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## **McaA and McaB control the dynamic positioning of a bacterial magnetic organelle**

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► **To cite this version:**

Juan Wan, Caroline L Monteil, Azuma Taoka, Gabriel Ernie, Kieop Park, et al.. McaA and McaB control the dynamic positioning of a bacterial magnetic organelle. *Nature Communications*, 2022, 13 (1), pp.5652. 10.1038/s41467-022-32914-9 . hal-03872974

**HAL Id: hal-03872974**

**<https://hal.science/hal-03872974>**

Submitted on 26 Nov 2022

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15 **Abstract**

16 Magnetotactic bacteria (MTB) are a diverse group of microorganisms that use intracellular chains  
17 of ferrimagnetic nanocrystals, produced within their magnetosome organelles, to align and  
18 navigate along the geomagnetic field. The cell biological and biochemical properties of  
19 magnetosomes make them a powerful model for studying the molecular mechanisms of  
20 biomineralization and compartmentalization in bacteria. While several conserved magnetosome  
21 formation genes have been described, the evolutionary strategies for their species-specific  
22 diversification remain unknown. Here, we demonstrate that the fragmented nature of magnetosome  
23 chains in *Magnetospirillum magneticum* AMB-1 is controlled by two genes named *mcaA* and  
24 *mcaB*. McaA recognizes the positive curvature of the inner cell membrane while McaB localises  
25 to magnetosomes. Along with the MamK actin-like cytoskeleton, they create space for addition of  
26 new magnetosomes in between pre-existing magnetosomes. Phylogenetic analyses suggest that  
27 McaAB homologs are widespread and may represent an ancient strategy for organelle positioning  
28 in MTB.

## 29 Introduction

30 Cellular compartmentalization results in the formation of different organelles, which need to be  
31 positioned correctly to fulfil their specific functions and ensure proper inheritance throughout cell  
32 division <sup>1</sup>. Organelle positioning in eukaryotic cells mainly relies on cytoskeletal and motor  
33 proteins <sup>1</sup>. Many bacteria also produce organelles <sup>2</sup>, and actively regulate their placement in the  
34 cell. For example, the protein-bounded carbon-fixation organelle, the carboxysome, uses the  
35 nucleoid as a scaffold with helper proteins that ensure equal distribution in the cell and proper  
36 segregation into daughter cells <sup>3</sup>. Similarly, it has been proposed that the carbon storage  
37 polyhydroxybutyrate (PHB) granules associate with nucleoids to mediate segregation during cell  
38 division <sup>4,5</sup>. A widely studied example of bacterial lipid-bounded organelles is the magnetosome  
39 compartment of magnetotactic bacteria (MTB). Magnetosomes mineralize ferrimagnetic  
40 nanoparticles composed of magnetite (Fe<sub>3</sub>O<sub>4</sub>) and/or greigite (Fe<sub>3</sub>S<sub>4</sub>) <sup>2,6</sup>, which are used as a  
41 compass needle for navigation along the geomagnetic field. Magnetic navigation is a common  
42 behaviour in diverse organisms, including bacteria, insects, fish, birds, and mammals <sup>7,8</sup>. MTB are  
43 the simplest and most ancient organism capable of magnetic navigation <sup>9</sup> and fossilized  
44 magnetosomes chains have been used as robust biosignatures <sup>10,11</sup>. Thus, magnetosome production  
45 in MTB is an ideal model system for studying mechanisms of organelle positioning, understanding  
46 the evolution of magnetic navigation, and connecting the magnetofossil record to the history of  
47 life on Earth.

48 To function as an efficient compass needle, individual magnetosomes need to be arranged into a  
49 chain. Various and complex magnetosome chains (single- or multi-stranded, continuous or  
50 fragmented) are found in diverse MTB groups <sup>12,13,14</sup>. The mechanisms leading to distinct chain  
51 configurations remain unknown, but may reflect strategies for adaptations to specific biotopes <sup>10</sup>.  
52 The most widely-studied model MTB strains *Magnetospirillum magneticum* AMB-1 (AMB-1)  
53 and *Magnetospirillum gryphiswaldense* MSR-1 (MSR-1) are closely related *Alphaproteobacteria*  
54 species sharing 96% identity in their 16S rRNA gene sequences <sup>15</sup>. However, their magnetosome  
55 chain organisation strategies are distinct. In AMB-1, magnetosomes containing magnetic crystals  
56 and empty magnetosomes are interspersed to form a chain that is fragmented in appearance,  
57 extends from pole-to-pole in the cell, and remains stationary during the entire cell cycle <sup>16</sup>. In  
58 contrast, in MSR-1, magnetic crystals are arranged as a continuous chain at the midcell and the  
59 divided daughter chains rapidly move from the new poles to the centre of the daughter cells after

60 cell division<sup>17,18</sup>. The actin-like protein MamK is conserved in all characterized MTB and forms  
61 a cytoskeleton that specifically regulates the stationary or moving behaviours of magnetosome  
62 chains in AMB-1 and MSR-1<sup>16,17</sup>. While the overall proteomes of AMB-1 and MSR-1 are on  
63 average 66% identical, MamK proteins from the two organisms are 90.8% identical at the amino  
64 acid level and *mamK*<sub>AMB-1</sub> complements the MSR-1  $\Delta$ *mamK* mutant<sup>19</sup>. However, the speed and  
65 spatial dynamics of MamK filaments are distinct in each organism<sup>16,17</sup>. The acidic protein MamJ  
66 is also a key regulator of chain organization. When *mamJ* is deleted in MSR-1, magnetosomes  
67 collapse into aggregates in the cell<sup>20</sup>. However, similar deletions in AMB-1 result in subtle defects  
68 with magnetosomes still organised as chains<sup>21</sup>. Additionally, *mamY* is critical for localizing the  
69 chain to the positive curvature of the cell in MSR-1<sup>22</sup> but does not have an impact on chain  
70 organization when deleted in AMB-1<sup>23</sup>. These observations suggest that unknown genetic  
71 elements may be needed for species-specific chain organisation phenotypes.

72 The genes for magnetosome production and chain assembly in MTB, such as *mamK*, *mamJ*, and  
73 *mamY*, are arranged into magnetosome gene clusters (MGCs) that are often structured as  
74 magnetosome gene islands (MAI)<sup>24</sup>. Unlike MSR-1, AMB-1 contains an extra genomic cluster,  
75 termed the magnetotaxis islet (MIS), outside of the MAI region<sup>25</sup>. The MIS is ~28kb long and  
76 contains 7 magnetosome gene homologs, including a very divergent copy of *mamK* and many  
77 genes of unknown function<sup>25</sup>. The MIS protein, MamK-like, partners with MamK in  
78 magnetosome chain formation, but does not contribute to the species-specific chain organisation  
79 phenotypes mentioned above<sup>26</sup>. Whether the other MIS genes are functional and play roles in  
80 magnetosome biosynthesis is unclear, especially given the presence of multiple transpose genes  
81 and pseudogenes<sup>25</sup>.

82 Here, we studied the MIS genomic region and identified two proteins (McaA and McaB) that  
83 mediate magnetosome chain assembly in AMB-1. McaA localises to the positively-curved  
84 cytoplasmic membrane as a dashed-line even in the absence of magnetosomes whereas McaB  
85 associates with magnetosomes. Together, McaA and McaB direct the addition of new  
86 magnetosomes to multiple sites between pre-existing magnetosomes to form a fragmented crystal  
87 chain. They also influence the dynamics of MamK filaments to control magnetosome positioning  
88 during the entire cell cycle. The action of McaA and McaB is sufficient to explain all of the known  
89 differences in chain organization between AMB-1 and MSR-1. Broader phylogenetic analysis  
90 reveals that McaA and McaB are specific to MTB with distant homologs in the vicinity of MGCs

91 in other MTB. We hypothesize that the MIS is a remnant of an ancient duplication event which  
92 paved the way for an alternative chain segregation strategy in AMB-1. This mode of chain  
93 segregation may lower the energy requirements for separating magnetic particles at the division  
94 septum and eliminating the need for rapidly centring the chain after cell division.

## 95 **Results**

### 96 **MIS genes control the location of and spacing between magnetosomes**

97 To investigate its possible role in magnetosome formation and placement, we deleted the entirety  
98 of the MIS from the AMB-1 genome. The deletion's effect on magnetosome production was  
99 assessed by measuring the coefficient of magnetism ( $C_{mag}$ ) using a differential  
100 spectrophotometric assay that quantifies the ability of MTB to orientate in an external magnetic  
101 field<sup>27</sup>. Unexpectedly, the  $C_{mag}$  values of  $\Delta$ MIS cultures are much higher than Wild-type (WT)  
102 cultures (Fig. 1a), indicating that, as a population,  $\Delta$ MIS cells better align with the applied external  
103 magnetic field. As expected, transmission electron microscopy (TEM) images of WT AMB-1  
104 cultures show that the magnetic crystals are organised into a chain with gaps from cell pole to pole  
105 (Fig. 1b). In contrast, the crystals in the  $\Delta$ MIS strain are organised into a continuous chain at the  
106 midcell (Fig. 1b). Analysis of TEM images shows that the number and length of crystals are similar  
107 (Supplementary Fig. 1a, b), but the shape factor of crystals (width/length ratio) differs between  
108 WT (0.82) and  $\Delta$ MIS (0.92) strains (Supplementary Fig. 1c, d). These data were collected from  
109 strains grown under microaerobic conditions. To ensure that the observed phenotypes were not  
110 generated by specific growth conditions, the experiment was repeated under anaerobic conditions  
111 and yielded similar results (Supplementary Fig. 2).

112 Magnetosome biogenesis in WT AMB-1 begins with the invagination of bacterial inner membrane  
113 to form empty magnetosomes (EMs), followed by the crystallisation of ferrimagnetic minerals to  
114 form crystal-containing magnetosomes (CMs)<sup>2, 6</sup>. To directly observe the organisation of  
115 magnetosome membranes, we imaged WT and  $\Delta$ MIS cells with whole-cell cryo-electron  
116 tomography (cryo-ET) (Fig. 1c, d). Similar to WT<sup>28</sup>, the magnetosome membranes of  $\Delta$ MIS  
117 mutant are invaginations of the inner membrane (Fig. 1d, lower left corner). We then measured  
118 the diameter of magnetosome membranes and the length of crystals. The size distribution of EMs  
119 and CMs, as well as the linear relationship between the sizes of crystals and magnetosome  
120 membrane diameters are similar between the WT and  $\Delta$ MIS mutant (Supplementary Fig. 3).  
121 Additionally, in both strains, the EMs are significantly smaller than the CMs (Supplementary Fig.  
122 3a). One major phenotypic difference between the two strains is that in WT EMs are present at  
123 multiple sites between CMs in the magnetosome chain (Fig. 1c) whereas EMs only localise at both  
124 ends of the continuous chain of in the  $\Delta$ MIS strain (Fig. 1d). Analysing the size and

125 biomineralization status of magnetosome membranes relative to their subcellular position shows  
126 that the location of EMs is random along the magnetosome chain in WT but is at both ends of the  
127 chain in  $\Delta$ MIS cells (Fig. 1 e, f). These results together suggest that MIS genes control the location  
128 of magnetosomes but not the size of magnetosome membranes.

129 Based on the different sizes and locations of magnetosomes, we hypothesised that the newly-made  
130 magnetosomes are added at multiple internal sites of the chain in WT, but only added at the ends  
131 of the chain in  $\Delta$ MIS. To test this hypothesis, we designed a pulse-chase experiment to label and  
132 follow a marker protein that incorporates into magnetosomes at the early steps of membrane  
133 invagination (Fig. 2a). We examined the magnetosome marker proteins MamI and MmsF<sup>29,30,31</sup>.  
134 Newly-synthesized MmsF proteins incorporate into both the new and old magnetosomes (see more  
135 details in supplementary results and supplementary Fig. 4), indicating that it is not suitable for the  
136 pulse-chase experiment.

137 The transmembrane protein MamI is needed for EM invagination from the inner membrane of  
138 AMB-1<sup>26,31</sup>. We first checked the localization of MamI-GFP in WT and  $\Delta$ MIS. Structured  
139 illumination fluorescent microscopy (SIM) imaging shows MamI-GFP localises as a continuous  
140 line from cell pole to pole in WT AMB-1 (Fig. 2b), indicating localization to both the EMs and  
141 CMs. In  $\Delta$ MIS, MamI-GFP only localises in the middle of the cells in a pattern reminiscent of  
142 magnetosome organisation as seen in cryo-ET images (Fig. 2b). We then performed pulse-chase  
143 experiments using MamI-Halo. The Halo-ligand JF549 was used as the pulse to mark old  
144 magnetosomes and the JF646 ligand was chased in to identify the newly-made magnetosomes (Fig.  
145 2a). JF646 signals do not colocalise with JF549 signals in WT AMB-1 (Fig. 2d and Supplementary  
146 Fig. 5a), indicating newly-synthesized MamI proteins are only added to the newly-made  
147 magnetosomes. Quantitative analysis shows very low colocalization coefficients of the pulse and  
148 chase signals in WT and  $\Delta$ MIS cells (Supplementary Table 1). As expected, JF549-marked old  
149 magnetosomes display gaps, which are filled with the JF646-marked newly-made magnetosomes  
150 in WT AMB-1 (Fig. 2d and Supplementary Fig. 5a). Conversely, the JF549-marked old  
151 magnetosomes still mainly show a continuous chain at the midcell of  $\Delta$ MIS, and the JF646-marked  
152 newly-made magnetosomes localise at both ends of the chain (Fig. 2d and Supplementary Fig. 5a).  
153 Together, these results confirm our hypothesis that the varying chain phenotypes between WT and  
154  $\Delta$ MIS strains are in part due to changes in the location where new magnetosomes are added.



