernate (soluble fraction) was removed, and the pellet (outer and cytoplasmic membranes) was further purified as follows: the pellet was resuspended in 20 mM TrisHCl, pH 7.1 containing 1 M NaCl to remove electrostatically associated proteins and ultracentrifuged as described above. The resulting new pellet was washed twice in 20 mM TrisHCl, pH 7.1 without NaCl, re-ultracentrifuged and finally resuspended in this same buffer. All cell fractions were stored at −20 °C.

After extensive washing, magnetosomes were suspended in 20 mM TrisHCl pH 7.1. Half of this suspension was not further processed and represents magnetosomes with magnetosome membranes. The other half was centrifuged in a microcentrifuge at approximately 10,000×*g* for approximately 5 min and the buffer removed. These magnetosomes were resuspended in 20 mM Tris·HCl pH 7.1 containing 1 % sodium dodecyl sulfate (SDS) incubated for 3 h at room temperature with occasional mixing to remove the magnetosome membranes. After incubation, the magnetosome magnetite crystals were recentrifuged and the supernate containing the extracted magnetosome membranes frozen at −20 °C. The magnetite crystals were washed several times with distilled, deionized H_2O and stored under N_2 gas at −20 °C to prevent oxidation.

Cloning, expression, purification and identification of MamC

The gene encoding the MamC protein in *Mc. marinus* is designated as Mmc1_2265 (Schübbe et al.2009). The following

primers were used to amplify this gene by the polymerase chain reaction (PCR): fw1 (5′-ATGGCTGCCTTTAATTTG GCACTG-3′) and rev2 (5′-TTACGGAGTTTCCAACTC CTGGGGATC-3′). PCR products were purified (MBL-PCR QuickClean Kit; Dominion-MBL) and sequenced (ABI PRISM BigDye Terminator Cycle Sequencing Kit; Life Technologies: Applied Biosystems).

The PCR product was cloned into pTrcHis-TOPO (Life Technologies: Invitrogen, Grand Island, NY) and the resulting construct sequenced (ABI model 3100 sequencer; Life Technologies: Applied Biosystems).

For protein expression, BL21CodonPlus (DE3) cells (Stratagene) previously transformed with the pTrcHis-TOPO *mamC* plasmid were grown overnight in Luria–Bertani (LB) broth and used to inoculate flasks containing LB broth containing 50 μg mL^{-1} ampicillin. This culture was incubated at 37 °C to reach an optical density (OD) of 0.6 at 600 nm. At this time, 1.5 mM isopropyl-1-thio-b-D-galactopyranoside (IPTG) was added, and the culture incubated for 4.5 h before cells were collected by centrifugation. Cells were resuspended in 50 mM Tris·HCl pH 8.0, 150 mM NaCl (buffer A) and lysed by sonication. This cell lysate was centrifuged at 106,979×*g* for 30 min at 4 °C. The supernate was loaded onto a HiTrap chelating HP column (GE Healthcare) previously equilibrated with buffer A. The recombinant protein was purified by using an Akta Prime FPLC System (Amersham Biosciences, Piscataway, NJ). Elution of the protein was performed by applying a continuous gradient increasing the concentration of imidazole up to 1 M). Fractions were collected and analyzed by SDS-PAGE.

To show unequivocally that the purified protein was MamC, the corresponding band in the SDS-PAGE gel of the cell lysate was excised and subjected to peptide mass fingerprinting as described by Henzel et al. (2003). Spectra were processed with the program Explorer Data (Applied Biosystems). Identification of MamC from the peptide masses was determined using MASCOT (Matrix Science, London, UK) and NCBI or Swiss-Prot databases.

Nanoscale liquid chromatographic tandem mass spectrometry (nLC-MS/MS)

The magnetosome membrane fraction was subjected to nLC-MS/MS in order to determine the presence of MamC. A portion of the magnetosome membrane fraction containing approximately 20 μg of protein was dissolved in Laemmli buffer and loaded in 15 % SDS–polyacrylamide gels. The gel was then stained with Coomassie Brilliant Blue R 250, and the bands were excised. The bands were cut in small pieces and destained in 50 mM ammonium bicarbonate/50 % acetonitrile (ACN), dehydrated with ACN and dried. The gel pieces were rehydrated with 12.5 ng mL^{-1} trypsin solution in 50 mM ammonium bicarbonate and incubated overnight at 30 °C. Peptides were extracted at 37 °C using 100 % ACN, followed by 0.5 % TFA, dried by vacuum centrifugation, purified using ZipTip (Millipore) and, finally, reconstituted in 0.1 % formic acid/2 % ACN for HPLC sample injection. The peptide mixtures from in-gel tryptic digestions were analyzed using nLC-MS/MS. The peptides were loaded onto a 2 cm C18-A1 ASY-Column, inner diameter = 100 μm, 5 μm precolumn (Proxeon, Thermo Scientific) and then eluted with a linear gradient of 2–99.9 % ACN in 0.1 % aqueous solution of formic acid.

