Genetic Dissection of the mamAB and mms6 Operons Reveals a Gene Set Essential for Magnetosome Biogenesis in Magnetospirillum gryphiswaldense

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Biosynthesis of bacterial magnetosomes, which are intracellular membrane-enclosed, nanosized magnetic crystals, is controlled by a set of >30 specific genes. In Magnetospirillum gryphiswaldense, these are clustered mostly within a large conserved genomic magnetosome island (MAI) comprising the mms6, mamGFDC, mamAB, and mamXY operons. Here, we demonstrate that the five previously uncharacterized genes of the mms6 operon have crucial functions in the regulation of magnetosome biomineralization that partially overlap MamF and other proteins encoded by the adjacent mamGFDC operon. While all other deletions resulted in size reduction, elimination of either mms36 or mms48 caused the synthesis of magnetite crystals larger than those in the wild type (WT). Whereas the mms6 operon encodes accessory factors for crystal maturation, the large mamAB operon contains several essential and nonessential genes involved in various other steps of magnetosome biosynthesis, as shown by single deletions of all mamAB genes. While single deletions of mamL, -P, -Q, -R, -B, -S, -T, and -U showed phenotypes similar to those of their orthologs in a previous study in the related M. magneticum, we found mamL and mamN to be not required for at least rudimentary iron biomineralization in M. gryphiswaldense. Thus, only mamE, -L, -M, -O, -Q, and -B were essential for formation of magnetite, whereas a mamL mutant still biominalized tiny particles which, however, consisted of the nonmagnetic iron oxide hematite, as shown by high-resolution transmission electron microscopy (HRTEM) and the X-ray absorption near-edge structure (XANES). Based on this and previous studies, we propose an extended model for magnetosome biosynthesis in M. gryphiswaldense.

Magnetotactic bacteria (MTB) orient along Earth’s magnetic field lines to navigate to their growth-favoring microoxic habitats within stratified aquatic sediments (1). This behavior is enabled by the synthesis of ferrimagnetic intracellular organelles termed magnetosomes (2). In the alphaproteobacterium Magnetospirillum gryphiswaldense and related MTB, magnetosomes consist of crystals of the magnetic iron oxide magnetite (Fe₃O₄) enclosed by the magnetosome membrane (MM), which contains a specific set of about 30 proteins (3, 4). The biosynthesis of magnetosomes is a complex process that comprises the (i) invagination of vesicles from the inner membrane (5, 6), (ii) sorting of magnetosome proteins to the MM (7), (iii) iron transport and crystallization of magnetite crystals (8), (iv) crystal maturation (7), and (v) assembly as well as positioning of mature crystals into a linear chain along a filamentous cytoskeletal structure (6, 9). Each step is under strict genetic control, and responsible genes were found to be located mostly within a genomic magnetosome island (MAI) (10, 11), comprising the mms6 operon (mms6₀), mamGFDC operon (mamGFDC₀), mamAB operon (mamAB₀), and mamXY operon (mamXY₀) (10–12). These operons were found to be highly conserved also in the closely related Magnetospirillum magnetica (13–17). It has been shown that the regions between and flanking the identified magnetosome operons have no functional relevance for magnetosome biosynthesis in M. gryphiswaldense and M. magnetica (7, 18). In M. gryphiswaldense, the mms6, mamGFDC, mamAB, and mamXY operons are transcribed as single polycistronic messengers under the control of the Pmms6, PmamDC, PmamAB, and PmamXY promoters, respectively (19, 20). A mutant with a deletion of mamGFDC₀, encoding the most abundant magnetos ines proteins, retained the ability to form magnetic, although smaller and less regular, magnetosomes, whil plasmidal overexpression of the entire mamGFDC operon yielded magnetite particles even larger than those produced by the wild type (WT) (21). Elimination of the corresponding region R3 in M. magnetica, comprising in addition parts of mms6₀, caused a severe biomineralization defect, resulting in cells with reduced magnetosome sizes and numbers (7). Deletion of the entire mamXY operon resulted in smaller and misshaped magnetosome particles in M. gryphiswaldense (18), whereas no obvious phenotype was observed for mamXY⁻ deletion in M. magnetica (7). mms6₀ of M. gryphiswaldense comprises the genes mgr4074, mms6, mmsF, mgr4071 (renamed here mms36), and mgr4070 (renamed here mms48; see Fig. S1 in the supplemental material), which was previously predicted to encode a TPR (tetratricopeptide repeat)-like protein (18). A mutant in which the entire mms6₀ was deleted (∆A10) was also severely impaired in the...
biomineralization of magnetite crystals, which exhibited defects in crystal morphology, size, and organization. However, the individual functions of mgr0474, mms6, mmsF, mms36, and mms48 as well as their contribution to the strong phenotype corresponding to Δmms6op have remained unknown. In M. magnetico, the mms6 cluster was described to comprise only amb0955 (mgr0474), amb0956 (mms6), and amb0967 (mmsF) and to lack homologs of mms48 and mms36 (22). Single gene deletions of mms6 in M. magnetico by different groups revealed inconsistent phenotypes. Whereas Tanaka et al. (23) reported an important regulatory function of Mms6 for magnetosome morphology, Murat et al. observed only minor effects on magnetosome biosynthesis after deletion of mms6 in vivo (22, 24). In vitro, the small (12.76 kDa in M. gryphiswaldense and 14.69 kDa in M. magnetico) Mms6 protein was shown to be tightly bound to isolated bacterial magnetite crystals as visualized by atomic force microscopy and transmission electron microscopy (TEM) (25, 26). In vitro crystallization experiments suggested that Mms6 and peptides mimicking it have iron-binding activity and affected the formation of cubo-octahedral crystal morphologies (27, 28).