155 Additionally, we found that the length ratio between the MamI-GFP marked magnetosome chain  
156 and the cell body is significantly larger in WT than in  $\Delta$ MIS (Fig. 2c). As mentioned above, the  
157 number of crystals in WT and  $\Delta$ MIS cells is similar, indicating the distance between the  
158 magnetosomes might be different in these two strains. We therefore measured the edge-to-edge  
159 magnetosomes distance and found that the distance between all magnetosomes in WT is about  
160 twice as long as in the  $\Delta$ MIS strain (Fig. 1g), while the distance between EMs in these two strains  
161 is similar (Fig. 1h), indicating the difference is mainly due to the distance between CMs.

162 To summarise, MIS genes control the shape of crystals, the distance between CMs, and the location  
163 for the addition of newly-made magnetosomes leading to the characteristic pattern of chain  
164 organisation in AMB-1.

### 165 **Comprehensive dissection of the MIS new chain organization factors**

166 To identify the key genes that control magnetosome positioning, we conducted conventional  
167 recombination mutagenesis to create unmarked deletions of selected segments in the MIS (Fig.  
168 3a). We first deleted large domains (LD1 and LD2) to narrow down the region of interest in LD1  
169 (Fig. 3b, c and Supplementary results). We then generated small islet region deletions ( $\Delta$ iR1,  $\Delta$ iR2,  
170  $\Delta$ iR3, and  $\Delta$ iR4) of LD1 to pinpoint specific genes involved in chain organisation. Genes in the  
171 iR2 region control magnetosome positioning, while those in iR3 contribute to crystal shape control  
172 (Fig. 3b, d and Supplementary results). iR2 contains a small putative operon with two hypothetical  
173 genes (*amb\_RS23835* and *amb\_RS24750*), which we have named **m**agnetosome **c**hain **a**ssembly  
174 genes A and B (*mcaA* and *mcaB*) (Fig. 3a). The reference genome in NCBI shows the iR2 region  
175 includes a third transposase gene (*amb\_RS23840*) (Fig. 3a), which does not exist in our lab strain.  
176 We then deleted these two genes individually. Both  $\Delta$ *mcaA* and  $\Delta$ *mcaB* strains have dramatically  
177 higher Cmag compared to WT and are similar to  $\Delta$ MIS (Fig. 3b). Similar to the  $\Delta$ MIS mutant,  
178  $\Delta$ *mcaA* and  $\Delta$ *mcaB* strains contain continuous crystal chains in the midcell region when viewed  
179 by TEM (Fig. 3d), indicating that both play essential roles in magnetosome positioning.

### 180 **McaA localises to the positively-curved cytoplasmic membrane as a dashed line**

181 We interrogated the localization of McaA and McaB in order to understand their role in controlling  
182 magnetosome positioning. McaA is predicted to contain a signal peptide, followed by a  
183 periplasmic von Willebrand factor type A (VWA) domain, a transmembrane (TM) domain, and a  
184 cytoplasmic C-terminus (Fig. 4a and Supplementary results). However, in some bioinformatic

185 predictions, the signal peptide region is predicted to be a TM domain with the N-terminus facing  
186 the cytoplasm (Supplementary Table 2). Using GFP fusions to either end of the protein, we predict  
187 that the C-terminus of McaA faces the cytoplasm (See more details in Supplementary results). SIM  
188 images show that, when expressed in WT AMB-1, McaA-GFP localises in a dashed-line pattern  
189 distributed along the positive inner curvature of the cell, forming a line covering the shortest  
190 distance from cell pole to pole (Fig. 4b). Since magnetosomes are also located to the positive inner  
191 curvature of AMB-1 cell<sup>22</sup>, we wondered if McaA is associated with magnetosomes. To test this,  
192 we expressed McaA-GFP in different genetic backgrounds and growth conditions, including  $\Delta$ MIS  
193 and  $\Delta$ iR2 mutants which contain continuous crystal chains, WT under iron starvation where only  
194 EMs are present, and the  $\Delta$ MAI $\Delta$ MIS mutant that is incapable of magnetosome production. McaA-  
195 GFP localises as a dashed line in all of the above strains and conditions (Fig. 4b), indicating that  
196 the association of McaA with the positive curvature of cytoplasmic membrane and its dashed-line  
197 localization are independent of magnetosome membrane formation, magnetite production, and  
198 chain organisation.

199 Using a series of truncations, we found that the N-terminus of McaA (including the predicted  
200 signal peptide and the VWA domain) is essential for its localization and magnetosome positioning.  
201 In contrast, the C-terminus conserved region (aa 530-665) is not essential for McaA localization  
202 but important for magnetosome positioning (Fig. 4c and Supplementary Fig. 8). The VWA domain  
203 is commonly involved in protein-protein interactions, largely *via* a noncontiguous metal ion-  
204 binding motif (DXSXS) called metal ion-dependent adhesion site (MIDAS)<sup>32, 33</sup>. McaA VWA  
205 domain contains an intact MIDAS motif. We investigated whether this MIDAS motif plays an  
206 important role using site-directed mutagenesis. McaA<sup>MIDAS</sup> mutant cannot complement  $\Delta$ mcaA  
207 and is evenly distribute within the cytoplasmic membrane (Fig. 4c and Supplementary Fig. 8),  
208 highlighting the important role of the MIDAS motif and divalent cations in the localization and  
209 function of McaA.

## 210 **McaB associates with crystal-containing magnetosomes**

211 McaB is predicted to contain one TM domain that is close to the N-terminus, which is mostly  
212 facing the periplasm (Supplementary Fig. 9a). We confirmed the cytoplasmic location of C-  
213 terminus McaB through the fluorescent signal of McaB-GFP (Fig. 4d and Supplementary results).  
214 SIM images show that McaB-GFP forms a dotted line from cell pole to pole along the positive

215 inner curvature of WT AMB-1 cells, whereas it exhibits a continuous line in the middle of the  
216  $\Delta$ MIS and  $\Delta$ iR2 cells (Fig. 4d and Supplementary Fig. 9b), indicating McaB might be associated  
217 with magnetosomes. Interestingly, McaB-GFP is not present at the magnetosome chain when WT  
218 cells are grown under low iron conditions that prevent magnetite production (Fig. 4d and  
219 Supplementary Fig. 9b), indicating that it does not localise to the EMs. These results together show  
220 that McaB might be specifically associated with CMs, resembling the localization pattern of the  
221 magnetosome protein Mms6<sup>34</sup>. Thus, we co-expressed McaB-GFP and Mms6-Halo in the same  
222 AMB-1 cell, and the SIM images, as well as quantitative determination of colocalization  
223 coefficients (Supplementary Table 1), show that the two proteins colocalise (Fig. 4e), further  
224 confirming the association of McaB with CMs.

225 We also examined the localization of McaA and McaB using cellular fractionation and  
226 immunoblotting analysis. McaA is mainly detected in the insoluble portion which includes the  
227 cytoplasmic membranes, while similar to Mms6, McaB is mainly detected in the magnetosome  
228 fraction (Fig. 4f). Thus, biochemical fractionation experiments confirm the association of McaA  
229 with cytoplasmic membrane and McaB with magnetosome membranes.

### 230 **McaA and McaB coordinate magnetosome positioning**

231 To explore the relationship between McaA and McaB, we co-expressed McaA-Halo and McaB-  
232 GFP in WT AMB-1 cells (see more details in supplementary results). Interestingly, SIM images  
233 show that McaB localises within the gaps of dashed McaA, indicating McaB-marked CMs are  
234 located in the gaps of dashed McaA (Fig. 4g and Supplementary Fig. 10d). Accordingly,  
235 quantitative analysis shows low colocalization coefficients for McaA and McaB signals in WT  
236 AMB-1 cells (Supplementary Table 1). Bacterial adenylate cyclase two-hybrid (BACTH) assays  
237 did not show any positive interactions between McaA and McaB (Table 1 and Supplementary Fig.  
238 11a), indicating that either the fusion proteins do not interact strongly, are nonfunctional in the  
239 context of BACTH, or unknown intermediate proteins are needed to facilitate their interactions.

240 We next investigated whether McaA and McaB directly affect the positioning of EMs when CMs  
241 are not produced. We grew WT,  $\Delta$ MIS, and  $\Delta$ iR2 strains expressing MamI-GFP under iron  
242 starvation conditions. SIM images show that MamI-marked EMs are located continuously in the  
243 midcell of all three strains (Fig. 5a), indicating that McaAB do not participate in the chain  
244 organisation under low iron conditions. We then examined the dynamics of magnetosome chain

245 organisation as WT and cells missing *mcaAB* transitioned from low to high iron conditions to  
246 trigger magnetite production in EMs (Fig. 5b). We performed pulse-chase experiments using  
247 MamI-Halo with cells growing from iron starvation (pulse with JF549) to standard iron growth  
248 conditions (chase with JF646). As expected, the pulse experiments show a continuous chain of  
249 EMs in the middle of all WT and *mcaAB* deficient cells under iron starvation conditions (Fig. 5c  
250 and Supplementary Fig. 5b). After iron addition to the growth medium, we observed the formation  
251 of gaps between JF549-marked old magnetosomes in WT, but not in  $\Delta$ MIS or  $\Delta$ iR2 cells (Fig. 5c  
252 and Supplementary Fig. 5b). In addition, the JF646-marked newly-made EMs filled the gaps  
253 between older magnetosomes in WT but were only added at both ends of the chain in  $\Delta$ MIS and  
254  $\Delta$ iR2 (Fig. 5c and Supplementary Fig. 5b). Accordingly, quantitative analysis shows a low  
255 colocalization coefficient of the pulse and chase signals in WT,  $\Delta$ MIS, and  $\Delta$ iR2 cells  
256 (Supplementary Table 1). Together, these results support the hypothesis that McaA serves as a  
257 landmark on the positively-curved inner membrane and coordinates with McaB to control the  
258 location and spacing between CMs, allowing the addition of newly-made EMs to multiple sites  
259 between pre-existing magnetosomes in the chain of WT AMB-1, which forms the fragmented  
260 crystal chain.

## 261 **McaA contributes to the differences of *mamJ* and *mamY* deletions between AMB-1 and** 262 **MSR-1**

263 As mentioned above, the phenotypes of *mamJ* and *mamY* deletion mutants in AMB-1 and MSR-1  
264 are distinct. MamJ is proposed as a linker to attach MamK filaments to magnetosomes and its  
265 deletion in MSR-1 causes the magnetosome chain to collapse and form an aggregate<sup>20</sup>. In contrast,  
266 the deletion of *mamJ* and its homolog *limJ* in AMB-1 still shows a magnetosome chain with some  
267 minor structural defects<sup>21</sup>. However, deletion of the entire MIS in a  $\Delta$ *mamJ* $\Delta$ *limJ* strain causes a  
268 dramatic chain collapse phenotype resembling those of MSR-1 $\Delta$ *mamJ* mutant<sup>10,20</sup>. MIS contains a  
269 second *mamJ* homolog called *mamJ-like* and our deletion analysis shows that it does not contribute  
270 to chain maintenance (Fig. 3a, Fig. 6a, c and more details in Supplementary results). To figure out  
271 the specific genes, we generated large domain and small region deletion mutants of the MIS in a  
272  $\Delta$ *mamJ* $\Delta$ *limJ* background. Cmag and TEM images show that *mcaA* is the specific gene that  
273 prevents magnetosome aggregation in the  $\Delta$ *mamJ* $\Delta$ *limJ* strain (Fig. 6a, c and Supplementary Fig.  
274 14).

275 MamY is a membrane protein that directs magnetosomes to the positively-curved inner membrane  
276 in MSR-1, thus aligning the magnetosome chain to the motility axis within a helical cell <sup>22</sup>. When  
277 *mamY* is deleted in MSR-1, the magnetosome chain is no longer restricted to the positively-curved  
278 regions of the membrane and can also be found at the negatively-curved membrane leading to a  
279 much lower Cmag compared to WT <sup>22</sup>. Surprisingly, when *mamY* is deleted in AMB-1, the Cmag  
280 is similar to WT (Fig. 6b), and the magnetosome chain still localises to the positively-curved  
281 membrane (Fig. 6d), indicating there might be other proteins that are functionally redundant to  
282 MamY in AMB-1. A  $\Delta$ *mamY $\Delta$ MIS mutant of AMB-1 has a much lower Cmag than  $\Delta$ MIS and  
283 produces magnetosome chains that localise to both positively- and negatively-curved cell  
284 membranes (Fig. 6b, d). Further deletion mutagenesis shows that McaA helps magnetosomes  
285 localise to the positive-curved membrane when MamY is lost in AMB-1 (Fig. 6b, d).*

286 Despite their genetic interactions, BACTH analysis does not show any direct interactions between  
287 MamJ and McaA, -B or between MamY and McaA, -B (Table 1 and Supplementary Fig. 11b-e).  
288 Nevertheless, our results indicate that the activity of McaA accounts for the distinct phenotypes of  
289 *mamJ* or *mamY* deletion mutants between AMB-1 and MSR-1.