Western blotting

Western blots were used to (1) identify the presence of MamC in both the induced culture and the purified protein fraction aliquots; and (2) detect the specific binding of the produced monoclonal antibodies to MamC; and (3) localize MamC in the different cell fractions (soluble, membrane and magnetosome membrane fractions). While anti-HisG-HRP (Invitrogen) was used in the first set of experiments, anti-MamC antibodies were used for the second and third sets of experiments. In both cases, two polyacrylamide gels were prepared and run in parallel following the protocol of Laemmli (1970). One was stained with Coomassie Brilliant Blue R 250 while the second gel was blotted to a nitrocellulose membrane (NC, pore size 0.45 μm; Whatman BA85 Schleicher & Schuell) according to Towbin et al. (1979). Membranes were blocked with 5 % bovine serum albumin (BSA) and labeled with the corresponding antibody (anti-HisG-HRP or anti-MamC, depending on the experiment). Anti-HisG-HRP was used at a concentration of 1:2,000, while anti-MamC was used at a concentration of 1:500. In the latter case, membranes were also labeled with a secondary anti-IgG mouse (Sigma-Aldrich) at a concentration of 1:1,000 by using the Clean-Blot IP Detection Kit HRP (Thermo-Pierce, Rockford, IL, USA). The membranes were scanned and imaged using an Eastman Kodak (Rochester, NY) Gel Logic 1500 imaging system using Kodak software.

Production of monoclonal antibodies against MamC

For the production of monoclonal antibodies, 4 Balb/c mice were inoculated with 40 μg of purified MamC. Anti-MamC was purified by Abyntek Biopharma S.L. The specificity of the antibodies produced at each stage was determined by Western blot and Elisa.

Immunogold labeling

Cells of *Mc. marinus* were harvested by centrifugation at 10,000×*g* for 20 min at 4 °C and then fixed with 2 % paraformaldehyde and 0.5 % glutaraldehyde in 0.2 M potassium phosphate buffer pH 7.2, for 2 h at 4 °C. Cells were then washed with potassium phosphate buffer, dehydrated

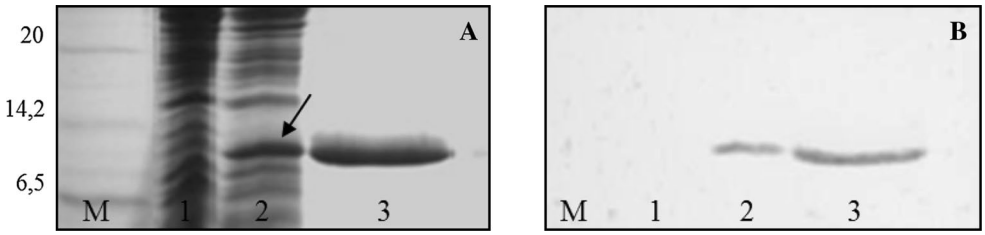

Fig. 2 One dimensional SDS-PAGE gel stained with Coomassie Brilliant Blue R 250 (**a**) and corresponding immunoblot (nitrocellulose membrane incubated with anti-HisG-HRP) (**b**) of proteins solubilized from transformed *E. coli* cells containing pTrcHis-TOPO *mamC* plasmid. *Lane 1*, non-induced cells as negative control; *lane 2* cells induced with 1.5 mM of IPTG for 4.5 h at 37 °C; and *lane 3*, purified MamC protein. *Lane labeled M*, molecular weight markers. MamC is indicated by the *arrow* in *lane 2* of the Coomassie Brilliant *Blue* R 250 stained gel. Note that it corresponds with the bands shown in the immunoblot in **b**

in ethanol (concentration ramp from 50 to 100 %) and embedded in LR White resin (Electron Microscopy Sciences, Hatfield, PA, USA). Resin blocks were polymerized in an oven at 55 °C for 24 h.