In contrast to the smaller accessory operons, mamABop was found to contain genes absolutely essential for magnetosome biosynthesis in M. gryphiswaldense and M. magnetico (18, 22). Whereas mamABop was found to be sufficient to support at least some rudimentary biomineralization of small magnetite crystals even in the absence of all other magnetosome operons in both strains (18, 22), the mamXY, mamGFDC, mms6, and mamAB operons were required all together for magnetosome biomineralization upon their transfer into the foreign host Rhodospirillum rubrum (29).

A recent comprehensive genetic dissection of mamABop in M. magnetico revealed that mamH, -P, -R, -S, and -T encode accessory functions for magnetosome synthesis, since mutants display various biomineralization defects, whereas mamU and -V had no obvious magnetosome phenotype (7). As in M. gryphiswaldense (see below), mamK and mamI were implicated in magnetosome chain assembly, but their loss did not affect biomineralization (30, 31). However, deletions of mamL, -E, -L, -M, -N, -O, and -Q as well as mamB (cotediated with their respective orthologs) fully abolished magnetosome synthesis in M. magnetico (7, 32). Whereas MamL, -L, -Q, and -B were suggested to be essential for vesicle genesis, MamE, -O, -M, and -N were classified to be mainly required only for magnetite crystallization (7). The discovery of a small “magnetosome islet” in the genome of M. magnetico with further copies of mamE, -J, -K, -L, -M, and -F as well as mamD suggested genetic redundancy that has to be clarified with respect to determination of the minimal essential gene set (33).

In M. gryphiswaldense, the 16.4-kb mamABop operon contains 17 genes (mamH, -I, -E, -J, -K, -L, -M, -N, -O, -P, -A, -Q, -R, -B, -S, -T, and -U) (see Fig. 3). Only a few genes of mamABop so far have been analyzed individually in this organism. The actin-like protein MamK forms a filamentous structure for magnetosome assembly and interacts with the acidic protein MamI, which is involved in connecting magnetosomes to the filament. Both proteins, however, have no or only minor effects on biomineralization (6). Deletion of mamH caused a moderate decrease of magnetosome number and size, whereas codeletion of mamH and its partial homolog mamZ had a considerably stronger effect, with only very few or no regular crystals detectable in the cells (20). Deletion of mamE, -O, -M, and -B either resulted in a total inhibition of crystal nucleation or prevented MM vesicle synthesis (18, 34, 35). However, mamL, -L, -N, -P, -A, -Q, -R, -S, -T, and -U have not yet been analyzed individually by mutagenesis, and it has remained unknown whether they have functions similar to or distinct from those of their corresponding orthologs in M. magnetico. Finally, it is not clear which genes constitute the minimal set of essential determinants for magnetosome biomineralization in M. gryphiswaldense.

In this study, we analyzed the functional relevance of proteins encoded by mms6op and mamABop for the biosynthesis of magnetic minerals in M. gryphiswaldense. We demonstrate that besides Mms6 and MmsF, mms6op of M. gryphiswaldense encodes two further important regulators (Mms36 and Mms48) for magnetosome biomineralization. Whereas deletions of mamA, -R, -S, -T, and -U resulted in phenotypes similar to those observed for deletion of homologous genes in M. magnetico, we show that in contrast to the case with M. magnetico, M. gryphiswaldense ΔmamN and ΔmamI mutants still synthesize particles, thus further shrinking the minimal gene set for iron biomineralization to mamE, -L, -M, -O, -Q, and -B. Finally, we propose an extended model for magnetosome biosynthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. WT and mutant strains of M. gryphiswaldense were grown in liquid modified flask standard medium (FSM) at 30°C under microaerophilic conditions if not otherwise specified (8, 36). Therefore, cells were cultivated in flasks, closed with butyl-rubber stoppers after incubation with a gas mixture of 2% O2 and 98% N2, or in purged jars. For anaerobic requirements, O2 was excluded from the gas mixture, while aerobic conditions were generated through free gas exchange with air. Escherichia coli strains were cultivated as previously described (37), and lysogenic broth medium was supplemented with 1 mM DL-α-diaminopimelic acid (DAP) for cultivation of E. coli strain BW29427 as well as WM3064. For selection of antibiotic-resistant cells, media were supplemented with 25 g/ml of kanamycin (Km), 12 g/ml of tetracycline (Tet), and 15 g/ml of gentamicin (Gm) for E. coli strains and 5 g/ml of Km, 5 g/ml of Tet, and 20 g/ml of Gm for M. gryphiswaldense strains.

Molecular and genetic techniques. Oligonucleotide sequences (see Table S2 in the supplemental material) were deduced from the working draft genome sequence of M. gryphiswaldense (GenBank accession number CU459003), and sequences were purchased from Sigma-Aldrich (Steinheim, Germany). Genetic fragments were amplified by standard PCR procedures with Phusion polymerase (NEB GmbH, Frankfurt am Main, Germany), and generated plasmids were sequenced with an ABI 3700 capillary sequencer (Applied Biosystems, Darmstadt, Germany), utilizing BigDye Terminator v3.1. Data were analyzed with Vector NTI Advance 11.5 (Invitrogen, Darmstadt, Germany) or MacVector 7.2.3 (Oxford Molecular, Oxford, United Kingdom) software.