## 290 **McaA and McaB control the dynamic positioning of magnetosomes by influencing the** 291 **MamK filaments**

292 In addition to the appearance of the chain, the dynamic movements and positioning of the chain  
293 differs between AMB-1 and MSR-1 <sup>16, 17</sup>. We reasoned that the McaAB system may contribute to  
294 the different dynamic chain positioning in these two strains. Using highly inclined and laminated  
295 optical sheet (HILO) microscopy, we performed live-cell imaging analysis to follow the dynamics  
296 of Mms6-GFP labelled magnetosomes during cell division in WT and McaAB deficient cells.

297 In WT AMB-1, magnetosomes are in static, spotty positions during cell division as indicated by  
298 the parallel lines in the kymographs of GFP fluorescence (Fig. 7a and Supplementary Movie 1),  
299 whereas every  $\Delta$ MIS and  $\Delta$ *mcaA* cells shows dynamic magnetosome chain segregation after cell  
300 division (Fig. 7b, c and Supplementary Movie 2, 3). In  $\Delta$ MIS and  $\Delta$ *mcaA*, magnetosomes are  
301 positioned at the midcell until the cell divides. After cytokinesis, magnetosomes are moved  
302 synchronously toward the centres of both daughter cells. Magnetosome migration to the middle of  
303 the daughter cells was completed within about 1 hour after cell division. Magnetosome chain  
304 displacement velocity in  $\Delta$ *mcaA* cells was about 20 nm/min, in line with what has been reported

305 previously for MSR-1<sup>17</sup>. Supplementary Movies 5 and 6 are long time-lapse videos of the  $\Delta$ MIS  
306 and  $\Delta$ *mcaA* cells, in which the magnetosomes are stably positioned at the middle of the daughter  
307 cells during the entire cell cycle after the migration of the magnetosomes. In contrast, in  $\Delta$ *mcaB*  
308 cells, magnetosome displacements are incomplete (Fig. 7d and Supplementary Movie 4, 7 (short  
309 and long time-lapse)). Magnetosomes do not move synchronously toward the middle of the cell  
310 and are randomly positioned in the cell. In other words, these results show that McaB does not  
311 impact daughter chain positioning in  $\Delta$ *mcaA* strain, while McaA impedes daughter chain  
312 positioning in  $\Delta$ *mcaB* strain, indicating McaA might have extra functions in magnetosome chain  
313 positioning during cell division.

314 It has been shown that the dynamics of MamK filaments is essential for magnetosome chain  
315 positioning in AMB-1 and MSR-1<sup>16, 17</sup>. To test whether the McaAB system influences the  
316 dynamics of MamK filaments, we performed fluorescence recovery after photobleaching (FRAP)  
317 assays on MamK-GFP filaments in WT and McaAB deficient cells. GFP-tagged MamK filaments  
318 localise as even thin lines from cell pole to pole in both WT and McaAB deficient AMB-1 strains.  
319 During FRAP experiments, sections of GFP-tagged MamK filaments are irreversibly  
320 photobleached and the recovery of fluorescence in the bleached area is tracked over time (Fig. 7e).  
321 The half-life ( $t_{1/2}$ ) of recovery represents the time point at which 50% of the fluorescence intensity  
322 returns to the bleached region relative to the whole filament at that same time point. The bleached  
323 area does not move in WT (Fig. 7e and Supplementary Fig. 15a), but it moves in a fraction of  
324  $\Delta$ MIS,  $\Delta$ *mcaA*, and  $\Delta$ *mcaB* cells (Fig. 7g, Supplementary Fig. 15b, and Supplementary Table 3).  
325 The  $t_{1/2}$  fluorescence recovery is similar in WT and McaAB deficient cells containing an immotile  
326 bleached spot (Fig. 7f and Supplementary Fig. 15a). For the McaAB deficient cells with a moving  
327 bleached spot, the  $t_{1/2}$  fluorescence recovery of the original bleached area is similar but much faster  
328 than in the cells containing an immotile bleached spot (Fig. 7h), indicating a similar moving speed  
329 of the bleached area. BACTH analysis did show MamK self-interactions but did not show any  
330 interactions between McaA, -B and MamK (Table 1 and Supplementary Fig. 11f, g). Together,  
331 these results indicate that McaA, -B influence the dynamics of MamK filaments which, in turn,  
332 leads to the AMB-1-specific pattern of magnetosome chain organization.

333 ***mcaA* and *mcaB* genes are specific to MTB**

334 To understand the evolutionary origins of the *mcaAB* system, we searched for homologs of these  
335 two genes in diverse species of MTB. Distant homologs were found in 38 MTB species. All of  
336 them belong to MTB strains either with characterized magnetosome chain phenotypes (Fig. 8a, b)  
337 or metagenomes obtained from a magnetic enrichment (supplementary Fig. 16 a, b), with the  
338 majority affiliated to the *Rhodospirillaceae* family in the *Alphaproteobacteria* class. Based on  
339 published reports, most studied MTB contain continuous crystal chains (supplementary dataset 1).  
340 However, some species show fragmented crystal chains, including the two *Alphaproteobacteria*  
341 species (*Ca. Terasakiella magnetica* PR-1 and *Terasakiella sp.* SH-1) that contain distant *mcaAB*  
342 homologs and two *Deltaproteobacteria* species that do not contain *mcaAB* homologs (Fig. 8a, b  
343 and supplementary dataset 1), which suggests that different mechanisms may exist for crystal chain  
344 fragmentation.

345 Besides the *mcaAB* genes of the MIS, AMB-1 contains two additional homologs of *mcaA* and  
346 *mcaB* with a similar domain architecture (named *mcaA-like* and *mcaB-like* here) that are present  
347 close to the MAI. McaA-like (encoded by *amb0908/amb\_RS04660*) has ~45% amino acid  
348 sequence identity over ~66% McaA sequence length, while McaB-like (encoded by  
349 *amb0907/amb\_RS24855*) has ~44% amino acid sequence identity over ~66% McaB sequence  
350 length. We deleted *mcaAB-like* genes in both WT and  $\Delta$ MIS AMB-1 strains but did not observe  
351 any obvious defects or changes in magnetosome formation or chain organisation (supplementary  
352 Fig. 16 c, d), indicating *mcaAB-like* genes are not functionally redundant with *mcaAB* genes in  
353 AMB-1. Based on comparative genomic analyses, *mcaA* and *mcaB* of AMB-1 form a distinct  
354 cluster, while *mcaA-like* and *mcaB-like* of AMB-1 cluster with a second group of homologs in the  
355 strains that do not show a fragmented crystal chain phenotype (Fig. 8a, b, clade in light blue),  
356 indicating those homologs might have functions more similar to *mcaAB-like* genes in AMB-1.

357 Molecular phylogenetics indicates that the last common ancestor to Mca proteins of AMB-1 and  
358 Mca-like proteins detected in *Magnetospirillum* species and *Ca. Magneticavibrio boulderlitore*  
359 LM-1 emerged before the first freshwater magnetotactic *Rhodospirillaceae* (Fig. 8a, b). High  
360 protein sequence identity percentages of Mca-like proteins between *Magnetospirillum* strains (85%  
361 up to 100%) compared to the average amino acid ID % at the genome scale<sup>35</sup> indicates that the  
362 *mca-like* genes are under purifying selection. Together these results may indicate that McaAB-  
363 mediated crystal chain fragmentation could be a trace of an ancient chain organisation strategy that  
364 is lost in most of the modern MTB species. However, the long external branches of McaA and

365 McaB (Fig. 8a, b and supplementary Fig. 16 a, b) suggest a recent acceleration of the evolution of  
366 Mca proteins which could be linked with their neofunctionalization in AMB-1.



## 367 **Discussion**

368 In this study, we uncovered the mechanisms of a novel magnetosome chain organisation strategy  
369 that explains phenotypic differences between closely related MTB. We demonstrated that the  
370 fragmented crystal chain organisation in WT AMB-1 strain is not growth condition dependent but  
371 genetically controlled by *mcaA* and *mcaB*. In their absence, magnetite crystals form a continuous  
372 chain similar to the chain phenotype of MSR-1<sup>22</sup>. The McaAB system also contributes to the  
373 differences in magnetosome dynamic positioning between AMB-1 and MSR-1 during the entire  
374 cell cycle.

375 Based on our study, we propose a model for McaAB-mediated dynamic positioning of  
376 magnetosomes (Fig. 8c). McaA localises to the positive inner curvature of the cell as a dashed line  
377 via its N-terminal periplasmic VWA domain. McaA serves as a landmark to regulate the placement  
378 and distance of McaB-marked CMs through its cytoplasmic C-terminal domain. As a consequence,  
379 neighbouring CMs are separated from each other allowing newly made EMs to be added at  
380 multiple locations of the chain. Without McaAB, the CMs are located closely together such that  
381 newly made EMs can only be added at the ends of the magnetosome chain. Furthermore, cryo-ET  
382 shows that MamK cytoskeleton is composed of short filaments and located along the magnetosome  
383 chain in both WT and  $\Delta$ MIS cells (Fig. 1c, d). Based on FRAP experiments, MamK filaments in  
384 WT display local recovery that can be caused by monomer turnover  
385 (depolymerization/polymerization), filament sliding, or formation of new filaments. In many  
386 McaAB deficient cells, MamK filaments recover and at the same time move across the cell, which  
387 might help position the magnetosomes in the midcell (Fig. 8c). We propose that the McaAB system  
388 localises the turnover of MamK filaments to allow for new magnetosome addition in between pre-  
389 existing magnetosomes in WT AMB-1 (Fig. 8c).

390 Beyond elucidating an unknown aspect of magnetosome chain formation, our findings raise new  
391 questions regarding the cell biological mechanisms of organelle formation and maintenance in  
392 bacteria. For instance, how McaA detects the positive curvature of cytoplasmic membrane and  
393 localises as a dashed pattern remains a mystery. We confirmed that the localization of McaA is  
394 dependent on its VWA domain. Eukaryotic VWA-containing proteins are involved in a wide range  
395 of cellular functions, but they share the common feature of being involved in protein-protein  
396 interactions, many of which depend on divalent cations coordinated by the MIDAS motif<sup>32</sup>.

397 Consistently, the MIDAS motif is essential for the location and function of McaA. VWA-domain  
398 proteins have been identified in some bacteria and archaea with different functions, but they are  
399 not well characterised<sup>36, 37, 38</sup>. These data suggest that there might be unknown proteins that partner  
400 with McaA to determine its specific localization. In addition to coordinating with McaB to control  
401 the fragmented crystal chain assembly, McaA also helps to prevent magnetosome chain  
402 aggregation when *mamJ* and its homologs are deleted. It also assists in keeping magnetosomes to  
403 the positively curved membranes when *mamY* is deleted, indicating that McaA contributes to  
404 multiple aspects of magnetosome chain organisation. Recently, a curvature-inducing protein CcfM  
405 has been identified and characterized in MSR-1<sup>39</sup>. CcfM localizes in a filamentous pattern along  
406 the positively curved inner membrane by its coiled-coil motifs, and it also functions as an  
407 intermediate protein that link the interaction between MamY and MamK<sup>39</sup>. Whether CcfM plays  
408 any role in McaA localization and its interactions with MamK still need to be investigated.

409 Although many MTB species from different taxa contain the McaA and McaB homologs, only  
410 AMB-1 and two other species of *Proteobacteria* form a fragmented magnetosome chain. Given  
411 our data, the evolutionary history of the fragmented chain formation strategy in AMB-1 may be  
412 explained by several scenarios. In the first one, the MIS could have been acquired after AMB-1  
413 emergence from an unknown magnetotactic *Rhodospirillaceae* donor through a bacteriophage-  
414 mediated lateral gene transfer. Indeed, the MIS is flanked with putative phage-related proteins that  
415 appears to be very well conserved in *Rhodospirillaceae* spp. Even if this scenario is parsimonious  
416 because it minimizes the number of evolutionary events, the presence of homologs of several  
417 magnetosome genes in the MIS with partial synteny conservation and many transposases could  
418 also be evidence for an old duplication event. In this case, the MIS would be a partial remnant of  
419 one of the duplicated versions. The duplication event at the origin would be even more ancestral  
420 to the one that led to the emergence of the *lim* cluster<sup>35</sup>. However, given the known  
421 *Magnetospirillum* evolutionary history<sup>35</sup>, this scenario would imply that many independent losses  
422 occurred over *Magnetospirillum* diversification. Assuming the latter scenario, fragmented chain  
423 formation would either be the trace of an ancestral strategy progressively replaced in the majority  
424 of lineages, or a recent one that emerged in AMB-1.

425 Our discoveries also highlight that previously undiscovered genes (*mca* and *mca-like* homologs)  
426 outside of MAI and conserved in diverse MTB species can play essential roles in magnetosome  
427 biosynthesis. The function of Mca-like proteins conserved in MTB remains to be elucidated; their

428 proximity to the MAI, the conservation of their synteny and the presence of the VWA domain in  
429 McaA-like proteins indicate that they probably play important role in magnetosome positioning  
430 along the magnetoskeleton.

