Magnetic bacteria synthesize magnetite crystals with species-dependent morphologies. The molecular mechanisms that control nano-sized magnetite crystal formation and the generation of diverse morphologies are not well understood. From the analysis of magnetite-crystal-associated proteins, several low molecular mass proteins tightly bound to bacterial magnetite were obtained from Magnetospirillum magneticum strain AMB-1. These proteins showed common features in their amino acid sequences, which contain hydrophobic N-terminal and hydrophilic C-terminal regions. The C-terminal regions in Mms5, Mms6, Mms7, and Mms13 contain dense carboxyl and hydroxyl groups that bind iron ions. Nano-sized magnetic particles similar to those in magnetic bacteria were prepared by chemical synthesis in the presence of the acidic protein Mms6. These proteins may be directly involved in biological magnetite-crystal formation in magnetic bacteria.

Magnetic bacteria synthesize nano-sized magnetic particles of an iron oxide, magnetite (Fe₃O₄); an iron sulfide, greigite (Fe₃S₄); or a combination of greigite and iron pyrite (Fe₃S) (1–3). These particles are individually covered with a stable lipid bilayer membrane that mainly consists of lipid and protein (4). The mineral size, type, and morphology of bacterial magnetic particles (BMPs) are highly controlled within bacterial species or strains (5). The species-specific control of BMP formation has focused attention on the possible roles of the surrounding membrane structures.

The molecular mechanism of BMP synthesis is a multistep process, including vesicle formation, iron transport, and magnetite crystallization (5, 6). Recent molecular studies have postulated the steps of BMP synthesis (7–9). Several proteins located on or in the BMP membrane have been isolated and analyzed in Magnetospirillum magneticum strain AMB-1. The first event of BMP synthesis is the formation of vesicles. Invagination of the cytoplasmic membrane is primed by a BMP membrane-specific GTPase (Mma16) to form the intracellular vesicle (7). MpsA, a homolog of an acetyltransferase containing a CoA-binding motif, is also considered to be involved in this process (8). The second process in BMP synthesis is iron transport into the BMP vesicles. It appears that ferric iron is reduced on the cell surface, taken into the cytoplasm, transported into the BMP vesicle, and finally oxidized to produce magnetite. The magA gene was isolated through transposon mutagenesis in strain AMB-1 (9). This gene encodes an integral membrane protein that is involved in the transport of iron into the BMP vesicles. The last process is crystallization of magnetite within the vesicle, but the process remains unclear.

Other proteins associated with the BMP membrane have been partially characterized in magnetic bacteria to date. Gorby et al. (4) observed two specific proteins in the BMP membrane from Magnetospirillum magnetotacticum MS-1. Okuda et al. (10) identified three additional specific proteins and determined the nucleotide/amino acid sequence of a 22-kDa protein. From motif analysis, this is considered to function as a receptor interacting with associated cytoplasmic proteins (11). Recently, Grünberg et al. (12) cloned and sequenced four genes that were assigned to two different genomic regions coding for BMP membrane-specific proteins in Magnetospirillum gryphiswaldense MSR-1.

To understand the molecular mechanism of magnetite crystallization in M. magneticum AMB-1, several proteins tightly bound to the bacterial magnetite crystals were isolated and characterized. A new class of mineral-associated proteins that may have important roles in the initiation of nucleation and magnetite crystal growth is described.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions—**Escherichia coli strains DH5α and BL21 were used for gene cloning and protein expression, respectively. E. coli cells were cultured in Luria broth at 37 °C after adding appropriate antibiotics. M. magneticum AMB-1 (ATCC700264) was grown anaerobically in an 8-liter fermentor as described previously (13).