Generation of unmarked deletion mutants. Markerless single gene deletions within the mamAB, mms6, and mamGFDC operons were partially realized with the pORFM_galk plasmid (59). The vector was digested with BamHI and KpnI to insert the approximately 1-kb downstream and upstream fragments of mamL, -L, -N, -P, -Q, -R, -S, -T, and -U, mms6, mms48, and mmsFop. For integration of homologous regions of mamA and mamL, the plasmid was digested with BamHI/NotI and Nsi/SpeI, respectively. Oligonucleotides used to amplify the 5’ and 3’ flanking sequence from M. gryphiswaldense by PCR are listed in Table S3 in the supplemental material. Both fragments were linked by an overlap PCR with the first and last listed corresponding oligonucleotide, subcloned into pJet1.2/blunt, sequenced, and ligated into the digested pORFM_galk vectors. Generated plasmids were termed pALΔmamL,
pOR\(\Delta\mbox{mamL}\), pAL\(\Delta\mbox{mamN}\), pAL\(\Delta\mbox{mamP}\), pAL\(\Delta\mbox{mamQ}\), pAL\(\Delta\mbox{mamR}\), pAL\(\Delta\mbox{mamS}\), pAL\(\Delta\mbox{mamT}\), pAL\(\Delta\mbox{mamU}\), pAL\(\Delta\mbox{mms36}\), pAL\(\Delta\mbox{mms48}\), and pAL\(\Delta\mbox{mms6}\). Deletion of \(\mbox{mms6}\), \(\mbox{mmsF}\), and \(\mbox{mamF}\) and double deletion of \(\mbox{mms6}\) and \(\mbox{mamF}\) was accomplished by double-crossover method. Oligonucleotides for amplification of flanking sections are listed in Table S3. Regions were cloned into pLet1.2/blunt and sequenced. Plasmid pCM184 was digested with Apal/Sacl, and 3’ end regions were inserted for deletion of \(\mbox{mms6}\), \(\mbox{mmsF}\), and \(\mbox{mamF}\). Generated strains \(\mbox{mmsF}\) were termed \(\mbox{mmsF}\) and \(\mbox{mamF}\) mutants. Mutants generated by double crossover were cultivated in 10 ml of F5, and excision of the Km resistance gene was induced after conjugation with the Cre expression plasmid pCM157. Generated strains were termed \(\mbox{mmsF}\) and \(\mbox{mamF}\) mutants. For double deletion of \(\mbox{mms6}\) and \(\mbox{mamF}\), the plasmid pCM184 was introduced into \(\mbox{mmsF}\), and deletion was verified as described above, resulting in the \(\mbox{mmsF}\) \(\mbox{mamF}\) strain.

Complementation of generated mutants and GFP localization. For MamC-green fluorescent protein (GFP) localization experiments, plasmid pCM236 was integrated into the genomes of \(\mbox{mmsF}\), \(\mbox{mamN}\), \(\mbox{mamP}\), \(\mbox{mamQ}\), \(\mbox{mamR}\), \(\mbox{mamS}\), \(\mbox{mamT}\), and \(\mbox{mamU}\). For transcomplementation of the other mutant strains, derivatives of pBAM\(\mbox{mmsF}\), pBAM\(\mbox{mmsP}\), pBAM\(\mbox{mmsQ}\), and pBAM\(\mbox{mmsR}\) were also used for complementation studies in the WT. For complementation of the \(\mbox{mmsF}\), \(\mbox{mmsP}\), \(\mbox{mmsM}\), and \(\mbox{mamF}\) mutants, corresponding genes were amplified with oligonucleotides listed in Table S3. Genes were inserted into pAPI50 after digestion with BamHI/Ndel, resulting in pAL\(\mbox{mmsF}\), pAL\(\mbox{mmsP}\), pAL\(\mbox{mmsM}\), and pAL\(\mbox{mamF}\). For integration of genes \(\mbox{mms6}\), \(\mbox{mmsP}\), and \(\mbox{mamF}\) after amplification with AL152/AL136, generating pAL\(\mbox{mms6},\mbox{mmsP},\mbox{mamF}\), 4074. For transcomplementation studies, the plasmids were transferred to the respective deletion mutants by conjugation. \(\mbox{mms6}\) \(\mbox{mmsP}\) and \(\mbox{mamF}\) were complemented with pAL\(\mbox{mms6}\) and pAL\(\mbox{mmsP}\), respectively. Plasmids pAL\(\mbox{mms48}\), pAL\(\mbox{mamDC}\), and pAL\(\mbox{mamF}\) were used for complementation studies in the \(\Delta\mbox{mamF}\) mutant.

Analytic methods. Optical density and magnetic response (\(\text{C}_{\text{mag}}\)) were analyzed photometrically at 565 nm (38). The applied magnetic field for \(\text{C}_{\text{mag}}\) measurements was about 70 mT, which is able to magnetize very small or irregular magnetosomes within the superparamagnetic state. Intracellular iron concentrations were measured after incubation under anaerobic conditions as described previously (39).

Phase-contrast and fluorescence microscopy. M. gryphiswaldense strains with genomic egfp were grown in 5 ml of F5 medium in six-well plates for 16 h at 30°C and 2% O2 without agitation. Cells were immobilized on agarose pads (F5 salts in H2O, supplemented with 1% agarose) and imaged with an Olympus BX81 microscope equipped with a 100 UPLSAPO100XO objective (numerical aperture of 1.40) and a Hamamatsu Orca AG camera. Olympus cell software was used to capture and analyze images.