431 It is notable that the action of two proteins is sufficient to fundamentally alter the assembly and  
432 organisation of magnetosome chains in AMB-1 as compared to MSR-1, one of its closest relatives.  
433 We propose that the alternative mode of chain organisation in AMB-1 may provide advantages  
434 that have led to its selective maintenance. Since magnetic particles are arranged as sub-chains  
435 along the length of AMB-1, daughter cells are ensured to inherit equal numbers of magnetic  
436 particles that are centrally positioned. Additionally, the distribution and spacing of CMs and EMs  
437 may reduce the forces needed to separate magnetic particles. In contrast, MTB such as MSR-1,  
438 need to break the closely located continuous crystal chain in the middle and dynamically reposition  
439 the entire chain after cell division, which could be more energy-demanding than the stationary  
440 ones in WT AMB-1. However, as seen in our Cmag data, AMB-1 cells, as a population, align  
441 better in magnetic fields in the absence of *mcaAB*. Given the specific biological interventions  
442 required for their assembly, preserved magnetite or greigite chains are also considered an  
443 important criterion for magnetofossil recognition and characterization<sup>10, 11</sup>. Thus, understanding  
444 the selective pressures that dictate the species-specific mechanisms of chain organisation in  
445 modern day organisms can provide much needed insights into the conditional functions of  
446 magnetosomes across evolutionary time.

## 447 **Methods**

### 448 **Bacterial growth**

449 The 1.5-mL stock cultures of AMB-1 strains were prepared as described previously<sup>40</sup>. The stock  
450 cultures were then used for larger volume growth with a dilution of 1:100. For Cmag  
451 measurements, TEM, cryo-ET, and fluorescent microscopy, 100  $\mu$ L of stock cultures were added  
452 into 10 mL of MG medium in the 24-mL green-capped tubes and kept in a microaerobic glovebox  
453 (10 % oxygen) at 30 °C for 1 to 2 days. For anaerobic growth conditions, 100  $\mu$ L of stock cultures  
454 were added into 10 mL of MG medium in sealed Balch tubes. The MG medium was flushed with  
455 N<sub>2</sub> gas for 10 min and then was autoclaved. For both microaerobic and anaerobic growth  
456 conditions, 1/100 vol of Wolfe's vitamin solution and 30  $\mu$ M ferric malate were added to the MG  
457 medium just before inoculation with bacteria. For low iron growth condition, 100  $\mu$ L of stock  
458 cultures were added in the 10-mL MG medium supplied with only 1/100 vol of Wolfe's vitamin  
459 solution, but no ferric malate, in the green-capped tubes that treated with 0.375 % oxalic acid (to  
460 remove the trace iron on the wall of glass tubes).

461 *E. coli* strains DH5a, XL1 Blue, DHM1, and WM3064 were grown in Lysogeny broth (LB)  
462 medium with appropriate antibiotics. For *E. coli* strain WM3064, 300- $\mu$ M diaminopimelic acid  
463 was added to the LB medium before inoculation with bacteria.

### 464 **Genetic manipulation**

465 The genome sequence of *Magnetospirillum magneticum* AMB-1 (GenBank accession number  
466 NC\_007626.1) was used for oligonucleotide design. Oligonucleotides were purchased from Elim  
467 Biopharm or Integrated DNA technologies. All constructs were confirmed by sequencing in UC  
468 Berkeley DNA Sequencing Facility. Plasmids were constructed by PCR amplifying DNA  
469 fragments of interest with the Phusion High Fidelity DNA Polymerase (New England Biolabs) or  
470 CloneAmp HiFi PCR Premix (Takara). All plasmids were introduced into AMB-1 by conjugation.  
471 The details about generation of plasmids and strains are described in the supplementary methods.  
472 The strains, plasmids, and primers used in this study are described in Supplementary Tables 5 to  
473 12.

### 474 **Deletion mutagenesis**

475 A two-step homologous recombination method was used to generate deletion mutants in AMB-1  
476 strains as previously described<sup>41</sup>. Briefly, an approximately 800 to 1000 bp region upstream and  
477 downstream of the deleted gene or genomic region were PCR amplified from the AMB-1 genomic  
478 DNA using primer pairs (A, B) and (C, D), respectively (Supplementary Table 8). The two PCR  
479 fragments were cloned into the SpeI restriction site of the pAK31 suicide plasmid using Gibson  
480 assembly to generate the deletion plasmids (Supplementary Table 6). The deletion plasmid was  
481 conjugated into AMB-1 strain using *E. coli* WM3064 donor strain. Colonies that had successfully  
482 integrated the plasmid were selected on MG agar plates containing 15 µg/mL kanamycin. To  
483 select for colonies that had undergone a second recombination event to lose the integrated plasmid,  
484 a counter-selectable marker *sacB*, which is toxic in the presence of sucrose, was used. Colonies  
485 were then passed in 10 mL of growth media without kanamycin and plated on MG agar plates  
486 containing 2% sucrose. The resulting sucrose-resistant colonies were checked for the successful  
487 deletions at their native locus by colony PCR with primers listed in Supplementary Table 9.

#### 488 **Cellular magnetic response**

489 The optical density at 400 nm (OD<sub>400</sub>) of AMB-1 cultures in the green-caped tubes was measured  
490 at 24 hours and 48 hours using a spectrophotometer. A large magnet bar was placed parallel or  
491 perpendicular to the sample holder outside the spectrophotometer, the maximum and minimum  
492 OD<sub>400</sub> were recorded. The ratio of the maximum to the minimum was designated as AMB-1 cells'  
493 C<sub>mag</sub>.

#### 494 **Transmission electron microscopy (TEM)**

495 For imaging the whole AMB-1 cells by TEM, 1-mL AMB-1 cells were taken from the 10-mL  
496 cultures that grew under different conditions. The 1-mL cells were pelleted and resuspended into  
497 5-10 µL of MG medium. The resuspended cells were applied on a 400-mesh copper grid coated  
498 with Formvar and carbon films (Electron Microscopy Sciences). The grids were glow-discharged  
499 just before use. Then the air-dried cells were imaged on an FEI Tecnai 12 transmission electron  
500 microscope equipped with a 2k x 2k charge-coupled device (CCD) camera (The Model 994  
501 UltraScan®1000XP) at an accelerating voltage of 120 kV. Crystal size quantification and  
502 statistical tests were performed as described previously<sup>42</sup>.

#### 503 **Cryo-electron tomography (cryo-ET)**

504 Cryo-ET sample preparation and data collection were performed as described previously<sup>42</sup>. The  
505 two-dimensional (2D) images of WT and  $\Delta$ MIS cells were recorded using JEOL JEM-3100 FFC  
506 FEG TEM (JEOL Ltd.) equipped with a field emission gun electron source operating at 300 kV,  
507 an Omega energy filter (JEOL), and a K2 Summit counting electron detector camera (Gatan).  
508 Single-axis tilt series were collected using SerialEM software<sup>43</sup> from  $-60^\circ$  to  $+60^\circ$  with  $1.5^\circ$   
509 increments, at a final magnification of 6,000x corresponding to a pixel size of 0.56 nm at the  
510 specimen, and a defocus set to  $-15 \mu\text{m}$  under low dose conditions (a cumulative electron dose of  
511  $\sim 120 \text{ e}/\text{A}^2$ ). Tomogram reconstructions were visualized using the IMOD software package<sup>44</sup>.  
512 Amira was used for the 3D model segmentation (Thermo Fisher Scientific).

### 513 **Size and location analysis of magnetosome membranes**

514 The reconstructed tomograms were visualized using a 3dmod software package<sup>44</sup>. To evaluate the  
515 relative size and location of magnetosome membranes, the diameter of each magnetosome  
516 membrane was measured as described previously<sup>40</sup>. The size of magnetosome membranes in each  
517 cell was sorted from largest to smallest and then was nominated from 1 (largest) to 0 (smallest)  
518 accordingly. The first magnetosome membrane on the left of the chain was numbered as 0, the  
519 middle one was numbered as 1, and the last magnetosome membrane on the right of the chain was  
520 numbered as 2. The location of other magnetosome membranes was nominated accordingly.

521 For measuring the distance between individual magnetosome membranes, the shortest distances  
522 between neighbouring magnetosome membranes were found by working through the tomogram  
523 slices and manually measured by 3dmod.

### 524 **Structured illumination fluorescent microscopy (SIM)**

525 To stain the genomic DNA, AMB-1 cells growing in 10-mL MG medium of the green-capped  
526 tubes were collected by  $16,800 \times g$  for 3 min. The cell pellets were resuspended in a 1-mL fresh  
527 MG medium and stained with  $1.4 \mu\text{M}$  4',6-Diamidino-2-Phenylindole (DAPI) in dark at room  
528 temperature for 15 min, and then washed 3 times with fresh MG medium. After washing, the pellet  
529 cells were resuspended with 30-50  $\mu\text{L}$  of MG medium and were immediately imaged by Carl Zeiss  
530 Elyra PS.1 structured illumination microscopy with objective lens Plan-APOCHROMAT  
531  $100\times/1.46$ . DAPI, GFP, JF549, and JF646 were excited by 405 nm, 488 nm, 561 nm, and 642 nm  
532 lasers, respectively, and fluorescence from each fluorophore was acquired through 420-480 nm,  
533 495-550 nm, 570-620 nm, and LP655 nm bandpass filters, respectively. Raw images were acquired

534 and processed using ZEN software (Zeiss). The processed images were then visualized using  
535 Imaris (Bitplane).

536 MamI-GFP localization patterns in WT and  $\Delta$ MIS cells were manually measured using the Fiji  
537 software package <sup>45</sup>. The magnetosome chain showed a linear line across the cellular axis, so the  
538 end-to-end distance of the GFP fluorescence line was considered as the length of the magnetosome  
539 chain. A line that parallels the magnetosome chain was drawn from cell pole to pole, and this line  
540 was considered as the length of the whole AMB-1 cell.

#### 541 **Pulse-Chase analysis**

542 To study the addition of newly-made empty magnetosomes into magnetosome chains over time,  
543 we applied pulse-chase analysis using Halo-tagged magnetosome proteins as magnetosome  
544 markers. Halo ligands can irreversibly bind to Halo proteins. Under standard growth conditions,  
545 WT or mutated AMB-1 cells expressing Halo-tagged magnetosome proteins were grown to early  
546 exponential phase ( $OD_{400}$  is  $\sim 0.05$ ). Cells were pelleted and resuspended with 60- $\mu$ L MG medium  
547 and mixed with 120- $\mu$ L 5  $\mu$ M pulse Halo ligand JF549, kept at 30 °C for 2.5 hours in dark to make  
548 sure the Halo proteins were stained saturated with JF549. Then the extra JF549 ligand was washed  
549 away with MG medium (3 times, 10 min for each time). keep a few microliters of JF549-stained  
550 cells for SIM microscopy imaging. Put the rest cells back in a green-capped tube containing 10-  
551 mL MG medium, Kept in the 10% oxygen glove box at 30 °C for 4 hours in dark to allow new  
552 protein and new magnetosome production. Cells were then pelleted and resuspended with 60- $\mu$ L  
553 MG medium, and mixed with 120- $\mu$ L 5  $\mu$ M chase Halo ligand JF646, kept at 30 °C for 1 hour in  
554 dark. Then the extra JF646 ligand was washed away with MG medium (3 times, 10 min for each  
555 time) and imaged immediately with a SIM microscope. For the control group, after one quick wash  
556 with MG medium, pulse-stained cells were fixed with 4% paraformaldehyde for 1 hour at room  
557 temperature to prevent the production of new Halo-tagged proteins, then washed with MG medium  
558 three times and kept for the chase staining. For transition experiments from iron starvation to  
559 standard iron growth conditions, AMB-1 cells were first grown with an MG medium without added  
560 iron and stained with JF549, then were inoculated to the MG medium with iron and incubated in  
561 the 10% oxygen glove box for 4-5 hours before JF646 staining.

#### 562 **Quantitative colocalization analysis of fluorescent-labelled magnetosome proteins.**

563 After image collection from the SIM microscope, the subcellular distributions of GFP- and Halo-  
564 labelled proteins or different-coloured ligands during pulse-chase experiments were quantitatively  
565 analysed by Pearson's Correlation Coefficient (PCC) and Manders' Colocalization Coefficients  
566 (MCC)<sup>46</sup> using the ImageJ JaCoP plugin (Supplementary Table 1). To avoid background and noise  
567 signals, only the region of interest (magnetosome chain) was cropped from the original image and  
568 measured for colocalization analysis.

### 569 **Fluorescence recovery after photobleaching (FRAP)**

570 10 mL of AMB-1 cells in the early exponential phase (OD<sub>400</sub> is ~0.05) were pelleted and  
571 resuspended in ~20 µL of MG medium. 3 µL of concentrated AMB-1 cells were applied in a glass-  
572 bottom dish (MatTek Corporation) and then covered with 2% solidified agarose for cell  
573 immobilization. The 2% agarose covers were formed by spotting 400-µL melted 2% agarose  
574 prepared in MG medium to the hole of the glass-bottom dish and solidifying for more than 30 min.