**Isolation of Proteins Tightly Bound to BMPs—**BMPs were extracted from 8 liters of fed-batch culture and washed 20 times with HEPES (pH 7.0). The other cell fractions were also prepared as described previously (8). The purified BMPs were treated three times with 800 μl of 1% (w/v) SDS in a 100 °C water bath for 30 min. During this process, the sample was treated briefly in an ultrasonic bath. After several washings, BMPs were treated with 20 ml of 2 M hydrofluoric acid plus 8 M ammonium hydrofluoride solution (pH 5.0). The dissolved material was dialyzed several times against 4 liters of fresh HEPES. The sample was precipitated by the same volume of 20% (w/v) trichloroacetic acid and resuspended in 400 μl of 0.1% SDS solution. The amount of protein on BMPs was evaluated using a modified Lowry method (14). One-hundred microliters of 1 N NaOH and 0.1 M Tris base solution with weak sonication to remove membrane-associated proteins were washed three times with 800 μl of 1% (w/v) SDS in a 100 °C water bath for 30 min. During this process, the sample was treated briefly in an ultrasonic bath. After several washings, BMPs were treated with 20 ml of 2 M hydrofluoric acid plus 8 M ammonium hydrofluoride solution (pH 5.0). The dissolved material was dialyzed several times against 4 liters of fresh HEPES. The sample was precipitated by the same volume of 20% (w/v) trichloroacetic acid and resuspended in 400 μl of 0.1% SDS solution. The amount of protein on BMPs was evaluated using a modified Lowry method (14). One-hundred microliters of 1 N NaOH and 0.1 M Tris base solution with weak sonication to remove membrane-associated proteins were washed three times with 800 μl of 1% (w/v) SDS in a 100 °C water bath for 30 min. During this process, the sample was treated briefly in an ultrasonic bath. 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of the mms6 gene from Amb-1 genomic DNA. E. coli BL21 cells were cultured in 200 ml of Luria broth at 37 °C under isopropyl-1-thio-β-D-galactopyranoside induction. The recombinant protein was purified under denaturing conditions using a nickel-nitritolactric acid column (QIAGEN). The eluted protein was diluted in the same volume of refolding buffer (50 mM Tris-HCl, 1 mM EDTA, 0.1 M l-arginine, 1 mM reduced glutathione, 10% (v/v) glycerol, and 0.8 mM oxidized glutathione (pH 8.0)). The purified protein was renatured by dialysis in 0.5 liter of buffer (0.01 M Tris-HCl, 10% glyceral, and 0.01 mM EDTA (pH 8.0)) containing 4 and 2 μl urea for 3 h each. Final dialysis was performed overnight in 1 liter without urea. The protein was further dialyzed for 3 h several times against 1 liter of fresh Tris-HCl (pH 8.0). All dialysis steps were performed at 4 °C. The His tag of the recombinant protein was removed (QIAGEN) and then the sample was filtered using a miniprep Microcon YM-3 membrane (Millipore Corp.).

Iron Binding Analysis—The blotting technique for detecting the iron-binding ability of Mms6 was performed according to Chen and Driedsdale (18). 59FeCl3 was used instead of 59FeCl3 in the same procedure. After blotting 0.3 nmol of proteins, the nitrocellulose membrane was rinsed with metal-binding buffer (0.02 M Tris-HCl and 0.15 mM NaCl (pH 7.0)) for 30 min. The membrane was then immersed in 5 ml of the same buffer containing 0.05 μCi/ml 59FeCl3 (PerkinElmer Life Sciences). The total iron concentration in the solution was adjusted to 8.16 μg/ml by adding FeCl3. Unbound iron ion was removed by washing the membrane with metal-binding buffer three times for 30 min. The membrane was wrapped using exposed to x-ray film. Equine spleen apoferritin (Calbiochem) and bovine serum albumin were used as positive and negative controls, respectively. Competitive iron binding was performed using 0.1 nmol of proteins. The membrane was immersed in 2 ml of metal-binding buffer with 0.05 μCi/ml 59FeCl3 in the absence or presence of competing ions, including 1 mM FeCl3, 10 mM CaCl2, 10 mM MgCl2, 10 mM CuCl2, 10 mM NiCl2, and 10 mM ZnCl2. Autoradiography was done for 3 h.

Magnetite Synthesis in the Presence of Proteins Tightly Bound to BMPs—Purified recombinant Mms6 was used for magnetite formation by coprecipitation (19). Recombinant Mms6 (2 μg) was added to 100 μl of solution containing 33 mM ferrous sulfate and 33 mM ferric chloride. Transformation was titrated very slowly using 0.1 M NaOH solution with sparging argon gas.

Transmission Electron Microscopy and Electron Diffraction Analysis of Magnetic Proteins—A transmission electron microscopy (H7000-H, Hitachi, Tokyo) was used to observe the particle size and morphology. The sample was applied to carbon-coated 150-mesh copper grids (Nishin EM Co., Ltd., Tokyo), dried overnight at room temperature, and stored with silica gel desiccant. The magnetic particles were visualized at 150 keV. Electron diffraction was performed using an analytical microscope (JEM-2000FX, Jeol Ltd., Tokyo).

