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A protein-protein interaction in magnetosomes: TPR protein MamA interacts with an Mms6 protein

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ABSTRACT

Magnetosomes are membrane-enveloped bacterial organelles containing nano-sized magnetic particles, and function as a cellular magnetic sensor, which assist the cells to navigate and swim along the geomagnetic field. Localized with each magnetosome is a suite of proteins involved in the synthesis, maintenance and functionalization of the organelle, however the detailed molecular organization of the proteins in magnetosomes is unresolved. MamA is one of the most abundant magnetosome-associated proteins and is anchored to the magnetosome vesicles through protein-protein interactions, but the identity of the protein that interacts with MamA is undetermined. In this study, we found that MamA binds to a magnetosome membrane protein Mms6. Two different molecular masses of Mms6, 14.5-kDa and 6.0-kDa, were associated with the magnetosomes. Using affinity chromatography, we identified that the 14.5-kDa Mms6 interacts with MamA, and the interaction was further confirmed by pull-down, immunoprecipitation and size-exclusion chromatography assays. Prior to this, Mms6 was assumed to be strictly involved with biomineralizing magnetite; however, these results suggest that Mms6 has an additional responsibility, binding to MamA.

1. Introduction

In 1975, magnetotactic bacteria (MTB) were first discovered by Blakemore [1,2]. These bacteria contain unique organelles called magnetosomes that biomineralize magnetic minerals using specific proteins that are only associated with the organelle [3]. In 1996, the first magnetosome associated protein was isolated, sequenced and found to consist of tetratricopeptide repeat (TPR) motifs which are known to mediate protein-protein interactions [4]. Since 1996, this protein has been designated MamA (Mam22), and researchers in the field of biochemistry and genetics have studied it, and recently the structures of four different MamA proteins from four different MTB have been resolved [5–7]. However, even using cutting edge techniques, researchers have merely confirmed the idea that MamA interacts with other magnetosome-associated protein(s), but the function of MamA still remains enigmatic. We have made a major discovery towards identifying the binding partner of MamA, which gives a significant clue to its function.

MamA is conserved in all known MTB [8], and even though it is a soluble cytoplasmic protein [9], it localizes in the magnetosome matrix, a proteinaceous layer surrounding magnetosome vesicles of *Magnetospirillum* species [10,11]. The entire primary structure of MamA consists of five TPR motifs and one putative TPR motif [9]. These motifs consist of a helix-turn-helix fold, which has been known to promote protein-protein interactions [12]. Proteins with TPR motifs are important to cells which use them in a wide variety of ways such as protein transport, protein folding, transcription and splicing, and cell cycle control [13].

Two different functions for MamA have been proposed. Based on studies of a *mamA* deletion mutant, MamA appears to activate or prime preformed magnetosomes for biomineralization [14]. A different study used the atomic force microscopy (AFM) to observe chains of magnetosomes with and without MamA and proposed that MamA is anchored to the magnetosome membrane and may stabilize the magnetosome chain [11]. According to the MamA crystal structures, the five TPR motifs form a superhelix structure which has at least three putative protein binding sites, and one of the sites specifically binds to one of the magnetosome-associated proteins [5–7], However, the question remains as to which magnetosome-associated protein(s) interacts with MamA.

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In this study, we used MamA affinity chromatography to screen the proteins from the magnetosomes of *Magnetospirillum magneticum* AMB-1 which bind to MamA. We found that Mms6, a magnetosome membrane-bound protein, binds to MamA. We further confirmed this binding using immuno-precipitation, pull-down and size-exclusion chromatography experiments. In addition to this, we established that two different types of Mms6 exist in the magnetosome membrane, a 14.5-kDa and 6.0-kDa version. Until now, Mms6 was thought to be exclusively involved in biomineralization, however these new results imply an additional function of Mms6 within magnetosomes and provide a clue to answer the question of how MamA binds to magnetosomes in *M. magneticum* AMB-1.

2. Materials and methods

2.1. Microorganisms and cultures

Bacterial strains and plasmids are listed in Table S1. *M. magneticum* AMB-1 (ATCC 700,264) was cultured as described [15]. *Escherichia coli* strains were cultivated in LB broth [16] at 37 °C, unless specified otherwise. When necessary, the antibiotics kanamycin (20 μg/ml) or ampicillin (100 μg/ml) were added to the *E. coli* cultures.