X-ray absorption spectroscopy. Bacterial cultures (90 to 135 ml) were pelleted by centrifugation (5 min at 9,000 × g and 4°C) and washed 3 times by resuspension with 5 ml of Tris-buffered saline (TBS; pH 7.6) and centrifugation. Pellets were then resuspended in 100 μl of TBS plus 25 μl of glycerol and frozen in liquid nitrogen on sample holders with Kapton film support. Samples were shipped to the European Synchrotron Radiation Facility (ESRF) on dry ice, where they were stored at −80°C until measurement. Fe K-edge X-ray absorption near-edge structure (XANES) spectra were recorded at the undulator beamline ID26 of the ESRF. We used an Si (311) double-crystal monochromator and focusing mirrors with beam spot size of 200 by 200 μm2 on the samples. Data were recorded in fluorescence detection mode using a Rowland-type spectrometer equipped with 4 Ge (440) analyzer crystals and an Si photodiode. After all measurements, samples were cooled to around 10 K using a liquid He cryostat. XANES spectra were recorded with 0.1-eV steps from 7,100 to 7,200 eV. To improve data quality, 10 to 100 XANES scans were recorded for each sample. Data were averaged using PyMca 4.6.2 after evaluation for iron photoreduction. Averaged spectra were normalized and fitted using Demeter 0.9.16. As reference materials, we used spinach ferredoxin (Sigma-Aldrich), hematin (20- to 60-nm grain size; Alfa Aesar), magnetite, ferritohydrite, and phosphate-enriched ferric oxyhydroxides (described in reference 41).

RESULTS

Deletion mutagenesis of the \(\mbox{mms6}\) operon and \(\mbox{mamF}\). After re-assessment of annotation and correction of the MmsF N terminus (see Fig. S1 in the supplemental material), we generated various unmarked in-frame single and double deletions of all \(\mbox{mms6}\) genes as well as of \(\mbox{mamF}\) (localized in the adjacent \(\mbox{mamDC}\) operon), which is highly similar (61% amino acid [aa] identity between the products) to \(\mbox{mamF}\) (see Table 1 and Fig. 1 for overview of deletions and resulting phenotypes). We found the hypothesical \(\mbox{mamF}\) a pseudogene with no role in biomineralization, although further studies are needed to address the expression and putative localization of its gene product. The \(\mbox{mms6}\) strain had slightly smaller crystals (30 nm); wild type [WT], 36 nm) that were scattered throughout the cell, either aligned in irregularly spaced “pseudochains” (i.e., with <10 crystals per chain) or approximating WT-like chain configurations (Table 1 and Fig. 1). Crystals between 30 and 35 nm were predominant (WT, 40 to 45 nm), but particles larger than 60 nm were absent (WT, <70 nm; see Fig. S2 in the supplemental material). The average crystal number per cell was reduced to 30 (WT, 34 particles per cell), and the magnetic response of a \(\mbox{mms6}\) culture
TABLE 1 Characterization of the generated mutants

<table>
<thead>
<tr>
<th>Relevant genotype of strain</th>
<th>Deleted gene(s)</th>
<th>Magnetic response (Cmag*)</th>
<th>Avg magnetosome size (nm)</th>
<th>Magnetosome size (% of WT)</th>
<th>No. of magnetosomes per cell*</th>
<th>Maximum size (nm)</th>
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<tbody>
<tr>
<td>WT</td>
<td></td>
<td>2.0 ± 0.1</td>
<td>35.6 ± 13.0</td>
<td>100</td>
<td>34.3 ± 8.4</td>
<td>69.2</td>
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<td></td>
<td>Intermediate</td>
<td>19.7 ± 6.9</td>
<td>55.3</td>
<td>16.8 ± 6.2</td>
<td>39.6</td>
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<tr>
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<td>mms6</td>
<td>WT</td>
<td>30.4 ± 9.0</td>
<td>85.4</td>
<td>29.7 ± 6.2</td>
<td>57.3</td>
</tr>
<tr>
<td>Δmms6F</td>
<td>mms6F</td>
<td>WT</td>
<td>28.6 ± 8.0</td>
<td>80.3</td>
<td>30.4 ± 7.4</td>
<td>60.9</td>
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<tr>
<td>Δmms6G</td>
<td>mms48</td>
<td>WT</td>
<td>46.4 ± 14.8</td>
<td>130.3</td>
<td>16.0 ± 5.6</td>
<td>82.8</td>
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<tr>
<td>Δmms6C</td>
<td>mms36</td>
<td>WT</td>
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<td>mamF</td>
<td>WT</td>
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<td>33.6 ± 10.4</td>
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<td>mms6F, mamF</td>
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<td>24.1 ± 6.8</td>
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<td>104.8</td>
<td>31.8 ± 8.4</td>
<td>65.3</td>
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</table>

* Values are means ± standard deviations.

was slightly weaker than that of the WT (Cmag(Δmms6) 1.7 ± 0.1; Cmag(WT) 2.0 ± 0.1 [Table 1]).