RESULTS

Isolation of Proteins Tightly Bound to BMPs—After extraction and purification, ~200 mg of BMPs was obtained from an 8-liter fermentor. The presence of membranes encapsulating the BMPs was confirmed by transmission electron microscopy (Fig. 1A). Results from the protein assay showed that the membrane proteins constituted 3% of the total mass weight of BMPs. By treating BMPs with a solution containing 7 μm urea, 2 μM thiourea, and 4% CHAPS with weak sonication, 60% of the proteins were extracted. As previously reported, Mms24 (identical to MAM22) (20), MpsA (8), and Mms16 (7) were observed in this fraction (Fig. 2A, lane 1). The residual 40% of the proteins associated with BMPs could not be extracted using the same solution. To strip these tightly associated proteins, BMPs were immersed three times in boiling 1% SDS solution. Over 95% of the proteins were removed, and aggregations of naked BMPs were observed (Fig. 1B). The protein profile of the 1% SDS fraction showed that it consisted mainly of low molecular mass proteins (~<15 kDa) (Fig. 2A, lane 4). For amino acid sequencing, the same sample protein was subjected to two-dimensional gel electrophoresis. Four prominent spots were identified in the gel (Fig. 2B). The residual magnetite was dissolved in 2 mM hydrofluoric acid plus 8 mM ammonium hydrofluoride solution (pH 5.0) to identify any embedded proteins in the bacterial magnetite after protein removal with boiling 1% SDS solution. The magnetite was dissolved completely, and
some compounds were observed by transmission electron microscopy (Fig. 1D). No protein bands were observed by SDS-PAGE (Fig. 2A, lane 5), suggesting that proteins exist on or near the surface of the crystals, but not within the magnetites. The proteins tightly bound to BMPs were further characterized to understand their roles in magnetite crystallization.

N-terminal Amino Acid Sequencing—The amino acid sequences of the small proteins obtained by Edman degradation are shown in Table I. From the obtained sequences, a spot with an approximate molecular mass of 13 kDa (pI 7.2) was determined as a homolog of MamC in M. gryphiswaldense (12). The molecular size visualized by Tricine/SDS matched the reported size of MamC. The protein was designated as Mms13. The N-terminal amino acid sequences of the spots at 5 kDa (pI 6.1), 6 kDa (pI 4.5), and 7 kDa (pI 5.9) showed high degrees of homology to each other and were designated as Mms5, Mms6, and Mms7, respectively. These proteins have the common sequence LGILGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLG. However, from several sequencing trials, we concluded that these are different proteins. Mms7 is homologous to the C-terminal part of MamD in M. gryphiswaldense. It was reported that the apparent mass of MamD on the SDS gel and the molecular mass calculated from the predicted mamD gene are different (12). These results suggest the existence of proteolytic cleavage of MamD. The obtained sequence most probably is the remnant of MamD after proteolytic digestion.

Isolation of Genes Encoding Proteins Tightly Bound to BMPs—Based on the obtained amino acid sequences, primers were designed for the gene sequencing of Mms5 and Mms6. Complete DNA fragments coding open reading frames were obtained from the AMB-1 genome. Putative sequence for the ribosome-binding site and several promoter regions were found upstream of the start codon. FASTA and BLAST searches showed that the obtained nucleotide and deduced amino acid sequences of Mms5 and Mms6 have no significant similarity, except to MamD. Interestingly, mms6, mms7, and mms13 are located closely (within a 3.2-kilobase pair region) in the AMB-1 genome (Fig. 3). The gene homolog of mms6 was also found in the genome sequence of MS-1 obtained from the data base. The amino acid sequence alignment of Mms6, Mms7, and Mms13 between M. magnetotacticum AMB-1 and M. magnetotacticum MS-1 revealed 100, 81, and 80% similarities, respectively. No motifs were identified in all four proteins. The gene encoding Mms5 was not found in the genome sequence of MS-1 obtained from the data base.

The amino acid sequence of Mms6 deduced from the full-length 399-bp gene is shown in Fig. 4. The sequence encodes a 12.5-kDa premature polypeptide. The N-terminal sequence obtained directly from the purified protein is completely contained within this deduced sequence. A predicted signal peptide presumed to mediate secretion, followed by a propeptide, was found using the SOSUI program (21). However, there was a gap between the signal peptidase cleavage site and the N-terminal sequence obtained by Edman degradation. This may be due to digestion by some specific proteases after protein secretion. The N-terminal region contains hydrophobic amino acids, and computer analysis suggests that this is a transmembrane region. However, the C-terminal region of Mms6 is highly acidic, consistent with a pI of 4.5. Amino acids containing hydroxyl groups were also observed. Furthermore, the region between the middle and C-terminal regions contains basic amino acids such as Lys, Tyr, and Arg. These structural features were also observed in Mms5, Mms7, and Mms13. The consensus sequences among these proteins are shown in Fig. 5. The hydrophilic domains in mineral-associated proteins capture metal ions (22, 23) or interact with the mineral phase (24), as previously reported.

