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

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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 ¹. Organelle positioning in eukaryotic cells mainly relies on cytoskeletal and motor
33 proteins ¹. Many bacteria also produce organelles ², 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 ³. Similarly, it has been proposed that the carbon storage
37 polyhydroxybutyrate (PHB) granules associate with nucleoids to mediate segregation during cell
38 division ^{4,5}. 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 (Fe₃O₄) and/or greigite (Fe₃S₄) ^{2,6}, 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 ^{7,8}. MTB are
43 the simplest and most ancient organism capable of magnetic navigation ⁹ and fossilized
44 magnetosomes chains have been used as robust biosignatures ^{10,11}. 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 ^{12,13,14}. The mechanisms leading to distinct chain
51 configurations remain unknown, but may reflect strategies for adaptations to specific biotopes ¹⁰.
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 ¹⁵. 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 ¹⁶. 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^{17,18}. 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^{16,17}. 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*_{AMB-1} complements the MSR-1 Δ *mamK* mutant¹⁹. However, the speed and
65 spatial dynamics of MamK filaments are distinct in each organism^{16,17}. 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 cell²⁰. However, similar deletions in AMB-1 result in subtle defects
68 with magnetosomes still organised as chains²¹. Additionally, *mamY* is critical for localizing the
69 chain to the positive curvature of the cell in MSR-1²² but does not have an impact on chain
70 organization when deleted in AMB-1²³. 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)²⁴. Unlike MSR-1, AMB-1 contains an extra genomic cluster,
75 termed the magnetotaxis islet (MIS), outside of the MAI region²⁵. 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 function²⁵. 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 above²⁶. 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 pseudogenes²⁵.

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 field²⁷. Unexpectedly, the C_{mag} values of Δ MIS cultures are much higher than Wild-type (WT)
102 cultures (Fig. 1a), indicating that, as a population, Δ 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 Δ 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 Δ 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)^{2, 6}. To directly observe the organisation of
115 magnetosome membranes, we imaged WT and Δ MIS cells with whole-cell cryo-electron
116 tomography (cryo-ET) (Fig. 1c, d). Similar to WT²⁸, the magnetosome membranes of Δ 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 Δ 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 Δ 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 Δ 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 Δ 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^{29,30,31}.
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^{26,31}. We first checked the localization of MamI-GFP in WT and Δ 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 Δ 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 Δ 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 Δ 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 Δ 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 Δ MIS (Fig. 2c). As mentioned above, the
157 number of crystals in WT and Δ 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 Δ 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 (Δ iR1, Δ iR2,
170 Δ iR3, and Δ 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 Δ *mcaA* and Δ *mcaB* strains have dramatically
177 higher Cmag compared to WT and are similar to Δ MIS (Fig. 3b). Similar to the Δ MIS mutant,
178 Δ *mcaA* and Δ *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 cell²², we wondered if McaA is associated with magnetosomes. To test this,
192 we expressed McaA-GFP in different genetic backgrounds and growth conditions, including Δ MIS
193 and Δ iR2 mutants which contain continuous crystal chains, WT under iron starvation where only
194 EMs are present, and the Δ MAI Δ 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)^{32, 33}. 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^{MIDAS} mutant cannot complement Δ 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 Δ MIS and Δ 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 Mms6³⁴. 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, Δ MIS, and Δ 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 Δ MIS or Δ 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 Δ MIS and
254 Δ iR2 (Fig. 5c and Supplementary Fig. 5b). Accordingly, quantitative analysis shows a low
255 colocalization coefficient of the pulse and chase signals in WT, Δ MIS, and Δ 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 aggregate²⁰. In contrast,
266 the deletion of *mamJ* and its homolog *limJ* in AMB-1 still shows a magnetosome chain with some
267 minor structural defects²¹. However, deletion of the entire MIS in a Δ *mamJ* Δ *limJ* strain causes a
268 dramatic chain collapse phenotype resembling those of MSR-1 Δ *mamJ* mutant^{10,20}. 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 Δ *mamJ* Δ *limJ* background. Cmag and TEM images show that *mcaA* is the specific gene that
273 prevents magnetosome aggregation in the Δ *mamJ* Δ *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 ²². 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 ²². 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 Δ *mamY Δ MIS mutant of AMB-1 has a much lower Cmag than Δ 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 ^{16, 17}. 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 Δ MIS and Δ *mcaA* cells shows dynamic magnetosome chain segregation after cell
300 division (Fig. 7b, c and Supplementary Movie 2, 3). In Δ MIS and Δ *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 Δ *mcaA* cells was about 20 nm/min, in line with what has been reported