2.2. Expression and purification of MamA and Mms6 proteins

The MamA expression vector was generated as previously described [9]. Primer sequences are shown in Table S2. For C-terminal His-tagged full-length Mms6 expression, the plasmid pET29b-mms6$^{1-133}$ was constructed by cloning the entire PCR-amplified *mms6* gene (*mms6*$^{1-133}$; accession number: AB096081) fragment into the *Nde*I/*Kpn*I sites of pET-29b (Merck-Millipore) using a primer set (mms6–1f and mms6–1r). The pET29b-mms6$^{1-133}$ was also used as the template to create the expression plasmid of Mms6$^{75-133}$-His. For protein expression, *E. coli* strain BL21(DE3) containing these recombinant plasmids were grown at 30 °C until an $A_{600\ nm}$ of ~0.6, and then induced by 1 mM (final concentration) of isopropyl-β-D-thiogalactopyranoside (IPTG) for 7 h. The cells were then harvested by centrifuging at 8000 × *g* for 15 min.

The purification of recombinant MamA in *E. coli* was performed by Ni-affinity chromatography as previously described [9]. For purifying the recombinant Mms6$^{1-133}$-His and Mms6$^{75-133}$-His, cells were suspended in 10 mM Tris–HCl (pH 8.0) and disrupted using sonication (80 W for 15 min). The lysate was centrifuged at 8000 × *g* for 15 min to remove the cell debris, and then the supernatant was ultra-centrifuged at 100,000 × *g* for 1 h to separate the membrane and soluble protein fractions. The membrane fraction was suspended in 10 mM Tris–HCl (pH 8.0) containing 2% CHAPS and 200 mM NaCl, and then incubated at 4 °C for 2 h to solubilize the membrane proteins. The solubilized fraction was harvested by ultracentrifugation (100,000 × *g* for 1 h) and the supernatant was subjected to a Ni-NTA resin (QIAGEN) column. The proteins bound to the column were eluted with 50 mM NaH_2PO_4 containing 250 mM imidazole, 0.2% CHAPS and 300 mM NaCl (pH 8.0). The eluted protein fraction was dialyzed against 10 mM Tris–HCl (pH 8.0) containing 0.2% CHAPS.

2.3. MamA-affinity chromatography

Preparation of His-MamA affinity resin was performed as previously described [11]. Bovine serum albumin (BSA) was also immobilized to the CNBr-activated Sepharose resin and used as a control column. Magnetosomes (770 mg wet weight) were purified from *M. magneticum* AMB-1 cells (125 *g* wet weight collected from 600 L of medium) by magnetic separation as previously described [10]. The purified magnetosomes were treated by an alkaline buffer, 0.1 M CAPS-NaOH (pH 11.0), to remove MamA specifically from the magnetosomes [10,11] (Fig. S1). The proteins associated with the MamA-eliminated magnetosomes were solubilized with 10 mM Tris–HCl (pH 8.0) containing 2% sucrose monocaprate at 4 °C for 16 h. This suspension was centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant was dialyzed against the equilibration buffer (10 mM Tris–HCl (pH 8.0) containing 0.2% sucrose monocaprate), the protein solution (11 ml, 0.32 mg proteins/ml) was subjected to the His-MamA-column (1 × 10 cm^2) and BSA-column (1 × 10 cm^2) with a flow rate of 1 ml/h. After that, the columns were washed with 25 ml of equilibration buffer and the bound proteins were eluted with 0.1 M CAPS-NaOH buffer (pH 11.0) containing 0.2% sucrose monocaprate. The eluted protein fractions were concentrated approximately two hundred times by ultrafiltration. The concentrated samples were applied to SDS-PAGE, and the protein bands were analyzed by mass spectrometry and identified.

2.4. Physical and chemical measurements

SDS-PAGE was performed using the method of Laemmli [17] and tricine-SDS-PAGE was performed as previous described [18]. His-tagged protein bands were visualized using InVision His-Tag In-Gel Stain (Thermo Fisher Scientific). The protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). The in-gel protein identification was performed as previously described using the 4800Plus MALDI-TOF/TOF Analyzer (Applied Bioscience, Carlsbad, CA) and the results were analyzed using Protein PILOT software [19].