Purification of Recombinant Mms6—Recombinant Mms6 was produced in E. coli BL21. The gene encoding the mature

### Table I

| Protein | N-terminal amino acid sequence |
|---------|-------------------------------|
| Mms13 (MamC) | PFFILAPXLAKSVPGVGLALGAGAAAXXVNRVRLKERT |
| Mms7 (MamD) | AGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLGLG |
| Mms6 | GLITWTKGKLQGLQGLGLAGPVFLAGAGL |
| Mms5 | SLQGLGLGLGLGLGLGLGLGLGLG |

Fig. 1. Morphological changes in BMPs during protein extraction. A, no treatment; B–D, after treatment with urea, SDS, and hydrofluoride solutions, respectively.

Fig. 2. Identification of proteins tightly bound to BMPs by SDS-PAGE. A, profile of cell fractions. Lane 1, BMP protein fraction extracted by a solution containing 7 M urea, 2 M thiourea, and 4% CHAPS; lane 2, cell membrane lysates; lane 3, cytoplasmic fraction; lane 4, BMP protein fraction extracted with boiling 1% SDS solution; lane 5, demineralized material of BMPs treated with 2 M hydrofluoric acid plus 8 M ammonium hydrofluoride solution (pH 5.0). Ten micrograms of proteins for lanes 1–4 and 15 μg of sample for lane 5 were loaded. The gel was stained with SYPRO Ruby. B, two-dimensional gel electrophoresis of the BMP protein fraction extracted with boiling 1% SDS solution. Twenty micrograms of protein was loaded. The gel was stained with Coomassie Brilliant Blue R-250. The identified proteins are indicated by arrows. Numbers to the left of A and B indicate molecular mass markers.
peptide of Mms6 (6 kDa) was amplified by PCR and cloned into the pET15b vector. The pET15b-mms6 plasmid was then transformed into E. coli and overexpressed. The His-tagged protein was purified from the cell lysate using a nickel-nitrilotriacetic acid column. The N-terminal amino acid of the purified protein was sequenced and confirmed that the desired protein was expressed in E. coli. The protein was reconstituted, and the His tag was removed by thrombin digestion. The protein sample after each step was checked by gel electrophoresis and Western blotting using anti-His antibody (Fig. 6). The expected sizes of His-tagged (7.5 kDa) and thrombin-digested (6 kDa) proteins were determined.

**Iron-binding Activity and Specificity of Mms6**—The iron-binding ability of Mms6 was found both in the presence and absence of the His tag (Fig. 7A). This indicates that the observed iron binding is mainly derived from Mms6, not from the His tag. In contrast, bovine serum albumin did not show iron-binding capability. Addition of 10 mM nonradioactive Fe$^{3+}$/H$_{1001}$ blocked the binding of radioactive Fe$^{3+}$/H$_{11001}$ to both Mms6 and ferritin (Fig. 7B). Inhibition of binding of radioactive Fe$^{3+}$/H$_{11001}$ to Mms6 (but not to ferritin) was also observed in the presence of Ca$^{2+}$/H$_{11001}$ and Mg$^{2+}$/H$_{11001}$. This binding inhibition was scarcely observed in the presence of Ni$^{2+}$/H$_{11001}$, Cu$^{2+}$/H$_{11001}$, and Zn$^{2+}$/H$_{11001}$.

**Chemical Magnetite Synthesis in the Presence of Mms6**—To determine the effect of Mms6 on crystal formation, artificial magnetite was synthesized. At the beginning of titration, we observed a yellow-to-white precipitate, which changed to dark green and finally to black at neutral pH. The magnetic iron precipitates produced in the presence of Mms6 showed cuboidal morphology, with sizes ranging from 20 to 30 nm (Fig. 8A). The results of electron diffraction analysis indicated that the black particles were composed mainly of magnetites (data not shown). The shape of the crystalline magnetites was similar to that of BMPs synthesized in M. magneticum AMB-1. Furthermore, the magnetic particles produced in the absence of Mms6 were non-homogeneous in size (1–100 nm) and shape (Fig. 8B). The observed needle-shaped crystals are similar to $\alpha$-FeOOH.
Organic molecules acting as templates that facilitate crystal formation have been isolated from demineralized materials (25–27) and organic matrix associated with mineral surfaces (28, 29). Direct evidence of biomimetic formations by proteins has been reported in calcium carbonate (26, 27), silica (25, 30), and hydroxyapatite (24). Although extraction of the proteins associated with BMPs has been examined using SDS (4, 10, 12) and urea-based (8, 20) solutions, no direct evidence of magnetite crystal formation by the proteins has been shown. In this study, several different protein fractions were separated by sequential treatment with urea, boiling SDS, and hydrofluoric acid. Peripheral proteins such as Mms24 (MAM22) (10, 20), MpsA (8), and Mms16 (7) and transmembrane proteins were removed by treatment with a urea-based solution. Four proteins (Mms5, Mms6, Mms7, and Mms13) were observed in the same fraction as minor components upon two-dimensional gel electrophoresis. The four protein spots were dominant in the fraction obtained by boiling SDS treatment. The protein solution showed a yellowish color, and the presence of iron was confirmed. Transmission electron microscopy showed no differences in the size and shape of BMPs before and after this treatment, indicating that only the surfaces of the particles were degraded. The presence of some specific interaction is suggested between these proteins and BMP surfaces. Furthermore, because protein was not observed in the BMP crystal core, the proteins bound to the surface play important roles in crystal formation.