Resin blocks were thin-sectioned (~60 nm thick) using an ultramicrotome (Leica Ultracut-R Wetzlar, Germany). Thin sections were mounted on 200-mesh nickel grids and processed for colloidal gold immunolabeling as follows. Drops of 30 µL of (a) PBS (phosphate saline buffer, pH 7); (b) PBS + glycine 0.02 M; (c) PBS + 1 % BSA; (d) anti-MamC in PBS 1:750; and (e) anti-mouse IgG-Gold (10 nm colloidal gold, Sigma-Aldrich) in PBS 1:200 were deposited on a sheet of parafilm. The grids were placed down on top of these drops (one grid per drop) following the sequential order specified below and maintained for different time intervals at room temperature. Grids were first placed on drops of PBS for 10 min after which they were placed on PBS + 0.02 M glycine drops for 10 min. Then grids were rinsed by immersion in the PBS + 1 % BSA drops in a three-wash cycle (5 min of immersion each step). Next, the grids were immersed in the drops containing anti-MamC in PBS and incubated at 25 °C for 3 h. Excess antibodies were removed by immersing the grids in PBS drops in a five-wash cycle (5 min each step). Lastly, the grids were immersed in the drops containing anti-mouse IgG-Gold and incubated at 25 °C for 2 h. Again, excess secondary antibodies were removed by a three-wash cycle (5 min per step).

Grids were stained with 2 % aqueous uranyl acetate (Electron Microscopy Sciences) dried and then observed using a transmission electron microscope (TEM, Zeiss Libra 120, Oberkochen, Germany).

Results and discussion

Expression and identification of MamC

DNA sequence of the PCR product obtained using primers *mamC* fw1 and *mamC* rev2 corresponded to the known nucleotide sequence of *mamC* (Schübbe et al. 2009) excluding the signal peptide coding region (atggctgccttaatttggcactgtacttatcaaaaagcatcccaggcgttggtgttttgggtggcgttatagggggttctgcggcgctggccaaaaacttaaaagccaaa). Cloning of this PCR product into pTrcHis-TOPO yielded a heterologous expressed protein in cells of *Escherichia coli* as soluble protein.

The molecular weight of purified MamC was found to be approximately 13.9 kDa by SDS-PAGE (Fig. 2), which corresponds to the expected size of MamC fused to the polyhistidine tag. The 13.9 kDa Coomassie-stained band (Fig. 1a, Lane 3) was excised and analyzed using peptide mass fingerprinting. Fingerprinting data from the purified protein showed significant homology ($p < 0.05$) with the magnetosome membrane protein MamC from *Mc. marinus* (locus tag = Mmc1_2265).

To further confirm this result, a Western blot was performed using anti-His-tag antibodies against samples from (1) the non-induced transformed *E. coli* culture; (2) the transformed *E. coli* culture induced by treatment with 1.5 mM of IPTG for 5 h at 37 °C); and (3) purified MamC. Only two bands appeared in the Western blot shown in Fig. 1b: One in the induced culture (lane 2) and the other in the purified MamC (lane 3). The position of these bands, as shown in the Coomassie-stained gel (Fig. 2a), corresponds to the expected size of MamC fused to the polyhistidine tag (~13.9 KDa). No band was detected for the non-induced culture control (Fig. 2b, lane 1). Since the anti-His-tag binds specifically to the anti-His-tag that is fused to the protein, these results confirm that the protein MamC was expressed heterologously in *E. coli* and that it was purified to a strong degree.

Data from nLC-MS/MS analyses revealed that MamC was only present in the extract of magnetosome proteins from *Mc. marinus*. Several peptides obtained after trypsin digestion of the band from the magnetosome membrane extract corresponded to the part of MamC representing the signal peptide (Figs. 2b, 3a). Thus, mature native MamC in *Mc. marinus* contains the signal peptide.