2.5. Immunoblotting analyses

Anti-Mms6$^{1-133}$ polyclonal rabbit antibodies were raised against the purified recombinant Mms6$^{1-133}$-His. Immunoreactivity of anti-Mms6$^{1-133}$ and anti-MamA [10] antibodies was detected at dilutions of 1:50,000 for each. Goat anti-Rabbit IgG conjugated to horseradish peroxidase (GE Healthcare Bioscience) was diluted 1:10,000 using the Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific). The chemifluoresence data were collected using a Luminescent Image Analyzer, LAS 3000 (Fujifilm) and the band intensities were quantified using Multi Gauge software v. 2.2 (Fujifilm). The protein weights of the 14.5-kDa Mms6 and 6.0-kDa Mms6 from the purified magnetosomes were calculated according to the relative intensities for equal weights of these two protein bands in the immunoblot. The relative intensities for the 14.5-kDa Mms6 (Mms6$^{1-133}$) and 6.0-kDa Mms6 (Mms6$^{75-133}$) protein bands were calculated from the immunoblotting profiles of the two purified proteins, 0.1 μg Mms6$^{1-133}$-His and 0.9 μg Mms6$^{75-133}$-His, against anti-Mms6$^{1-133}$ polyclonal antibodies (Fig. S2).

2.6. Immunoprecipitation assay

The immunoprecipitation assay was performed as described [20] with some modification. A 200 μl mixture containing 2 μM His-MamA and 1 μM Mms6$^{1-133}$-His was incubated at 28 °C for 1 h. After incubation, 2 μl of anti-Mms6$^{1-133}$-antibody, *anti*-MamA antibody or normal serum were added to the mixture and incubated for 1 h. A slurry of protein A-Sepharose resin (GE Healthcare Bioscience) was added, and the proteins that co-precipitated with the protein A-Sepharose resin were analyzed by SDS-PAGE.

2.7. Pull-down assay

Prior to performing the pull-down assay, the N-terminal poly-His of MamA was removed by using the Biotinylated Thrombin Kit (Novagen). The solution of the 4 μM $Mms6^{1-133}$-His, was incubated with 3 μM MamA at 25 °C for 1 h. Afterwards, 15 μl of Ni-NTA agarose resin (QIAGEN), which had been equilibrated with buffer (50 mM NaH_2PO_4 containing 10 mM imidazole and 300 mM NaCl [pH 8.0]) was added to the solution. The resin was then washed five times with 400 μl of the same buffer. The bound proteins were eluted with 15 μl of elution buffer (50 mM NaH_2PO_4 containing 250 mM imidazole, and 300 mM NaCl [pH 8.0]) and the eluted proteins were analyzed by SDS-PAGE. In addition to this, $Mms6^{1-133}$ with the His-tag removed was mixed with His-MamA and was precipitated with Ni-NTA agarose resin and the protein-protein interaction was analyzed by SDS-PAGE.

2.8. Size-exclusion chromatography

Chromatography was performed at 4 °C in a high pressure liquid chromatography (HPLC) system (GE healthcare) using a Superose 6 Increase 10/300GL column equilibrated with 10 mM Tris–HCl (pH 8.0) containing 0.2% CHAPS and 200 mM NaCl with a flow rate of 0.4 ml/min. Three markers were used, 669-kDa thyroglobulin (bovine thyroid), 220-kDa β-amylase (*Ipomoea batatas*), and 29-kDa carbonic anhydrase (bovine erythrocytes). For the protein-protein interaction, a sample containing two proteins His-MamA (91 μg) and $Mms6^{1-133}$-His (116 μg) or cytochrome a_1-like hemoprotein (control) was incubated for 1 h at room temperature. All samples were centrifuged at 20,000 × *g* for 10 min prior to being injected into the column.