The four proteins (Mms5, Mms6, Mms7, and Mms13) showed no sequence similarities to known functional proteins. The only observed similarity was to the BMP membrane proteins MamC and MamD, reported in M. gryphiswaldense (12). Common features observed within their sequences may describe a structural property. The proteins are mainly composed of two domains, hydrophilic N-terminal and hydrophilic C-terminal regions. The organic matrix surrounding the BMP crystal consists of a lipid bilayer membrane (4, 7). The hydrophilic peptide of the N-terminal transmembrane region may be integrated into the lipid bilayer. The dense carboxyl and hydroxyl groups in the C-terminal regions may interact directly with the mineral surface.

For the proteins that directly interact with biominerals, two plausible functions are suggested: (i) initiation of crystal nucleation (25, 27, 30) and (ii) inhibition and regulation of crystal growth (24, 31) and determination of morphology (32, 33). The initiation starts from the interaction and accumulation of metal ions on or in the organic molecules. The acidic groups (carboxylic acid derived from aspartate and glutamate) are known to have strong affinity with metal ions and to act as bridging ligands. The hydroxyl groups in serine, threonine, and tyrosine also possess metal-binding capability. These side chains of amino acids preferentially bind to metal ions such as Fe$^{3+}$, Ca$^{2+}$, and Mg$^{2+}$ (23). The binding of these groups to Cu$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$ is unlikely. The competitive iron binding assay supports that the observed iron binding is derived from the carboxyl and hydroxyl groups. Therefore, the hydrophilic C-terminal region in Mms6 is considered to initiate crystal nucleation in magnetite formation. It has been reported that a small acidic protein called statherin possesses dense negative charges in the N-terminal region that are responsible for the mineral-adsorbing ability, inhibiting crystal growth (24). Although the interaction between organic molecules and solid phases is dependent upon the metal (34), the observed iron-binding ability of Mms6 is also thought to inhibit iron mineralization, controlling morphology. However, a control mechanism of morphology is still unknown.

In magnetic bacteria, the origin of BMP membrane vesicles has been suggested to arise through invagination of the cytoplasmic membrane (4, 7). The biological compartmentalization through the formation of enclosed vesicles enables the chemical process of magnetite formation to be optimized and regulated. On the basis of the high resolution transmission electron microscopy and Mössbauer spectroscopy results, mineral transformation through BMP synthesis was hypothesized (35). The first step involves the accumulation of iron, followed by the precipitation of hydrated iron oxide and, finally, phase transformation of amorphous iron to magnetite during the nucleation stage and surface-controlled growth similar to the formation of magnetoferritin cores (36). Magnetite films have been synthesized using –OH groups of arrayed lipid layers as molecular templates for crystal synthesis by controlling gas-phase oxygen concentrations at room temperature (37). The densely arrayed –OH groups derived from carboxylate adsorb iron ions and stimulate magnetite growth. Similarly, magnetite has been synthesized in iron solutions by adding recombinant Mms6. The observed precipitate color change suggests the phase transformation of iron oxides, Fe(OH)$_3$ and γ-FeOOH, through magnetite. Significant shape similarity was observed between BMP and the crystals obtained from in vitro mineralization using Mms6. The –OH-rich C-terminal parts of the proteins tightly bound to BMPs might act as templates for magnetite crystal formation and direct the shape of magnetite crystals formed.

In this study, the existence of proteins tightly associated with BMP crystals and their iron-binding ability have been shown. Mms6 provides nucleation sites for precipitation of iron oxide in the BMP vesicle.

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A Novel Protein Tightly Bound to Bacterial Magnetic Particles

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A Novel Protein Tightly Bound to Bacterial Magnetic Particles in *Magnetospirillum magneticum* Strain AMB-1
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