A

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1          11          21          31          41          51
MAAFNLALYL SKSIPGVGVL GGVIGGSAAL AKNLKAKQRG EITTEEAVID TGKEALGAGL
6          71          81          91          101         111
ATTVSAYAAG VVGGGLVVSL GTAFAVAVAG KYAWDYGMEQ MEAKLQEKKH QEQGGQTYGD
121        131
NPDPFDPQEL ETP
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B

Sequence	XCorr
QRGEITTEEAVIDTGK	5.67
GEITTEEAVIDTGK	4.82
SIPGVGVLGGVIGGSAALAK	4.79
HQEQGGQTYGDNPDPFDPQELETP	4.47
YAWDYGMEQMEAK	3.96
KHQEQGGQTYGDNPDPFDPQELETP	3.83

Fig. 3 Identification of MamC from *Mc. marinus* using nanoscale liquid chromatographic tandem mass spectrometry (nLC-MS/MS). **a** Amino acid sequence of MamC from *Mc. marinus*. Sequences of the peptides obtained after trypsin digestion of the protein correspond with fragments of the amino acid sequence of MamC shadowed in *gray*. **b** Table with the sequence of several peptides and their Xcorr (obtained from nLC-MS/MS) corresponding to MamC; one of these peptides (*shadowed in gray*) corresponds to the signal peptide

Subcellular localization of MamC

The subcellular localization of MamC was determined using two different approaches: first by Western blotting the different cell fractions (soluble, outer and cytoplasmic membranes and magnetosome membrane) with anti-MamC and by comparing electrophoresis gels of these three fractions (Fig. 4); and second, by immunogold labeling and TEM (Fig. 5).

Gel electrophoresis of the soluble, cytoplasmic membrane and magnetosome membrane fractions of *Mc. marinus* shows a band of about 15 kDa corresponding to the molecular mass of the MamC protein only in the magnetosome membrane fraction and not in the others (Fig. 4a). To confirm whether this band actually represents MamC and that, in fact, was only present in the magnetosome membrane fraction, a Western blot of all cell fractions was performed using anti-MamC (Fig. 4b). These results taken together clearly show that MamC is only located in the magnetosome membrane in *Mc. marinus*.

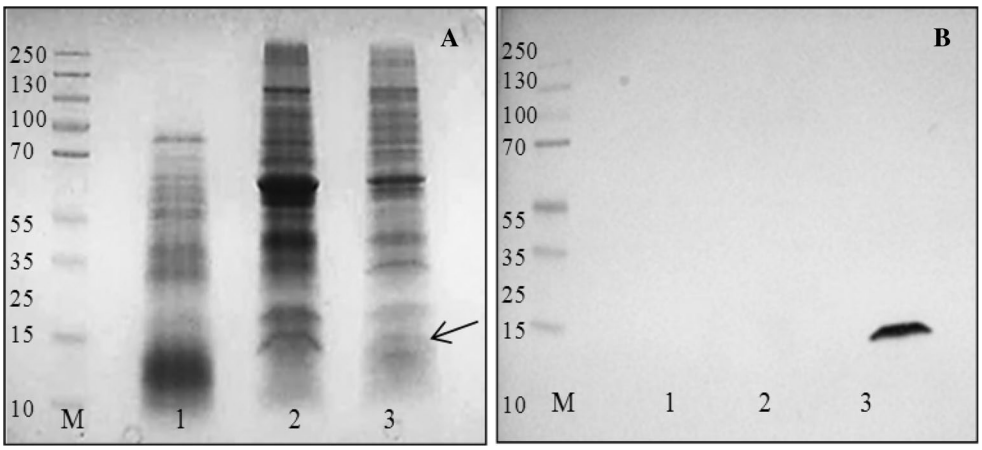

Fig. 4 One dimensional SDS-PAGE gel stained with Coomassie Brilliant Blue R 250 (**a**) and immunoblot (nitrocellulose membrane incubated with anti-MamC) (**b**) of proteins solubilized from different fractions of *Mc. marinus*. Lanes each contain 20 μg protein. *Lane 1*, soluble cell fraction (contains soluble cytoplasmic and periplasmic proteins); *lane 2*, membrane cell fraction (contains cytoplasmic and outer membrane proteins but not magnetosome membrane proteins); *lane 3*, magnetosome membrane fraction. *Lane M*, molecular weight markers