3. 3. Results and discussion

3.1. Screening MamA binding proteins in magnetosomes

Previous studies demonstrated that when MamA was removed from magnetosomes, purified MamA could be added back to the MamA-eliminated magnetosomes and bind to them [10,11]; our objective was to identify which magnetosome-associated proteins were binding to MamA. We began by extracting proteins from MamA-eliminated magnetosomes (Fig. S1), running them through a MamA-affinity chromatography column and loading the eluted fractions onto an SDS-PAGE gel (Fig. S3). Thirteen protein bands were detected and analyzed by tandem mass spectrometry (Fig. 1). Six different MamA bands were detected (bands 7–11 and 13) which represent recombinant MamA and truncated recombinant MamA that detached from the column. Bands 2, 4, and 6 were identified as proteins derived from *E. coli*. These proteins were contaminated in the purified His-MamA sample, which was used to make the column, and probably detached from the affinity column during the elution process. Five of the bands were identified as proteins from *M. magneticum* AMB-1; band 1 is a methyl-accepting chemotaxis protein (amb1418), band 3 is a hypothetical protein (amb3421), band 5 is a porin (amb0025), and band 12 is two proteins, the ATP synthase epsilon chain (amb4138) and Mms6 (amb0956). Of these five proteins, Mms6 is the only magnetosome-associated protein. The other four proteins are inner or outer membrane-bound proteins, which are contaminants, derived during the magnetosome purification process [21]. A control was performed using BSA to screen for MamA binding proteins. The SDS-PAGE gel profile showed no proteins specifically bound to the BSA column. The 63.7 and 54.0-kDa protein bands that were found in all lanes in both columns represent contaminated proteins from the electrophoresis procedure (Fig. S3). Our goal was to understand which magnetosome associated protein binds to MamA, therefore we focused on Mms6. However, the other four membrane proteins could also be MamA binding candidates specifically, the cytoplasmic membrane proteins, amb1418, amb3421, and amb4138, which come in direct contact with MamA in the cell. There is also the possibility that other MamA binding magnetosome-associated proteins still remain, because some magnetosome membrane proteins may not have been solubilized by sucrose monocaprate prior to the affinity chromatography step.

3.2. Presence of a 14.5-kDa Mms6 in magnetosomes

After we screened MamA binding proteins and analyzed them by SDS-PAGE, we determined that the 14.5-kDa Mms6 is the primary binding candidate (Fig. 1). Previous to this result, Mms6 is generally known as a 6.0-kDa peptide that is tightly bound to magnetite crystals and is involved in the biomineralization of cubo-octahedral magnetite crystals both *in vitro* [22,23] and *in vivo* [24–26]. Arakaki et al. [22] identified Mms6 as a 6.0-kDa mature protein consisting of 59 amino acids (from a. a. 75–133), but the *mms6* gene sequence shows that the full-length Mms6 protein is 133 amino acids (deduced from the 14.5-kDa peptide) (Fig. S4). The 6.0-kDa Mms6 could have been present during the elution of MamA bound proteins but was not detected because the gel that

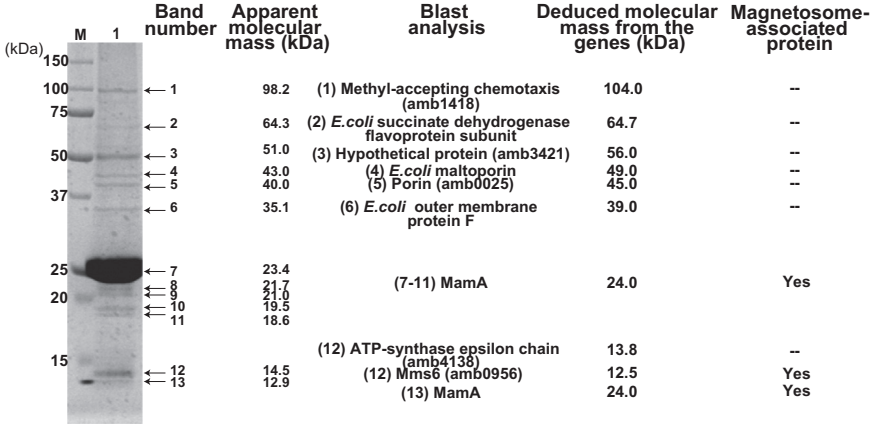

Fig. 1. SDS-PAGE gel profile of proteins eluted from the His-MamA column and their apparent molecular masses; lane M, protein markers (Precision Plus protein standards; Bio-Rad); lane 1, eluted proteins. The eluted fractions were concentrated approximately 200 times for SDS-PAGE. These 13 bands were analyzed using tandem mass spectrometry and identified. Bands 2, 4, and 6 were proteins belonging to *E. coli*; bands 1, 3, 5, and 12 were proteins belonging to *M. magneticum* AMB-1; and bands 7–11, and 13 were recombinant MamA proteins. Only two of the bands were identified as magnetosome associated proteins, Mms6 and MamA. The gel was stained with Coomassie Brilliant Blue G-250.

was used could not separate low molecular mass proteins.