The Cmag of ΔmmsF cells was similar to that of Δmms6 cells. Magnetosomes displayed variable intracellular arrangements, such as one or more short chains, partially scattered crystals, or the absence of any chain-like alignment (Fig. 1). Mean crystal sizes were reduced to <30 nm, whereas the particle number was only slightly lower than in the WT (Table 1; see also Fig. S2 in the supplemental material). Since the high similarity, 61%, between Mms36 and MamF suggested possible functional redundancy, mamF was eliminated both alone and in combination with mmsF. In the ΔmamF mutant, MamC, encoded downstream of mamF in the same operon, was found to be properly expressed by immunodetection, indicating that deletion of mamF had no polar effect (data not shown). The mean crystal sizes (34 nm) and numbers (34 per cell) were similar in the ΔmamF mutant to those in the WT. However, the combined excision of both genes within the Δmms6F ΔmamF mutant resulted in a more drastic decrease in size (25 nm) and number (27 crystals per cell [Fig. 1 and Table 1; see also Fig. S2]). Thus, loss of Mms6F had a more pronounced effect on crystal size, number, and alignment than loss of MamF, and the additive effect of their combined deletion suggested that both proteins are involved in size control.

Double deletion of mms6F and mms6 reduced the size to 24 nm and the number to 24 crystals per cell (Table 1). However, iron content, size, and number of magnetite particles as well as Cmag (1.6 ± 0.1) of the Δmms6F Δmms6 strain were still higher than in the ΔA10 strain (in which the entire mms6 operon has been deleted) (Cmag 1.0 ± 0.1), with particles of 20 to 25 nm prevailing in both strains (Fig. 1 and Table 1; see also Fig. S2 in the supplemental material). HRTEM images of particles from the ΔA12 strain, in which the entire mms6 and mamGFDC operons had been deleted together (18), revealed fringes spaced in correspondence to magnetite. This indicates that the deleted genes alone do not have a critical role in magnetite formation (Fig. 2). In contrast to the strong size reduction observed in all other mutant strains, deletions of mgr4070 and mgr4071 (renamed into mms48 und mms36 according to their predicted protein masses of 48 and 36 kDa, respectively) unexpectedly caused a substantial increase in mean crystal size. Particles synthesized by both strains resembled WT crystals in shape but were significantly larger in the Δmms6 (39 nm) and Δmms48 (46 nm [Fig. 1 and Table 1; see also Fig. S2]) mutants. This is equivalent to a mean size increase of about 30% compared to that of the WT for the latter strain, in which crystals between 50 and 60 nm were most abundant, with a maximum size of up to 85 nm (see Fig. S2). However, both strains synthesized fewer particles than the WT (Δmms6 mutant, 22 per cell; Δmms48 mutant, 16 per cell), and whereas in the WT magnetosome chains of larger particles at midchain are usually flanked by numerous smaller crystals, those characteristic small crystals (15 to 25 nm) were less frequent at the chain ends in the Δmms6 and Δmms48 mutants (Fig. 1 and Table 1; see also Fig. S2). Thus, the predominance of larger (>30 nm) particles partly accounted for the substantially increased mean crystal size. However, despite the reduced particle numbers per cell, overall magnetite biomineralization was increased, as evident by the increased iron content of both deletion strains (21% more iron than for the WT). Genomic expression of additional copies of mms48 and mms36 did not significantly change mean particle size (WT::mms36 strain, 35 nm; WT::mms48 strain, 33 nm [Table 1]), but the size distribution was shifted toward smaller crystals for both strains. Crystals between 30 and 45 nm were predominant in the WT::mms36 and WT::mms48 strains, whereas particles larger than 60 nm were not observed, unlike for WT crystals, which were most frequently between 40 and 45 nm, with a maximum size up to 70 nm (data not shown). Whereas particle number was WT-like for overexpression of Mms36 (32 per cell), crystal number was increased for the WT::mms48 strain (40 per cell [Table 1]). Interestingly, cells con-
taining double chains were more abundant for the WT::mms48 strain (67%, compared to 28% for the WT::mms36 strain and 32% for the WT; see Fig. S3 in the supplemental material).

In summary, all proteins encoded by mms6\textsubscript{op} are involved in control of magnetosome size and/or number. The previously observed severe biomineralization defects in the \textit{H9004}mms6\textsubscript{op} strain are thus due to the loss not of a single gene but of several genes, which points toward a cumulative effect on magnetosome synthesis by various proteins encoded by mms6\textsubscript{op}.