575 FRAP experiments were carried out on an inverted Carl Zeiss LSM880 FCS laser scanning  
576 confocal microscope with an objective lens Plan-Apochromat 100x/1.40 Oil DIC. MamK-GFP  
577 filaments were imaged using 488 nm excitation at 0.4 % laser power and fluorescence was acquired  
578 through a 490-600 nm bandpass filter. A small area of the filaments was bleached using 488 nm  
579 laser light at 100 % laser power for 5 iterations with intervals of 30 s and 30 cycles. For each strain,  
580 images were captured through the LSM880 Zen software (Zeiss) and analysed using Fiji<sup>45</sup>.

### 581 **Time-lapse imaging using HILO microscopy**

582 For sample preparation, round coverslips (Matsunami, 25-mm diameter, 0.12–0.17 mm thick)  
583 were used as the imaging support. The coverslip was coated with poly-L-lysine and 500 µL of  
584 culture was added to an Attofluor cell chamber (Thermo Fisher Scientific). Then, a 5-mm thick  
585 gellan gum pad (containing 0.55% gellan gum and 0.08 mM MgCl<sub>2</sub> in MG liquid medium) on the  
586 top of the coverslip to sandwich the cells against the bottom coverslip during time-lapse imaging.  
587 After removing excess culture by a pipette, the chamber was filled with fresh MG liquid medium,  
588 and the top of the chamber was covered with another coverslip to allow adequate microaerobic  
589 conditions to support the growth of AMB-1 cells. The sample was set up under about 10% oxygen  
590 atmosphere. Bacteria imaging and processing were then performed as previously described<sup>16</sup>.

### 591 **Protein secondary structure prediction Methods**



592 Membrane topology prediction method CCTOP (ref or <http://cctop.enzim.ttk.mta.hu>) was used  
593 with the TM Filter and Signal Prediction. The CCTOP prediction is a consensus of 10 different  
594 methods enhanced with available structural and experimental information of any homologous  
595 proteins in the TOPDB database. Signalp 4.1 (<http://www.cbs.dtu.dk/services/SignalP-4.1/>) and  
596 Phobius (<https://phobius.sbc.su.se>) were used for signal peptide prediction. SMART  
597 (<http://smart.embl-heidelberg.de>) and InterProScan  
598 (<https://www.ebi.ac.uk/interpro/search/sequence/>) were used for protein domain prediction.

### 599 **Cellular fractionation**

600 WT AMB-1 cells expressing pAK1255 were first grown in 50-mL MG medium in conical tubes  
601 with a 1:100 dilution from stock cultures at 30°C for two days and then grown in 2-L MG medium  
602 in a microaerobic glove box (10% oxygen) for two days. These 2-L cells were pelleted by  
603 centrifugation at  $8,000 \times g$  for 15 min and kept at -80 °C freezer for future use. Cell pellets were  
604 thawed on ice and resuspended in 5-mL ice-cold 25 mM Tris buffer (pH 7.0). Pepstatin A and  
605 leupeptin were added to a final concentration of 1 µg/mL, and PMSF was added to a final  
606 concentration of 1 mM. The resuspension was passed through a French press two times at 1000  
607 psi. From this step, all samples were kept on ice or at 4 °C. 20 µg/mL DNase I and 2 mM MgCl<sub>2</sub>  
608 were added to the homogenate and incubated at 4 °C for 30 min. To separate the magnetosome  
609 fraction, the cell lysates were passed through a magnetized MACS LS column (Miltenyi Biotec  
610 Inc.) that was surrounded by magnets. After washing the column 3 times with 25 mM Tris buffer  
611 (pH 7.0), the magnets were removed and the magnetosome fraction was eluted in 5-mL of 25 mM  
612 Tris buffer (pH 7.0). To separate soluble and insoluble non-magnetic fractions, the column flow-  
613 through was centrifuged at  $160,000 \times g$  for 2 hours. The sedimented membrane fraction was  
614 resuspended with 100 mM Tris buffer (pH 7.0) and both fractions were centrifuged a second time  
615 at  $160,000 \times g$  for 2 hours. The resulting supernatant contained the non-magnetic soluble fraction  
616 and the resuspended pellet contained the non-magnetic insoluble fraction.

617 Cellular fractions were analysed by SDS-PAGE as described previously <sup>42</sup>. In brief, different  
618 fractions were mixed with 2x Laemmli Sample Buffer (Bio-Rad) and heated for 15 min at 95°C.  
619 Proteins were resolved by Bio-rad stain-free any KDs gels before transfer to nitrocellulose  
620 membrane by electroblotting. Immunological detection was performed with primary antibodies,  
621 including anti-GFP polyclonal antibodies (Abcam), anti-Mms6 polyclonal antibodies (Produced

622 by ProSci Inc), or anti-HaloTag monoclonal antibody (Promega), and HRP-Conjugated secondary  
623 antibodies (Bio-Rad).

#### 624 **Bacterial adenylate cyclase two-hybrid assay (BACTH)**

625 The assay was performed as described in the Euromedex BACTH system kit manual. N- and C-  
626 terminal T18 and T25 fusions of McdA, McdB, or MamY proteins were constructed using plasmid  
627 pKT25, pKNT25, pUT18C, and pUT18 in *E. coli* K12 recA strain XL1-Blue. T18 and T25 fusions  
628 with MamK or MamJ were generated previously <sup>47</sup>. Sequence-verified constructs expressing  
629 T18/T25 magnetosome protein fusions were co-transformed into competent *E. coli* DHM1 cells  
630 (lacking endogenous adenylate cyclase activity) in all pairwise combinations <sup>48</sup>, then plated on LB  
631 agar plates containing 100 µg/mL carbenicillin and 50 µg/mL kanamycin, and incubated at 30°C  
632 overnight. Several colonies of T18/T25 cotransformants were isolated and grown in LB liquid  
633 medium with 100 µg/mL carbenicillin and 50 µg/mL kanamycin overnight at 30°C with 220 rpm  
634 shaking. Overnight cultures were spotted on indicator LB agar plates supplemented with 40 µg/mL  
635 X-gal, 100 µg/mL carbenicillin, 25 µg/mL kanamycin, and 0.5 mM IPTG. Plates were incubated  
636 24 - 48 hours at 30°C before imaging. Bacteria expressing interacting hybrid proteins will show  
637 blue, while bacteria expressing non-interacting proteins will remain white.

#### 638 **Comparative genomics and molecular phylogenetics**

639 McaA and McaB homologs were searched in other bacterial genomes available in public databases.  
640 Protein sequences were aligned against reference proteins and non-redundant protein sequences of  
641 the *refseq\_protein* and *nr* NCBI databases respectively in October 2021 using the BLASTP  
642 algorithm, a word size of 6 and default scoring parameters. A similar task was performed using  
643 public genomic assemblies of MTB annotated with the Microscope platform <sup>49</sup>. BLAST hits with  
644 an expectation value below  $5 \times 10^{-2}$  were further analysed. First, pairwise sequence comparisons  
645 were performed using BLASTP (BLAST+ version 2.10.0). Sequence clustering was then  
646 performed with the Mmseqs2 <sup>50</sup> clustering algorithm version 13.45111 to define groups of distant  
647 homologs using the default parameters, a sequence identity threshold of 30% and an alignment  
648 coverage of 80% for the longer sequence and for the shorter sequence.

649 A first phylogenetic tree was built to determine the monophyly of the different clusters and  
650 evaluate their taxonomic and phenotypic composition. For this task, the total 38 McaA and 31  
651 McaB homologous sequences retrieved were aligned using MAFFT version 7.487 <sup>51</sup>. Relaxed

652 trimming on the alignments was then performed using BMGE <sup>52</sup>, selecting the BLOSUM30  
653 substitution matrix, a minimum block size of 2 and removing characters with more than 50% gaps.  
654 Maximum likelihood trees were built using IQ-TREE <sup>53</sup> version 2.1.3, the substitution model for  
655 each protein was selected by ModelFinder <sup>54</sup> with the Bayesian Information Criterion. The  
656 statistical support of the branches was estimated by a standard nonparametric bootstrapping  
657 approach implemented in IQ-TREE applying 500 replicates. All sequences retrieved belong to  
658 magnetotactic bacteria from *Proteobacteria* classes and *Nitrospirae*. Non-Alphaproteobacteria  
659 members were used to form external groups and infer the ancestry of McaA and McaB genes  
660 compared to other clusters.

661 Guided by these first phylogenies, a second set of trees was built following the same approach  
662 using genome sequences of strains for which transmission electron microscopy images of the  
663 magnetosome chains are available and for which organisation could be compared with that of  
664 *Magnetospirillum magneticum* AMB-1. Relationships between sequences and chain features were  
665 then inferred after collecting all metadata including transmission electron microscopy images  
666 published previously (Table S1). The synteny analyses were further explored using the tools  
667 implemented in the Microscope <sup>49</sup> platform.

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859



## 860 **Acknowledgments**

861 We thank members of the Komeili lab for helpful discussions and suggestions. We thank Pranami  
862 Goswami for helping generate the plasmid pAK1037, Julia Borden for helping generate the  
863 plasmids pAK1101 and pAK1102, Pedro Leão for helping generate the plasmid pAK1121. We  
864 thank Elizabeth Montabana and Kenneth H. Downing for assistance with cryo-ET data collection  
865 using the cryo-EM facility at Lawrence Berkeley National Laboratory. We also thank Danielle  
866 Jorgens, Reena Zalpuri, and Guangwei Min from the UC Berkeley Electron Microscope  
867 Laboratory for assistance in electron microscopy data collection. We thank Steven Ruzin and  
868 Denise Schichnes from the CNR Biological Imaging Facility at UC Berkeley for their technical  
869 support with fluorescent microscopes. The Zeiss Elyra PS.1 Super-Resolution microscope for SIM  
870 was supported in part by the National Institutes of Health S10 program under award number  
871 1S10OD018136-01. We thank the INRA MIGALE bioinformatics platform  
872 (<http://migale.jouy.inra.fr>) for providing computational resources. We thank Michelle C. Chang  
873 from Department of Chemistry at UC Berkeley for the kind gift of anti-Mms6 antibodies. Finally,  
874 We thank Luke D. Lavis from Howard Hughes Medical Institute Janelia Research Campus for  
875 providing free JF549 and JF646 Halo ligands before they are commercialized.

876

## 877 **Author Contributions**

878 J.W. conceived, performed, and analysed most of the experiments. A.T conducted the live-cell  
879 imaging analysis with HILO microscopy. C.L.M and C.T.L. performed the phylogenetic analysis  
880 of McaA and McaB. G.E. designed and generated the small region (iR1-iR4) deletion plasmids,  
881 generated the deletion mutants of  $\Delta iR1-\Delta iR4$  and  $\Delta mamJ\Delta limJ\Delta iR1-\Delta mamJ\Delta limJ\Delta iR4$ , and  
882 collected the TEM images of these mutants. K.P. generated the deletion mutants of  $\Delta MIS$  and  
883  $\Delta mamJ\Delta limJ\Delta MIS$ , collected the TEM images of these two mutants. M.A. discovered the crystal  
884 shape difference between WT and  $\Delta MIS$ . E.T-C. helped to establish the Halo-based pulse-chase  
885 experiment system. A.K. supervised the experimental design, data analysis, and data presentation.  
886 J.W., A.T., C.L.M., C.T.L., and A.K. wrote the manuscript, and all co-authors edited the  
887 manuscript.