In order to resolve the conflict of why we found an abundance of 14.5-kDa Mms6 instead of 6.0-kDa Mms6, we first wanted to confirm the presence of the 14.5-kDa version of Mms6 in magnetosomes. To do this, we generated anti-Mms6$^{1–133}$ polyclonal antibodies which were used for the immunoblotting analysis of cellular fractions. We found two bands that were specifically localized in the magnetosome fraction, one at 14.5-kDa and another at 6.0-kDa (Fig. 2(A)). As a control experiment, we performed the immunoblotting with an excess amount of Mms6$^{1–133}$ (antigen), confirming that the cross-reactions of these two bands, 6.0-kDa and 14.5-kDa, were specific (Fig. 2(A)). Also, we confirmed that the anti-Mms6$^{1–133}$ antibodies could recognize both recombinant protein bands of Mms6$^{1–133}$ and Mms6$^{75–133}$ (Fig. S2). Using immunoblotting, we quantified the ratio of 14.5-kDa and 6.0-kDa Mms6 bands in the magnetosome extracts using two different preparation methods. Method 1: incubating in 2% SDS at 37 °C for 1 h, method 2: incubating in boiling 1% SDS for 1.5 h and taking an aliquot every 30 min (the same method used by Arakaki et al. [22]) (Fig. 2(B)). In each method, both types of Mms6 were detected, but are present in different amounts. We calculated the ratio of 14.5-kDa and 6.0 -kDa Mms6 amounts from the intensities of the protein bands in the immunoblots. The signal intensity for the Mms6$^{1–133}$ band was 23 times stronger than that for the Mms6$^{75–133}$ band for an equal weight of proteins (Fig. S2). The ratios were 63% and 37% for 14.5-kDa Mms6 and 6.0-kDa Mms6, respectively for method 1; and 38% and 62% for 14.5-kDa Mms6 and 6.0-kDa Mms6, respectively for method 2. This result showed, for the first time, that two different sizes of peptides of Mms6 exist in the magnetosome, and they are present in roughly equal amounts depending on the method of preparation. Previous studies demonstrated that the 6.0-kDa Mms6 (Mms6$^{75–133}$) binds magnetite crystals and controls the crystal morphology [23–26]. Furthermore, peptides mimicking the C-terminal region of Mms6 formed cubo-octahedral shaped crystals [27]. These insights indicate that the purpose of the 6.0-kDa version of Mms6 is for magnetite biomineralization, but there may be a separate function for the 14.5-kDa version of Mms6.

3.3. Confirmation of the interaction between MamA and 14.5-kDa Mms6

We confirmed the protein-protein interaction between MamA and 14.5-kDa Mms6 (Mms6$^{1–133}$) by immunoprecipitation and pull-down assay (Fig. 3). Immunoprecipitation was performed using His-tagged Mms6$^{1–133}$ and His-tagged MamA, and two different antibodies, *anti*-MamA and anti-Mms6$^{1–133}$, in different combinations to prove the binding between the two peptides (Fig. 3(A)). This demonstrated that Mms6$^{1–133}$ co-precipitated with MamA (Fig. 3(A)). In the control experiment, there was no interaction (Fig. 3(A)). Additionally, the Ni-NTA pull-down assay designed to test the specific interaction between MamA and Mms6$^{1–133}$ demonstrated that they did co-precipitate (Fig. 3(B)). We also confirmed the interaction between MamA and Mms6$^{1–133}$ using size-exclusion chromatography (SEC) (Fig. S5). According to SEC, MamA (Fig. S5A) and Mms6$^{1–133}$ (Fig. S5B) were individually eluted in different fractions from the column. For example, MamA and Mms6$^{1–133}$ formed large oligomers with different molecular mass, ~500-kDa and >1000-kDa, respectively, which is consistent with previous studies [11,28]. Whereas, when we applied the mixture of MamA and Mms6$^{1–133}$ to the column they were eluted in the same fractions at near the void volume of the column (Fig. S5C). Even though these results show the interaction between MamA and Mms6$^{1–133}$, the question remains as to whether the interaction is due to the nonspecific binding between the hydrophobic regions of the putative TPR motif in MamA [9] and the