Deletion analysis of the \textit{mamAB} operon: \textit{mamE}, -L, -M, -O, -Q, and -B are essential for iron biomineralization. First, annotations of all 17 \textit{mamAB}\textsubscript{op} genes were reassessed. Sequences corresponding to the N termini that were conserved between all three closely related magnetospirilla \textit{M. gryphiswaldense}, \textit{M. magnetotacticum}, and \textit{M. magnetotacticum} were considered the most likely translation starts. Annotations were corrected accordingly for \textit{mamI} and \textit{mamL} (see Fig. S4 in the supplemental material) and experimentally confirmed by the ability of genes to complement their respective gene deletions. In addition to the previous deletions of the \textit{mamAB}\textsubscript{op} genes, we constructed 10 single in-frame deletions comprising \textit{mamI}, -L, -N, -P, -A, -Q, -R, -S, -T, and -U. As expected, all resulting deletion strains displayed WT-like growth and morphologies. However, deletion mutants were impaired in magnetosome biomineralization to variable extents. Based on their magnetic responses, mutants were considered either (i) magnetically responsive, with variable but significant \(C_{\text{mag}}\) (\(\Delta\textit{mamN}, -P, -A, -R, -S, -T,\) and -U mutants), or (ii) entirely nonmagnetic, without any detectable \(C_{\text{mag}}\) (\(\Delta\textit{mamI}, -Q,\) and -L mutants) (Table 1). TEM analysis confirmed that the first group of strains were still able to synthesize magnetosome-like particles but displayed various distinct phenotypes with respect to crystal morphology, size, and number per cell (Fig. 3). \(\Delta\textit{mamU}\) cells were hardly distinguishable from WT cells and produced 32 cubo-octahedral crystals per cell with a size of 37 nm. All other mutants showed a drastically decreased magnetosome size, number, and/or alignment. Magnetosomes of the \(\Delta\textit{mamA}\) strain had a WT-like size, 35 nm, but their number was substantially decreased, to 10 per cell. \(\Delta\textit{mamS}\) particles exhibited a widely spaced linear chain-like arrangement within the cell. Whereas the crystal size was strongly decreased (22 nm), the crystals were present in about same numbers as in the WT (35 particles per cell). The \(\Delta\textit{mamT}\) strain also synthesized irregularly spaced magnetosome chains, whereas in some cells larger magnetosomes appeared at the chain center and formed condensed “pseudochains.” Due to the prevalence of smaller crystals, the mean particle size was decreased to 29 nm, whereas their number was WT-like (32 particles per cell). Several \(\Delta\textit{mamR}\) cells showed scattered magnetosomes lacking any chain-like alignment or showed short, densely spaced chains flanked by smaller particles with irregular morphologies or WT chains (average size, 29 nm; number per cell, 34). \(\Delta\textit{mamP}\) cells at first glance seemed to contain only a few (i.e., not more than six magnetosomes; mean, three) particles larger than for the WT (59 nm on average). However, at closer inspection, numerous very small and irregularly shaped crystals flanking the larger crystals became apparent, with an average size of 16 nm (see arrows in Fig. 3). In total, \(\Delta\textit{mamP}\) cells synthesized, on average, only 19

![FIG 1. Molecular organization of the mms6 and mamGFDC operons in M. gryphiswaldense and TEM micrographs of generated deletion mutants. Dark blue bars indicate extent of gene deletions generated in this study. Light blue bars indicate gene deletion mutants generated previously (18, 21). Scale bars, 1 \(\mu\text{m}.\)](http://jb.asm.org/2662)
crystals, with a mean size of 22 nm. HRTEM of the two distinct particle types revealed that the lattice fringes for the larger crystals corresponded clearly to magnetite, whereas in contrast, the smaller and poorly crystalline particles produced lattice fringes characteristic for hematite (Fig. 2). The \( \Delta \text{mamN} \) mutant showed a very weak but detectable magnetic response (C_{mag} 0.1). TEM confirmed the presence of a few (11 per cell) tiny, widely spaced crystals with a size of only 18 nm. HRTEM images of these particles and their Fourier transforms indicated that these crystals have the structure of magnetite.

The \( \Delta \text{mamI} \), \( \Delta \text{mamL} \), and \( \Delta \text{mamQ} \) mutants represent the second class of mutants with no detectable magnetic response (C_{mag} 0).

The \( \Delta \text{mamQ} \) and \( \Delta \text{mamL} \) mutants were entirely devoid of any clearly recognizable crystalline electron-dense structures (Fig. 2). In the \( \Delta \text{mamL} \) mutant, occasionally a few tiny (around 10 nm) structures were observed (not shown); however, these were difficult to discern unambiguously from the cellular body and the background. The relevance and identity of these structures remain to be verified in future studies with higher resolution. Careful analysis of \( \Delta \text{mamI} \) cells, however, revealed the presence of a few (10 per cell) electron-dense particles with highly irregular or elongated morphologies and a size of 15 nm (Fig. 3). As shown by HRTEM, the nuclei within \( \Delta \text{mamI} \) cells were composed of several small grains that formed thin aggregates (Fig. 2). Lattice fringes were observed in only two particles, and according to the Fourier transforms of the HRTEM images, the spacing between the fringes is very close to the d (012) and d (014) spacing in hematite. XANES (X-ray absorption near-edge structure) spectra obtained from whole \( \Delta \text{mamI} \) and \( \Delta \text{mamN} \) cells were clearly distinct from those of pure magnetite as in the WT and suggest that the ferrous compounds are predominantly Fe-S clusters (proteins) that account for around 40% of the total iron content in the cells (see Fig. S5 in the supplemental material). Magnetite was clearly present in \( \Delta \text{mamN} \) cells (around 50% of total iron), whereas the low fit quality for \( \Delta \text{mamI} \) did not allow us to reliably determine the structure of the Fe present in the bacteria apart from Fe-S (see supplemental material for more detailed information). However, the overall line shape appears most consistent with an amorphous or only poorly ordered Fe compound as suggested by HRTEM.

Intracellular localization of the magnetosome chain marker MamC-GFP. All mutant strains could be complemented by either genomic reintegration or plasmidal transfer (see the supplemental material). We studied the ability of all mutants to properly localize the abundant MamC magnetosome protein, which served as a marker for magnetosome chain localization in previous studies (6, 35, 42). To this end, MamC was tagged by a chromosomal in-frame enhanced GFP (EGFP) insertion on pFM236 that shows in WT cells a continuous straight-line fluorescence signal (see Fig. S6 in the supplemental material). Within \( \Delta \text{mamN} \), -P, -S, and -T strains, MamC-GFP localized as shorter structures but still...
showed a linear localization running along the inner curvature of the cell. Within ΔmamI cells, a short but still elongated fluorescence signal at midcell was observed. Thus, MamI, -N, -P, -R, -S, and -T are not required for proper MamC localization. On the contrary, in ΔmamA, -Q, and -L cells, no defined position of the MamC-GFP signal was detectable, but instead a diffuse spot like accumulation within the cell was predominant (see Fig. S6). In the ΔmamA mutant, the magnetosome formation was not inhibited, even though MamC is misplaced within this strain, which suggests that MamA may interact with MamC.