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889 **Competing interests**

890 The authors declare no competing interests.

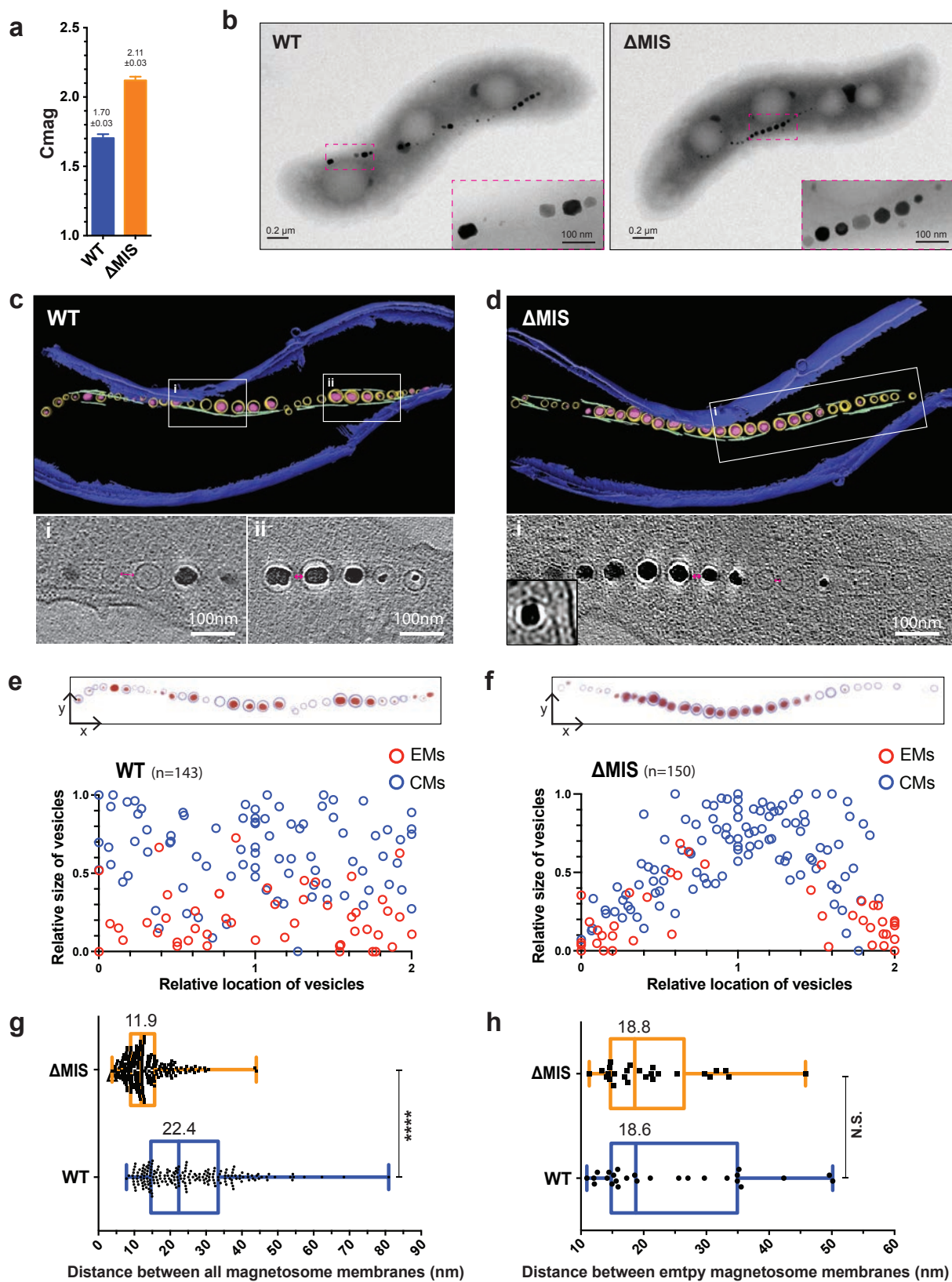
891 **Tables**

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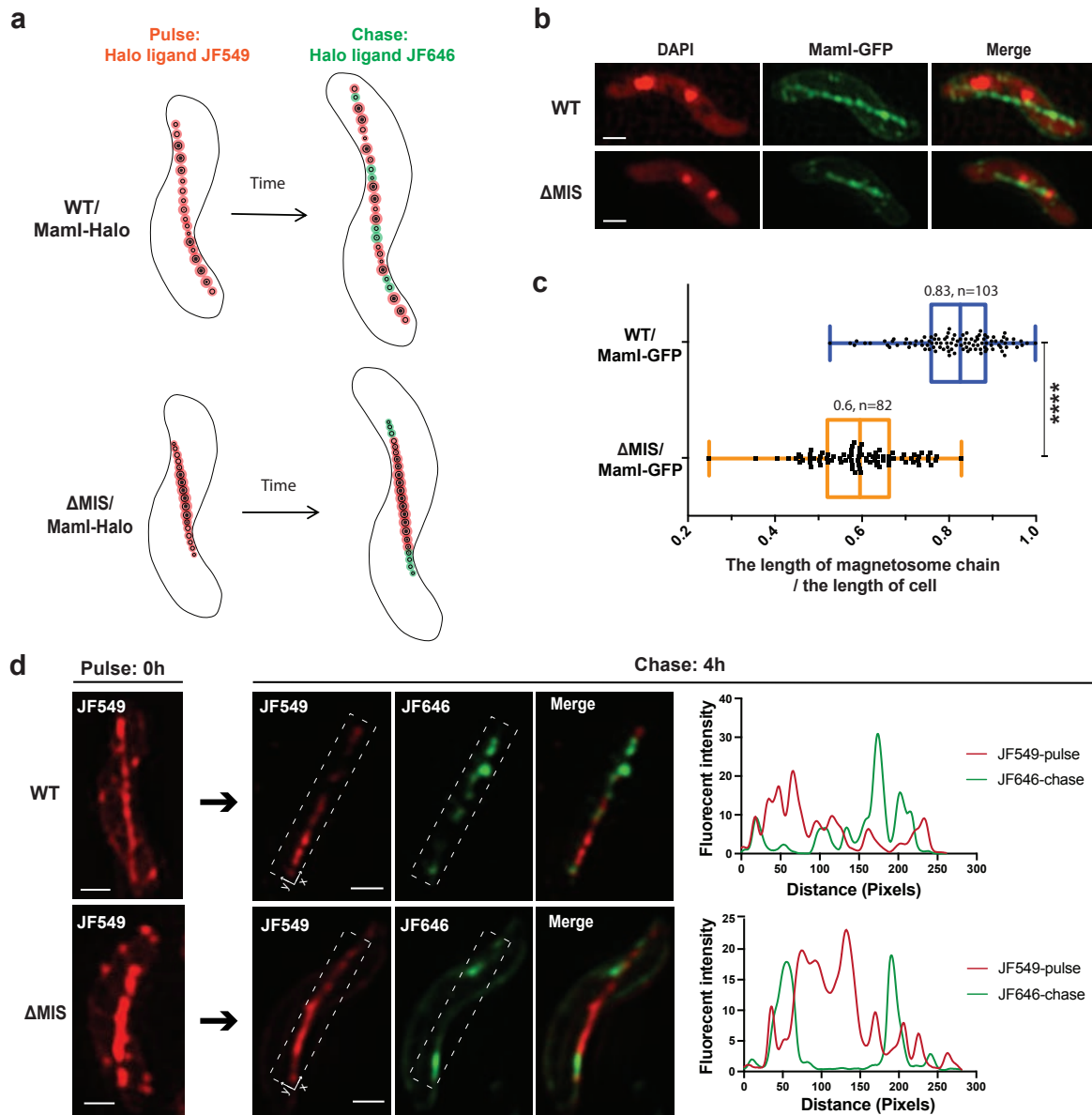
Table 1. The interaction results of BACTH.

	Zip-T25	T25-McaA	McaA-T25	T25-McaB	McaB-T25	T25-MamK	MamK-T25	T25-MamJ	T25-MamY	MamY-T25
<b>Zip-T18</b>	+	NA	NA	NA	NA	NA	NA	NA	NA	NA
<b>T18-McaA</b>	NA	-	-	-	-	-	-	-	-	-
<b>McaA-T18</b>	NA	-	-	-	-	-	-	-	-	-
<b>T18-McaB</b>	NA	-	-	-	-	-	-	-	-	-
<b>McaB-T18</b>	NA	-	-	-	-	-	-	-	-	-
<b>T18-MamK</b>	NA	-	-	-	-	+	+	NA	NA	NA
<b>MamK-T18</b>	NA	-	-	-	-	+	+	NA	NA	NA
<b>MamJ-T18</b>	NA	-	-	-	-	NA	NA	-	NA	NA
<b>T18-MamY</b>	NA	-	-	-	-	NA	NA	NA	-	-
<b>MamY-T18</b>	NA	-	-	-	-	NA	NA	NA	-	+

893 NA, No analysis was performed. -, Negative Interaction (White Colonies), +, Positive Interaction (Blue Colonies).

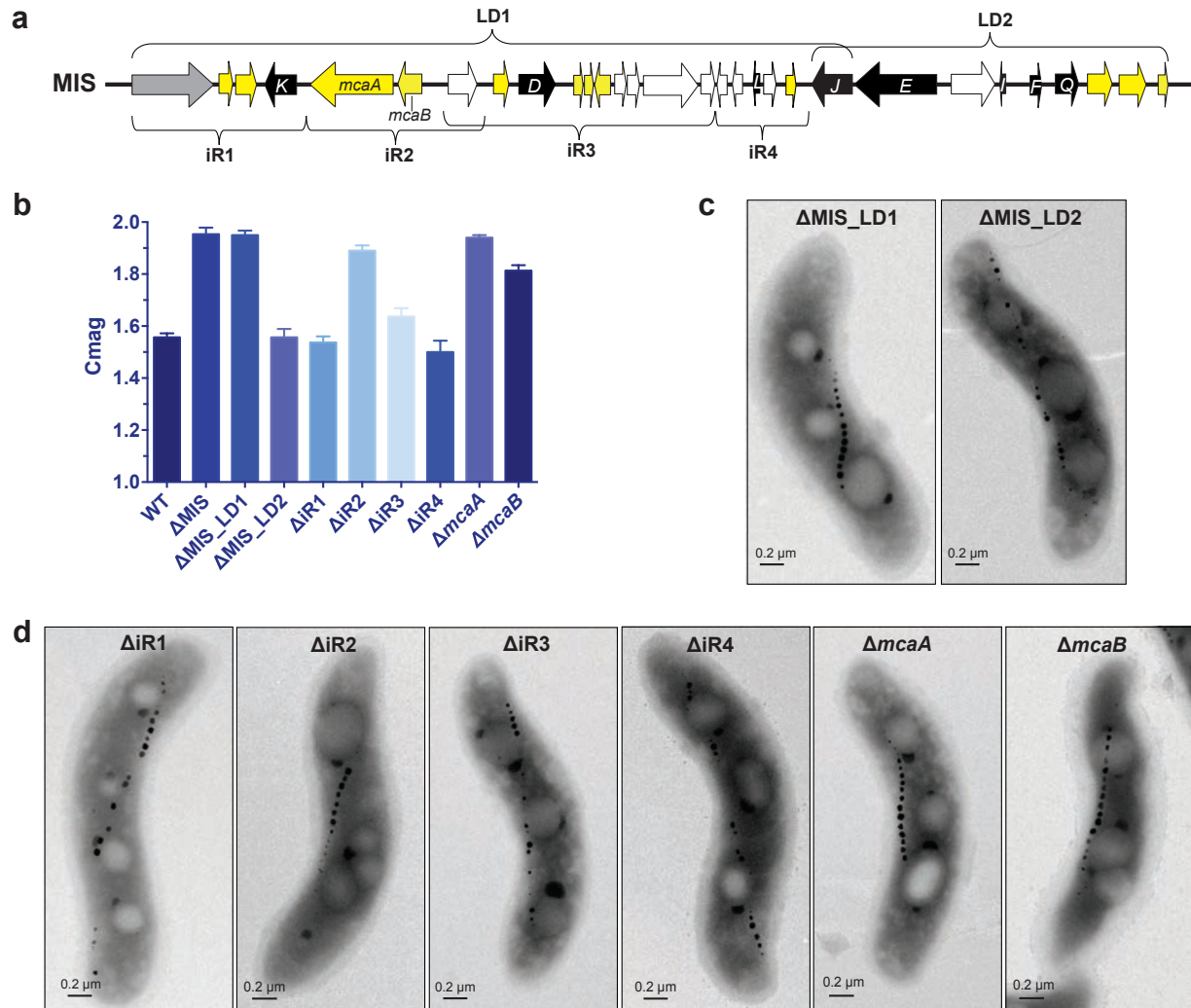


**Fig. 1: MIS genes contribute to the magnetosome chain assembly.** (a) Magnetic response ( $C_{mag}$ ) of WT and  $\Delta$ MIS cultures grown under microaerobic conditions. Each measurement represents the average and standard deviation from three independent growth cultures. (b) TEM micrographs of WT and  $\Delta$ MIS cells. Insets: magnification of the magnetic crystals in magenta rectangles. (c) and (d) Segmented 3D models (upper panels) and selected area of tomographic slices (lower panels, Box i and ii) showing phenotypes of WT (c) and  $\Delta$ MIS (d) strains. The outer and inner cell membranes are depicted in dark blue, magnetosome membranes in yellow, magnetic particles in magenta, and magnetosome-associated filaments in green. Full tomograms are shown in Movies S1 and S2. (e) and (f) Relative size and location of magnetosome vesicles in WT and  $\Delta$ MIS cells, respectively (lower panels). Upper panels are 2D projections of magnetosomes from the 3D models in (c) and (d). The magnetosome membranes are shown in light blue and magnetic particles are shown in red. (g) and (h) Edge-to-Edge distance between all of the magnetosomes (g) and the EMs (h) measured from neighbouring magnetosome membranes. Values represent the median. The box plots show the median (line within box), the first and third quartiles (bottom and top of the box). P-values were calculated by the Mann-Whitney test. No statistically significant difference ( $P > 0.05$ , N.S.), significant difference ( $****P < 10^{-4}$ ).