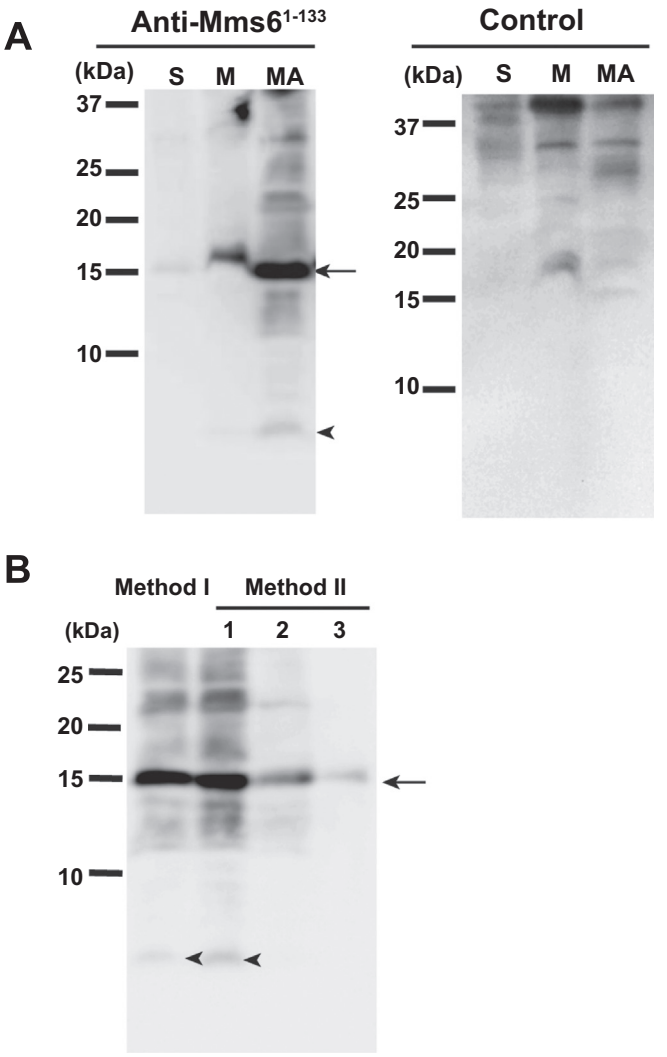

Fig. 2. (A) Immunoblotting of *M. magneticum* AMB-1 extracts labeled with anti-Mms6$^{1–133}$ polyclonal antibodies [left]. Two different Mms6 bands are evident, one at 14.5-kDa (arrow) and the other at 6.0-kDa (arrowhead). The 14.5-kDa Mms6 has a higher intensity than the 6.0-kDa Mms6. In the control experiment, the immunoblotting was carried out with an excess amount of Mms6$^{1–133}$ antigen. In the control, the 14.5-kDa and 6.0-kDa bands were not detected [right]. S: soluble fraction; M: membrane fraction; MA: magnetosome fraction. (B) Two methods were used to extract Mms6 from the magnetosomes and then analyzed using immunoblotting. Method I used 2% SDS at 37 °C for 1 h to extract Mms6 which produced two Mms6 bands, one at 14.5-kDa and another at 6.0-kDa, but the 14.5-kDa band has higher intensity. Method II was performed by Arakaki et al. [22] which extracted Mms6 by boiling magnetosomes in 1% SDS for 1.5 h with three aliquots taken every 30 min lane 1, 2, and 3. This resulted in two distinct Mms6 bands (14.5-kDa [arrow] and 6.0-kDa [arrowhead]) in the first aliquot but only one band (14.5-kDa) in the second two aliquots.

transmembrane region in Mms6$^{1–133}$ [22]. To reconcile this, we examined the interaction between MamA and a hydrophobic transmembrane protein, cytochrome a_1-like hemoprotein [29], by SEC. The MamA elution profile was not affected by adding the cytochrome a_1-like hemoprotein (Fig. S6), indicating that the MamA-Mms6$^{1–133}$ interaction is specific.