**DISCUSSION**

The Mms6 operon encodes nonessential magnetosome proteins crucial for proper crystal growth. As in *M. magneticum* (reduction of size of crystals by 19% [22]), we observed only minor biominalerization defects upon deletion of *mms6* in *M. gryphiswaldense* (15% size reduction). Only a 20% size reduction was also seen after *mmsF* deletion in *M. gryphiswaldense*, which, however, is weaker than its deletion phenotype in *M. magneticum* (52% size reduction [22]). However, double deletion of *mms6* and *mmsF* resulted in an almost 32% size reduction, which suggests a certain functional overlap between Mms6 and MmsF. We found functional redundancy between *mmsF* and *mamF* (carried on the adjacent *mamGFDC* operon), since their double deletion exacerbated defects in crystal maturation (30% size reduction). Hence, the loss of several genes together contributed to the strong magnetosome defect (45% size reduction) observed after deletion of the entire *mms6* operon (ΔA10), which indicates a cumulative regulation of magnetosome biomineralization.

*mms6*op of *M. gryphiswaldense* contains two additional genes, named *mms36* (mgr4071) and *mms48* (mgr4070), that are expressed under magnetosome-forming conditions [18] but had not yet been studied by deletion analysis in either *M. gryphiswaldense* or *M. magneticum*. Surprisingly, their deletion caused the synthesis of larger magnetite crystals instead of size reduction. Since no conserved domains or motifs are present in Mms36 and -48, apart from weak similarity to proteins involved in porphyrin synthesis (Mms36, 29% to uroporphyrinogen III synthase of *Rhodospirillum rubrum*; Mms48, 28% to HemY-like proteins, possibly involved in porphyrin biosynthesis [43, 44]), their precise in vivo functions are difficult to infer. They might either be inhibitors of crystal growth (32) or recruit other inhibitory proteins to the MM in order to prevent excessive crystal growth.

**Genetic analyses of the mamAB operon:** MamU, -T, -S, -R, -A, -N, and -I are not essential for iron biomineralization in *M. gryphiswaldense*. We found that deletion of several genes (*mamU*, -T, -S, -R, -A, -P, -Q, and -L) from *mamAB*op of *M. gryphiswaldense* essentially phenocopied the deletions of their orthologs in *M. magneticum* (7). Loss of *mamU*, -T, -S, -R, -A, -P, and -N did not entirely abolish biominalerization of magnetite crystals. As in *M. magneticum*, deletion of MamU did not have any detectable magnetosome phenotype in *M. gryphiswaldense*. MamT of *M. magneticum* as well as of *M. gryphiswaldense* contains a double cytochrome *c* motif (CXXCH), referred to as the magnetochrome domain, necessary for heme binding (45). It was speculated that MamT therefore transfers electrons to balance the ferric-to-ferrous iron ratio form required for magnetite formation (45). Deletion of *mamT* in *M. gryphiswaldense* resulted in smaller magnetite particles as in *M. magneticum*, supporting its previously predicted function in crystal maturation. As in the ΔmamT mutant, deletion of *mamR* in *M. gryphiswaldense* resulted in smaller crystals and partially modified chain formation, similar to the phenotype observed for ΔmamR AMB. Thus, MamR is involved in controlling particle number and size, as also suggested for MamR of *M. magneticum* (7). We also confirmed a key role for MamS in *M. gryphiswaldense*, which has similarity to the putative serine proteases and magnetochrome domain-containing proteins MamE and -X (20, 32). However, MamS itself lacks a magnetochrome domain, which argues against its direct participation in redox control. The TPR domain-containing protein MamA was speculated to play a role in activation of biomineralization (5). It was suggested that MamA self-assembles through its putative TPR domain and concave site to form a large homoooligomeric scaffold surrounding the magnetosomes (46, 47), whereas its convex site interacts with other magnetosome-associated proteins, like MamC, and several unidentified proteins (46, 47). However, as in *M. magneticum*, the deletion of *mamA* in *M. gryphiswaldense* had only a weak effect (5), suggesting that these interactions are not essential or can be partly compensated by other proteins. In Δ*mamP* *M. gryphiswaldense*, particles larger than those synthesized by the WT were flanked by smaller and poorly crystalline particles, similar to the case with *M. magneticum* (48). MamP contains two closely spaced magnetochrome domains and was speculated to interact with MamE, -X, and -T through its PDZ domain and to somehow regulate the electron transport required for biomineralization of the mixed-valence iron oxide magnetite (20, 32, 48). Magnetosomes in Δ*mamP* *M. gryphiswaldense* show a crystallization defect (magnetite crystals flanked by flakes) similar to that of the mamX mutant of *M. gryphiswaldense* (20), which might indicate involvement of this gene in the same step of magnetosome biosynthesis. In contrast, phenotypes of Δ*mamT* (smaller particles) and Δ*mamE* (total loss of electron-dense particles) of *M. gryphiswaldense* are distinct from the phenotype of the Δ*mamP* strain, suggesting that some or all of the magnetochrome proteins have different or additional functions. However, MamE also contains, besides the magnetochrome domains, a pro tease and double PDZ domains, which might cause the differences between the generated mutants upon their deletion (32). Thus, further analyses are needed to explain the different observed mutant phenotypes, such as the specific deletion of the different magnetochrome domains. MamP from *M. magneticum* catalyzed the formation of ferrihydrite and magnetite from iron solutions *in vitro*, indicating that MamP binds and oxidizes iron (48). However, this ability of MamP is not essential *in vivo*, as the Δ*mamP*