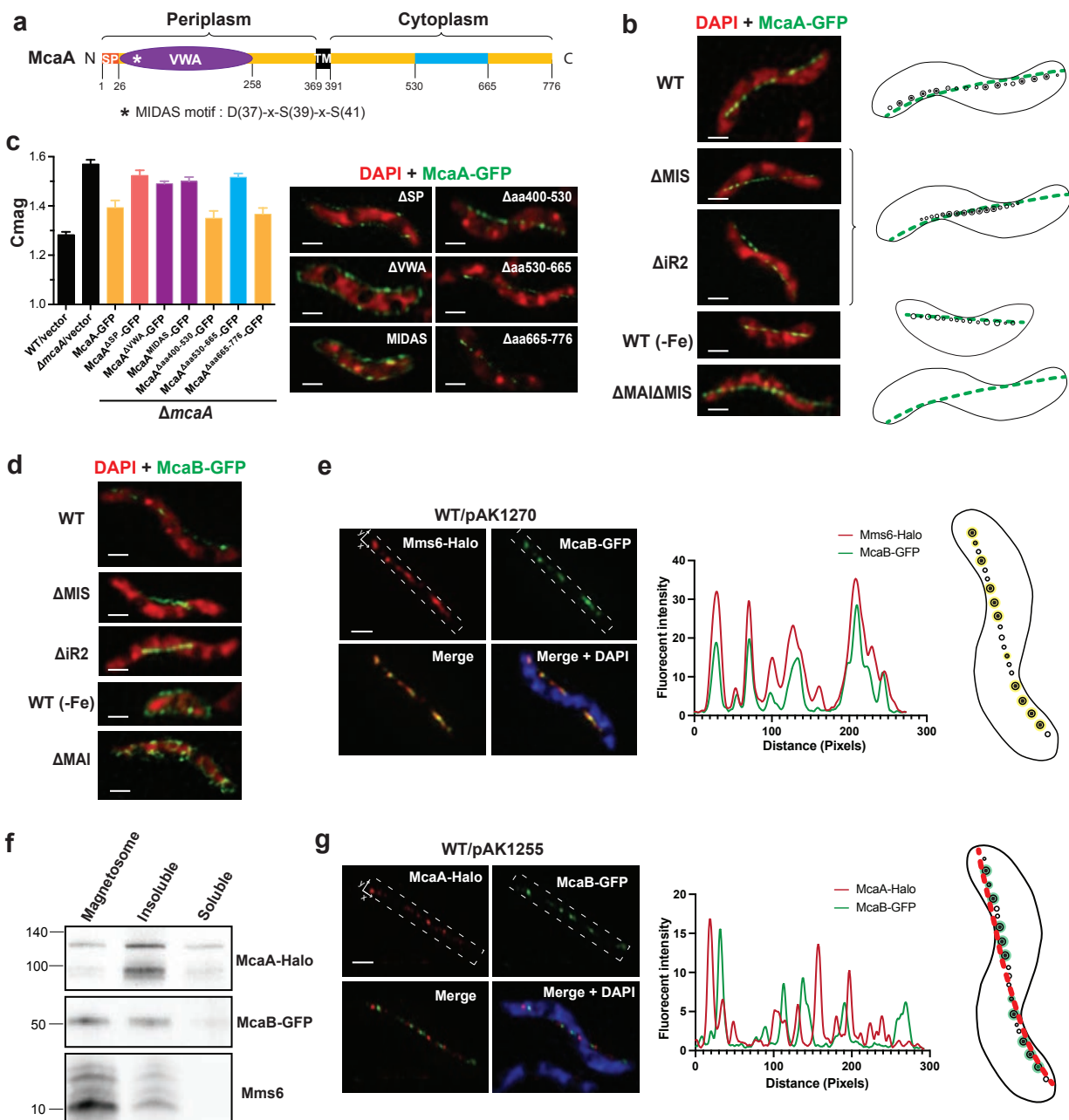
**Fig. 2: MIS genes determine the location of newly formed magnetosomes in the chain.** (a) Model of the pulse-chase experiment shows how the newly-formed EMs are added to the magnetosome chains in WT and  $\Delta$ MIS. (b) SIM micrographs of WT and  $\Delta$ MIS cells expressing Mami-GFP under standard growth conditions. The DAPI staining is shown in false-colour red, Mami-GFP is shown in green. (c) Quantification of the length of magnetosome chain versus the length of cell in WT and  $\Delta$ MIS strains. (d) Left and middle: SIM micrographs from the pulse-chase experiments with Mami-Halo fusion protein for analysing the addition of newly-formed magnetosomes in WT and  $\Delta$ MIS. The JF549 staining is shown in red, and the JF646 staining is

shown in green. Right: fluorescent intensity map of the dashed rectangular area on the SIM micrographs. Scale bars are 0.5  $\mu\text{m}$  in (b) and (d).



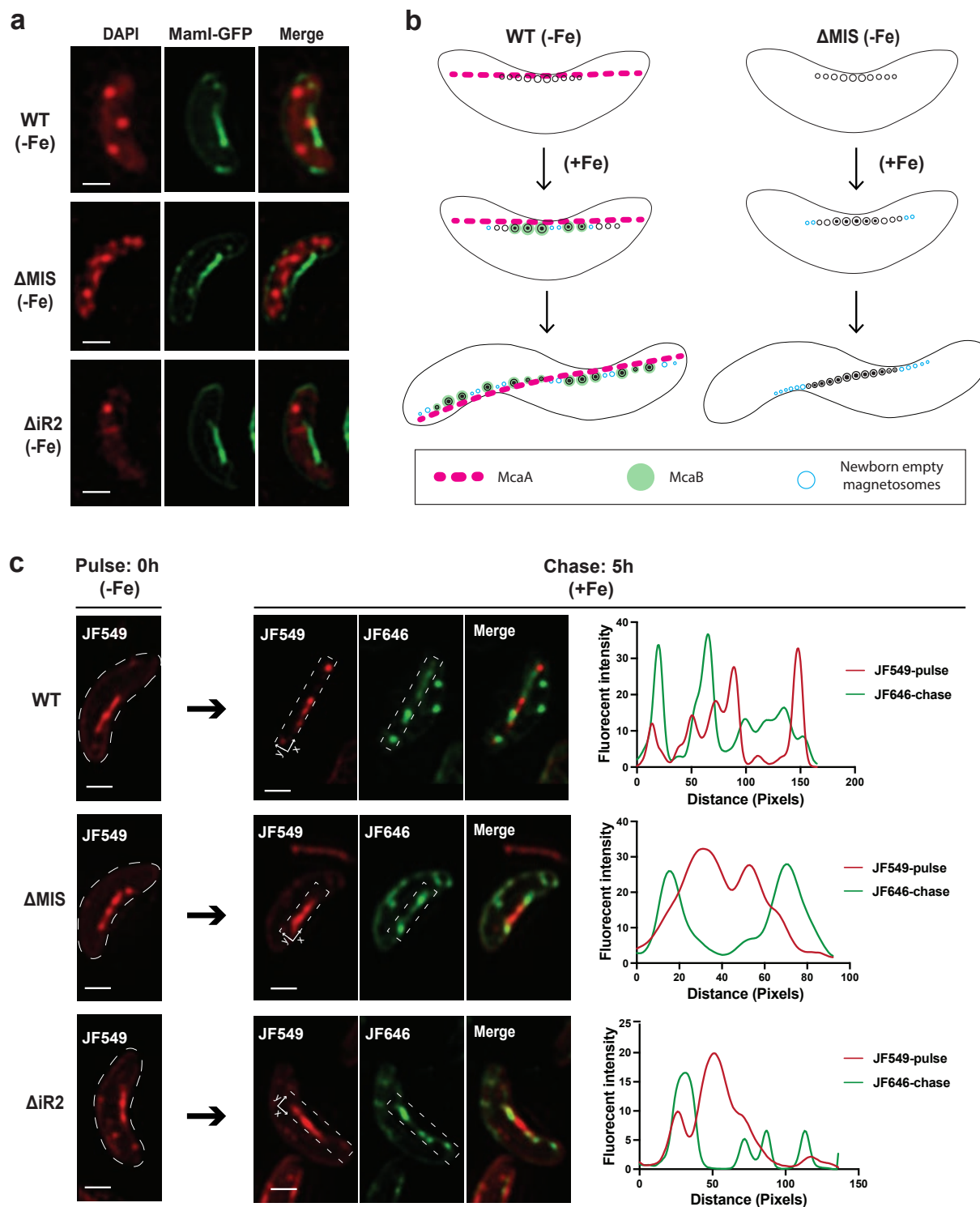
**Fig. 3: Comprehensive genetic dissection of the MIS identified *mcaA* and *mcaB* as chain assembly factors.** (a) Schematic depicting the MIS region of AMB-1, including predicted magnetosome gene homologs (black), a phage-associated protein (grey), transposases (white), and hypothetical genes (yellow). (b) Cmag of WT and different mutants in the MIS region. Each measurement represents the average and standard deviation from three independent growth cultures. (c) TEM micrographs of  $\Delta$ MIS\_LD1 and  $\Delta$ MIS\_LD2 cells. (d) TEM micrographs of  $\Delta$ iR1-  $\Delta$ iR4,  $\Delta$ *mcaA*, and  $\Delta$ *mcaB* cells.





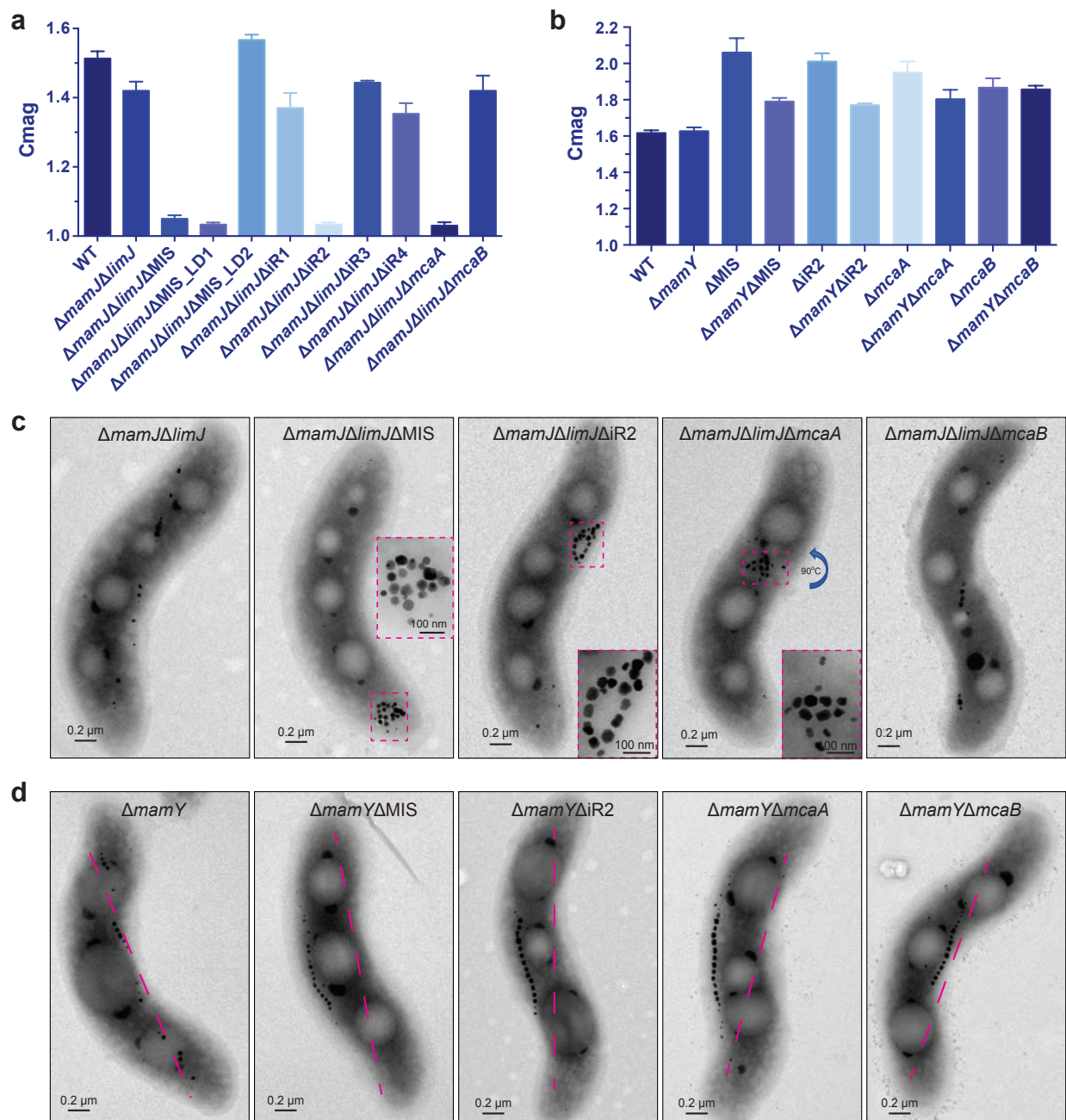
**Fig. 4: Localization of McaA and McaB.** (a) Predicted secondary structure and topology of McaA. SP, signal peptide. VWA, von Willebrand factor type A domain. TM, transmembrane domain. (b) SIM micrographs of cells expressing McaA-GFP (left) and models of magnetosome production (right) in WT and different genetic backgrounds or growth conditions. (c) Cmag (left) and SIM micrographs (right) of mutated McaA-GFP expressed in  $\Delta mcaA$  cells. (d) SIM micrographs show the localization of McaB-GFP in WT and different genetic backgrounds. From (b) to (d), DAPI staining is shown in false-colour red and the GFP fusion proteins are shown in green. (e) Left: SIM

micrographs show the localization of Mms6-Halo and McaB-GFP in WT. Middle: fluorescent intensity map of the dashed rectangular area on the SIM micrographs. Right: model of the colocalization (yellow) of Mms6 and McaB at CMs. (f) Western blotting shows McaA and McaB are enriched in different cellular fractionations. (g) Left: SIM micrographs show the localization of McaA-Halo and McaB-GFP in WT. Middle: fluorescent intensity map of the dashed rectangular area on the SIM micrographs. Right: model of the association of McaA (red) and McaB (green) with magnetosomes. In (e) and (g), the DAPI staining is shown in blue, the JF549-stained Halo proteins are shown in red, GFP fusion proteins are shown in green. Scale bars: 0.5  $\mu$ m.