We determined the interaction between MamA and 14.5-kDa Mms6 by affinity chromatography, immunoprecipitation, pull-down, and size-exclusion chromatography. The 14.5-kDa Mms6 (Mms6$^{1–133}$) has a larger N-terminal soluble domain (a. a. 1–88) (Fig. S4), therefore it could extend into the cytosolic space and might anchor the MamA at the magnetosome surface. It is possible that MamA can bind to 6.0-kDa Mms6, however due to the sample

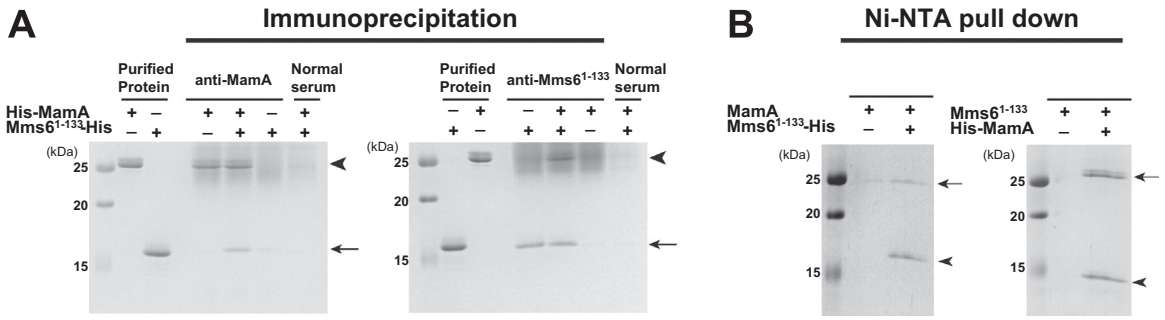

Fig. 3. (A) SDS-PAGE analyses of the immunoprecipitation assays. A mixture containing His-MamA and Mms6$^{1-133}$-His was precipitated with *anti*-MamA (left) or anti-Mms6$^{1-133}$ (right) antibodies and clearly show that Mms6 (arrow) co-precipitates with MamA (arrowhead). When normal serum was used, there was no band for either MamA or Mms6 (right lanes). (B) SDS-PAGE analyses of the Ni-NTA agarose pull-down assay. The arrows indicated the His-MamA and the His-tag removed MamA protein bands; the arrowheads indicated the Mms6$^{1-133}$-His and His-tag removed Mms6$^{1-133}$ protein bands. Both the immunoprecipitation and pull-down assays confirm the interaction between MamA and Mms6$^{1-133}$. The molecular mass standards (Precision Plus protein standards; Bio-Rad) are indicated on the left side of the gels. The gels were stained with Coomassie Brilliant Blue G-250.

limitation, we are unable to confirm this by the affinity chromatograph experiment.

MamA was shown to cover the outside of the magnetosome and to play a role in maintenance processes such as protein sorting or activating magnetosome vesicles [11,14]. Our results suggest a direct interaction between MamA and Mms6. We propose that Mms6 localizes in the magnetosome membrane and is a factor controlling MamA localization. Because MamA homogenously surrounds the magnetosomes and are attached to Mms6, these proteins must also be homogenously spaced around the magnetosome as well. This homogeneous localization of Mms6, which controls the magnetite crystal shape, may affect the growth of the magnetite crystals. Therefore, in cells with the *mamA* gene deleted, the magnetite crystals may be altered. This may account for the results shown by Komeili et al. [14] who demonstrated that Δ*mamA* AMB-1 cells contained fewer crystals in the magnetosomes vesicles.

There are at least 30 proteins associated with the magnetosome, one of which is MamA, a key protein for the process of constructing the organelle. By identifying that Mms6 is the binding partner of MamA, we found a major piece of the puzzle, which allows other researchers to continue the work on MamA and other magnetosome-associated proteins. Over the 40 year history of research on magnetotactic bacteria, a great deal of progress has been made, however many questions remain. For example, does MamA bind to the 6.0-kDa Mms6 and is it the same binding site as the 14.5-kDa Mms6? An important next step involves performing *in vivo* studies to examine the function of the MamA-Mms6 interaction. On the other hand, even though MamA is conserved in all known MTB, Mms6 exits only in MTB belonging to *Alphaproteobacteria*. Therefore, MamA in MTB belonging to non-*Alphaproteobacteria* should bind to a different magnetosome-associated protein in order to be anchored to the magnetosome. This hypothesis gives a new view that should inspire further studies into the protein-protein interactions in magnetosome bacterial organelles.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2016.05.010. This supplemental file contains Table S1, S2, and Figure S1, S2, S3, S4, S5, and S6.

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