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**FIG 3** Molecular organization and deletion analysis of the *mamAB* operon of *M. gryphiswaldense* as well as representative TEM micrographs of cells and magnetosome morphologies observed within the generated excision mutants. The highly conserved *mamAB* operon encodes 17 magnetosome proteins (*MamH*, -L, -E, -J, -K, -L, -M, -N, -O, -P, -A, -Q, -R, -B, -S, -T, and -U) in *M. gryphiswaldense* and was found to be essential and sufficient to maintain magnetite biogenesis (18, 22). Red arrows, genes essential for magnetosome crystal formation; blue arrows, genes nonessential for particle formation; gray bars, nonessential genes for which deletion strains were previously generated (6, 9, 20); blue bars, nonessential genes for which deletions were generated in this study; dark red bars, essential genes for which deletions were generated previously (34, 35); light red bars, essential genes for which deletions were generated in this study; white arrows, magnetosome chains or particles; black arrows, PHB granules.
mutant of *M. gryphiswaldense* continued to biomineralize particles of magnetite. Potentially this might be due to unchecked mineral growth after deletion of a major redox regulator (49) and/or the ability of other magnetochrome proteins, like MamX, -E, and -T, to partially compensate the loss of MamP.

In addition to the strong similarities between several AMB and *M. gryphiswaldense* mutants, we also found several striking differences between the two species. MamN was described to be essential for magnetosome biosynthesis, as indicated by the absence of electron-dense crystals in *M. magneticum* (7). However, our TEM, HRTEM, and XANES analyses revealed the presence of magnetite particles within ∆mamN *M. gryphiswaldense*. Because of its similarity to the human permease P, which is predicted to regulate precipitation of iron oxide particles (32), the crystal growth is affected by several magnetosome proteins, including MamE, which proteolytically remove a growth inhibitor(s) or activate growth-promoting proteins (32). Based on the conserved CXXCH heme-binding motifs within MamE, MamT, MamP, and MamX, it has been speculated that the proteins form a complex for electron transport to regulate electron flow (20, 45). MamS and MamR control crystal size by an unknown mechanism. MamN exhibits similarity to H⁺ translocating proteases and might be involved in crystal growth by regulating intramagnetosomal pH (51). Mms6 is tightly bound to the magnetosome crystals (26, 28) and assembles into coherent micelles for templating crystal growth (57). mms48 (Mms48) and mms36 (Mms36) act as inhibitors of crystal growth or recruit inhibiting proteins of particle growth by an unknown mechanism. The small, hydrophobic proteins MamG, MamF, MamD, and MamC control in a cumulative manner the growth of magnetite crystals (21). Magnetosomes were assembled into chains by the interaction of Mam with the actin-like MamK filament that is also involved in chain positioning (6, 9, 58). OM, outer membrane; IM, inner membrane; MP, magnetosome protein. Underlined proteins are proteins analyzed in this study by single gene deletion of encoding genes. Proteins in brackets are nonessential proteins encoded by *mamX1*op′.
shares homology with the LemA protein, which is conserved in several bacteria but whose function is uncertain (12, 55). MamQ has a high content of α-helices that are somewhat reminiscent of the EFC/BAR domain of formin binding protein 17 (7). BAR domains have the ability to sense and stabilize membrane curvatures (56), and their weak similarity to MamQ might hint towards related functions in MM vesicle genesis of the protein. The small protein MamL has no predicted function but was shown to be essential for magnetosome membrane genesis in _M. magneticum_ (7).

Despite the metabolic and genetic similarities between _M. magneticum_ and _M. gryphiswaldense_, previous studies already suggested that the functions of orthologous genes might be somewhat distinct in these organisms (6, 14, 18). Apart from the possibility that the tiny magnetite and hematite particles in _MamNandued MamM_ and _MamN_ of _M. gryphiswaldense_ simply had escaped detection in the corresponding mutants of _AMB_ (7), this might possibly due to the different genetic context, with only about 50% of all genes shared by the genomes of these two strains (14). In fact, it cannot be excluded that further genes outside the MAI partially compensate the loss of deleted genes, as observed in a recent study, in which a magnetosome islet outside the MAI compensated the deletion of _mamK_ in _M. magneticum_ (6, 33).

In summary, whereas in _M. magneticum_ eight proteins (MamL, -E, -L, -M, -N, -O, -B, and -Q) were found to be essential for magnetosome biomineralization, in _M. gryphiswaldense_ only six proteins (MamE, -M, -O, -B, and -Q, and most probably MamL) are essential for at least some rudimentary iron biomineralization and, if including MamL, seven proteins for the biosynthesis of magnetite-containing magnetosomes. This leads to an expanded model of magnetosome biosynthesis in _M. gryphiswaldense_ (Fig. 4). However, it remains to be shown whether these essential magnetosomes proteins are also sufficient for vesicle formation and crystallization even in the absence of the other factors encoded by the _mamAB_ and other magnetosome operons.

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