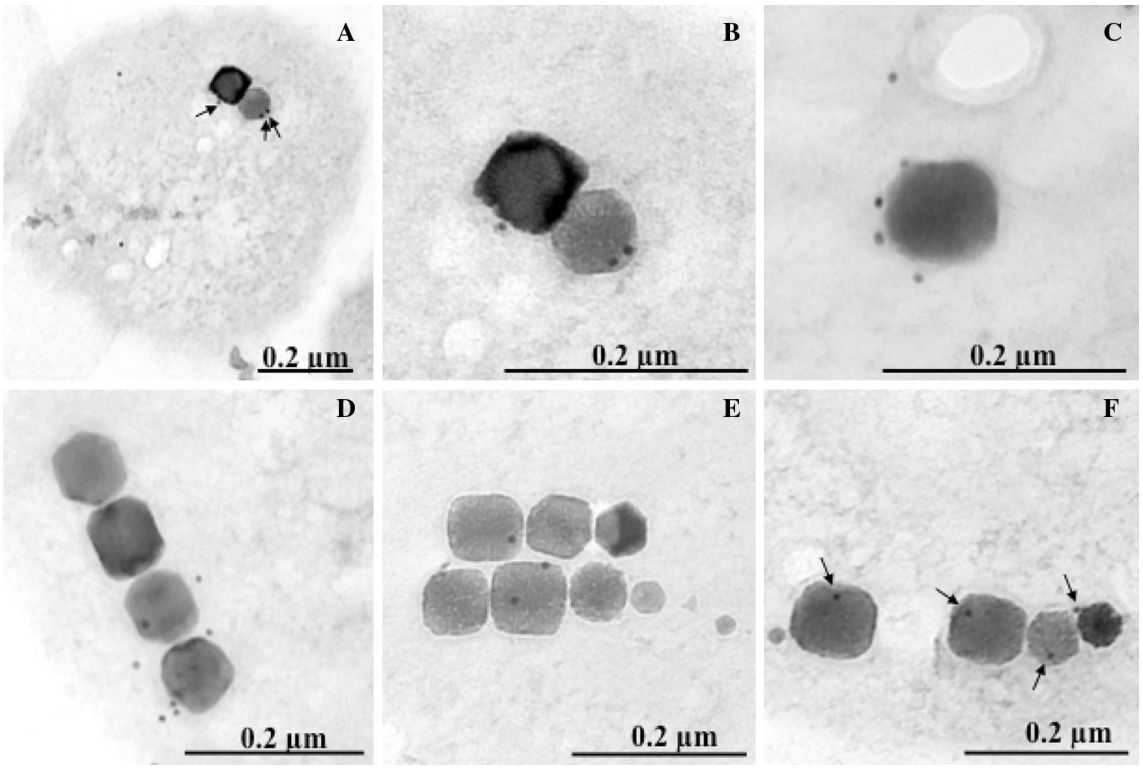

Fig. 5 Transmission electron microscope (TEM) images of immunogold labeled thin sections of cells of *Mc. marinus*. **a** A cell of *Mc. marinus* showing magnetosomes labeled with anti-MamC (1:750) and then labeled with rabbit anti-mouse antibody (1:200) conjugated with 10 nm colloidal gold (shown at *arrows*). **b**–**e** High-magnification TEM images of magnetosomes in cells of *Mc. marinus* treated as described in **a**. Note that in all cases, the nanogold particles detected in the bacteria are at or very close to the magnetosome membrane. They were not found in any other part of the cell. *Arrows* indicate nanogold particles that difficult to discern because of the density of the magnetite crystal

The subcellular localization of MamC was also determined by immunogold labeling TEM. TEM images of immunogold staining of anti-MamC-treated thin sections of cells of *Mc. marinus* (Fig. 5) show colloidal gold particles mainly localized very close to the magnetosome membrane and/or attached to the magnetite crystal. Significant amounts of colloidal gold particles were not detected anywhere else.

Our results, taken together, unequivocally show that MamC is localized only to the magnetosome membrane and the magnetite crystal to which it may be partially bound. In addition, the results show that MamC maintains the signal peptide in its native form in the cell.

Our findings with *Magnetococcus* are consistent with previous studies that examined the subcellular localization of MamC in *Magnetospirillum* species. Using immunoblotting and fluorescence microscopy, Lang and Schüler (2008) demonstrated that MamC, such as MamF and MamG, strongly localizes with the magnetosome membrane in *Ms. gryphiswaldense*. Although some fluorescence in the cytoplasm of cells expressing MamF-GFP and MamG-GFP was observed, no significant cytoplasmic fluorescence was detected when MamC-GFP was expressed. Taoka et al. (2006) found similar results for MamC (Mam12) in *Ms. magnetotacticum* using immunogold staining of both purified magnetosomes and ultrathin sections of the bacterial cells. Our results with *Mc. marinus* suggest that MamC is exclusively localized to the magnetosome membrane in all magnetotactic bacteria.

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