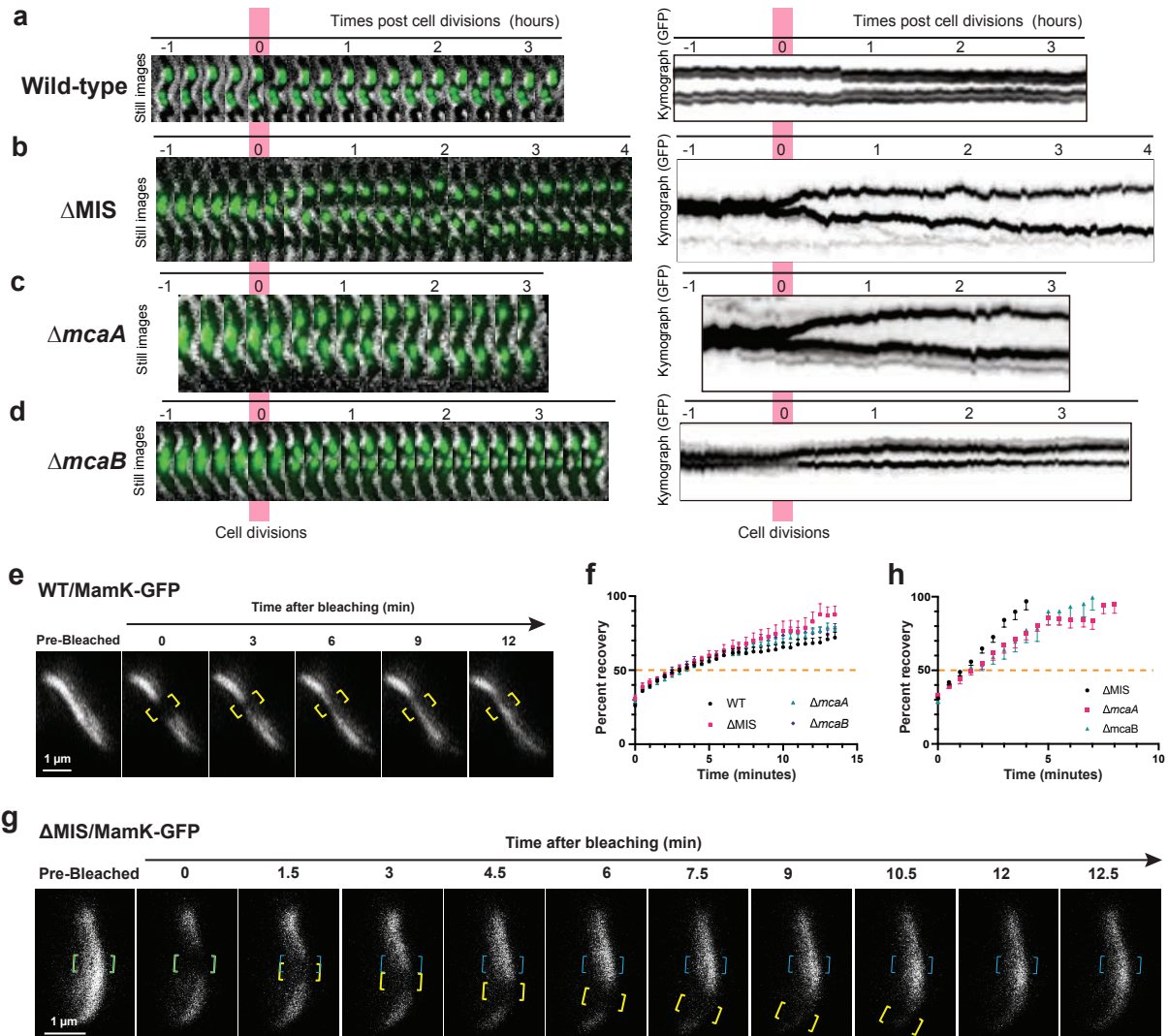


**Fig. 5: Addition of newly formed magnetosomes to the chain from iron starvation to normal iron growth conditions.** (a) Representative SIM micrographs of WT,  $\Delta$ MIS, and  $\Delta$ iR2 cells expressing MamI-GFP under iron starvation growth conditions. The DAPI staining is shown in

false-colour red, MamI-GFP is shown in green. (b) Model of how McaA and McaB coordinate to control the addition of newly formed magnetosomes in WT and  $\Delta$ MIS cells. A continuous chain of EMs is generated in the middle of both WT and McaAB deficient cells under iron starvation conditions. After iron is added to the medium, magnetic crystals are mineralized in the existing EMs<sup>20, 22</sup>. Once EMs become CMs, the McaB is recruited to CMs and help locating CMs to the gaps of dashed McaA, thereby increasing the distance between CMs, which allows for the addition of newly formed EMs inbetween CMs in WT AMB-1. In the absence of McaAB, CMs are located close to each other, and newly formed EMs are added at both ends of the chain. (c) Left and middle: SIM micrographs from pulse-chase experiments with MamI-Halo fusion protein for analysing the addition of newly formed magnetosomes in different AMB-1 genetic backgrounds from iron starvation to normal iron growth conditions. Right: fluorescent intensity map of the dashed rectangular area on the SIM micrographs. JF549 staining is shown in red, and JF646 staining is shown in green. Scale bars are 0.5  $\mu$ m in (a) and (c).



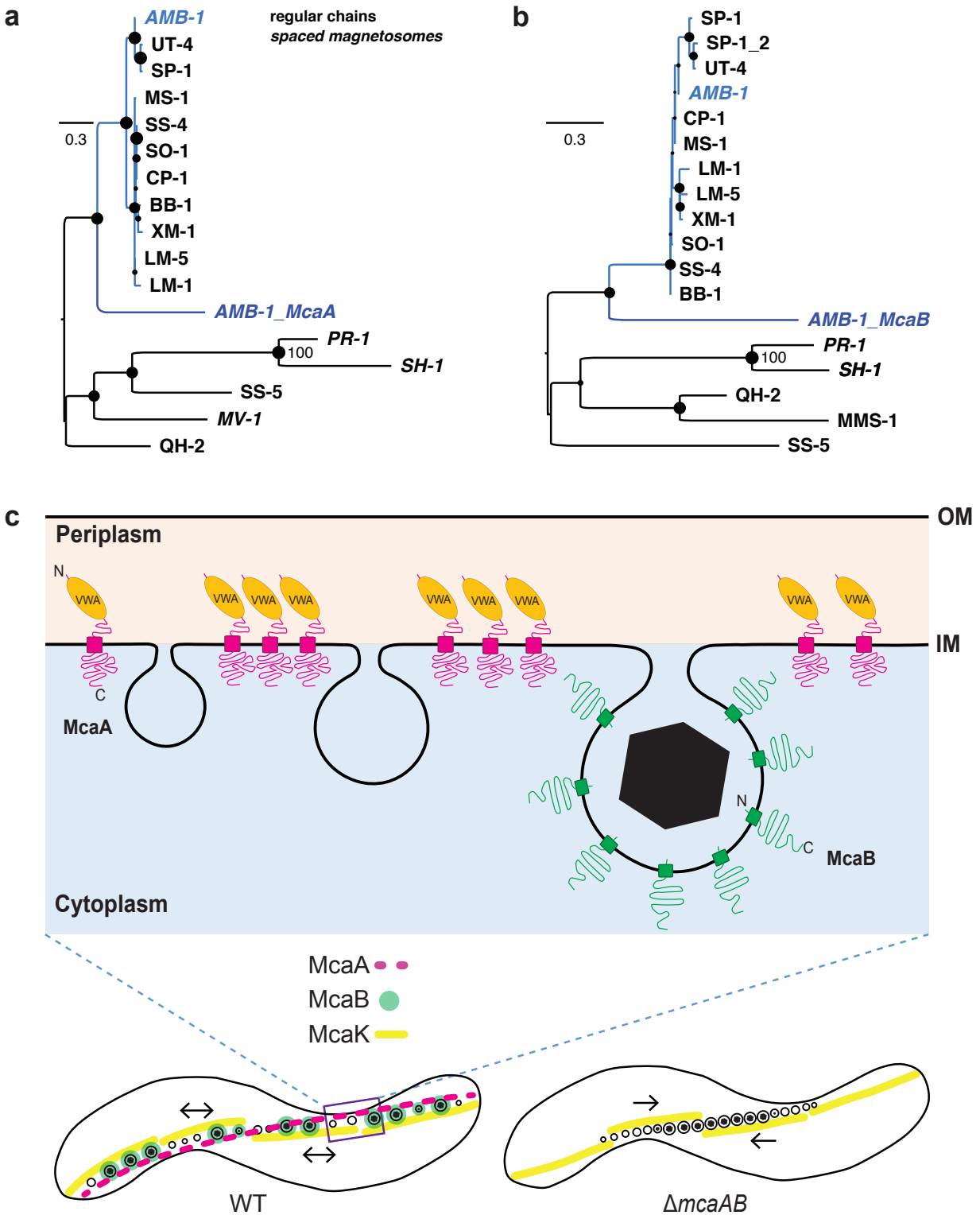
**Fig. 6: McaA prevents magnetosome chain aggregation and directs the chain to the positively curved regions of the cytoplasmic membrane.** (a) and (b) C<sub>mag</sub> of WT and different mutants in the  $\Delta mamJ\Delta limJ$  (a) and  $\Delta mamY$  (b) backgrounds. Each measurement represents the average and standard deviation from three independent growth cultures. (c) TEM micrographs of  $\Delta mamJ\Delta limJ$ ,  $\Delta mamJ\Delta limJ\Delta MIS$ ,  $\Delta mamJ\Delta limJ\Delta iR2$ ,  $\Delta mamJ\Delta limJ\Delta mcaA$ , and  $\Delta mamJ\Delta limJ\Delta mcaB$  cells. (d) TEM micrographs of  $\Delta mamY$ ,  $\Delta mamY\Delta MIS$ ,  $\Delta mamY\Delta iR2$ ,  $\Delta mamY\Delta mcaA$ , and  $\Delta mamY\Delta mcaB$  cells.



**Fig. 7: McaAB control the segregation of magnetosomes and dynamics of MamK filaments.**

(a) to (d) Effects of *mcaA* and *mcaB* deletions on magnetosomes segregation. Live-cell time-lapse imaging of magnetosome segregation in wild-type (a),  $\Delta$ MIS (b),  $\Delta$ *mcaA* (c), and  $\Delta$ *mcaB* (d) cells during cell division. Magnetosomes are marked with Mms6-GFP. Left: GFP fluorescence and bright field merged time-lapse still images. Right: kymographs of Mms6-GFP signals in maximum projection. (e) A FRAP experiment time course with WT AMB-1 expressing MamK-GFP. Yellow brackets indicate the portion of the MamK-GFP filament designated for photobleaching. (g) A FRAP experiment time course with  $\Delta$ MIS expressing MamK-GFP where the bleached area moved from its original position toward the cell pole. Yellow and blue brackets indicate the portion of the MamK-GFP filament designated for photobleaching. Blue brackets indicate the original bleaching area, and yellow brackets track the movement of the bleached area. The MamK-GFP is shown in

false-colour white in (e) and (g). (f) and (h) Normalized (average mean and standard error of mean [SEM]) percent recovery of each strain's recovering cells with non-moving (f) and moving (h) bleached area. The 50% mark is noted with a dashed orange line.



**Fig. 8: Phylogenetic analysis and model of McaAB-mediated magnetosome chain assembly.**  
(a) and (b) Maximum likelihood trees showing the ancestry of McaA (a) and McaB (b) proteins in



relation to their homologs in freshwater magnetotactic *Rhodospirillaceae* (blue clade) and the external groups of other *Proteobacteria*. All strains' accession numbers are given in supplementary dataset 1. Trees were drawn to scale and branch length refers to the numbers of substitution per site. Robustness of the internal branches is symbolized by a circle whose size is proportional to the bootstrap value estimated from 500 non-parametric replicates. The magnetosome chains in these strains were previously characterized. If magnetosomes are spaced from each other similarly to strain AMB-1, names are in italics. (c) McaA serves as a landmark on the positively curved inner membrane and coordinates with McaB to control the location of CMs to the gap region of dashed-McaA. As a consequence, the neighbouring CMs are separated from each other, which allows the addition of newly formed EMs to multiple sites of the magnetosome chain in WT AMB-1. Alternatively, the CMs are located closely together without McaAB, leaving no space for the addition of newly formed EMs between CMs but at both ends of the magnetosome chain. McaAB also influence the dynamics of MamK filaments to control the dynamic positioning of magnetosomes during the whole cell cycle. OM, outer membrane. IM, inner membrane.