305 previously for MSR-1¹⁷. Supplementary Movies 5 and 6 are long time-lapse videos of the Δ 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^{16, 17}. 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 Δ 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 Δ 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 scale³⁵ 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-1²². 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 Δ 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 motif³².

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^{36, 37, 38}. 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-1³⁹. 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 MamK³⁹. 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* cluster³⁵. However, given the known
421 *Magnetospirillum* evolutionary history³⁵, 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^{10, 11}. 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 previously⁴⁰. 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 μ 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 μ L of stock cultures
454 were added into 10 mL of MG medium in sealed Balch tubes. The MG medium was flushed with
455 N₂ 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 μ M ferric malate were added to the MG
457 medium just before inoculation with bacteria. For low iron growth condition, 100 μ 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- μ 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 described⁴¹. 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 (OD₄₀₀) 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 OD₄₀₀ were recorded. The ratio of the maximum to the minimum was designated as AMB-1 cells'
493 C_{mag}.

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 previously⁴².

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

504 Cryo-ET sample preparation and data collection were performed as described previously⁴². The
505 two-dimensional (2D) images of WT and Δ 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 software⁴³ from -60° to $+60^\circ$ with 1.5°
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 package⁴⁴.
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 package⁴⁴. To evaluate the
515 relative size and location of magnetosome membranes, the diameter of each magnetosome
516 membrane was measured as described previously⁴⁰. 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 μL 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 Δ MIS cells were manually measured using the Fiji
537 software package ⁴⁵. 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 ~ 0.05). Cells were pelleted and resuspended with 60- μ L MG medium
547 and mixed with 120- μ L 5 μ 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- μ L
553 MG medium, and mixed with 120- μ L 5 μ 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)⁴⁶ 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 (OD₄₀₀ 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 Fiji⁴⁵.

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 MgCl₂ 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 described¹⁶.

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 MgCl₂
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 ⁴². 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 ⁴⁷. 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 ⁴⁸, 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 ⁴⁹. BLAST hits with
644 an expectation value below 5×10^{-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 ⁵⁰ 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 ⁵¹. Relaxed

652 trimming on the alignments was then performed using BMGE ⁵², 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 ⁵³ version 2.1.3, the substitution model for
655 each protein was selected by ModelFinder ⁵⁴ 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 ⁴⁹ platform.

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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 ΔMIS 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 ΔMIS . 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.

888

889 **Competing interests**

890 The authors declare no competing interests.

891 **Tables**

892

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
Zip-T18	+	NA	NA	NA	NA	NA	NA	NA	NA	NA
T18-McaA	NA	-	-	-	-	-	-	-	-	-
McaA-T18	NA	-	-	-	-	-	-	-	-	-
T18-McaB	NA	-	-	-	-	-	-	-	-	-
McaB-T18	NA	-	-	-	-	-	-	-	-	-
T18-MamK	NA	-	-	-	-	+	+	NA	NA	NA
MamK-T18	NA	-	-	-	-	+	+	NA	NA	NA
MamJ-T18	NA	-	-	-	-	NA	NA	-	NA	NA
T18-MamY	NA	-	-	-	-	NA	NA	NA	-	-
MamY-T18	NA	-	-	-	-	NA	NA	NA	-	+

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

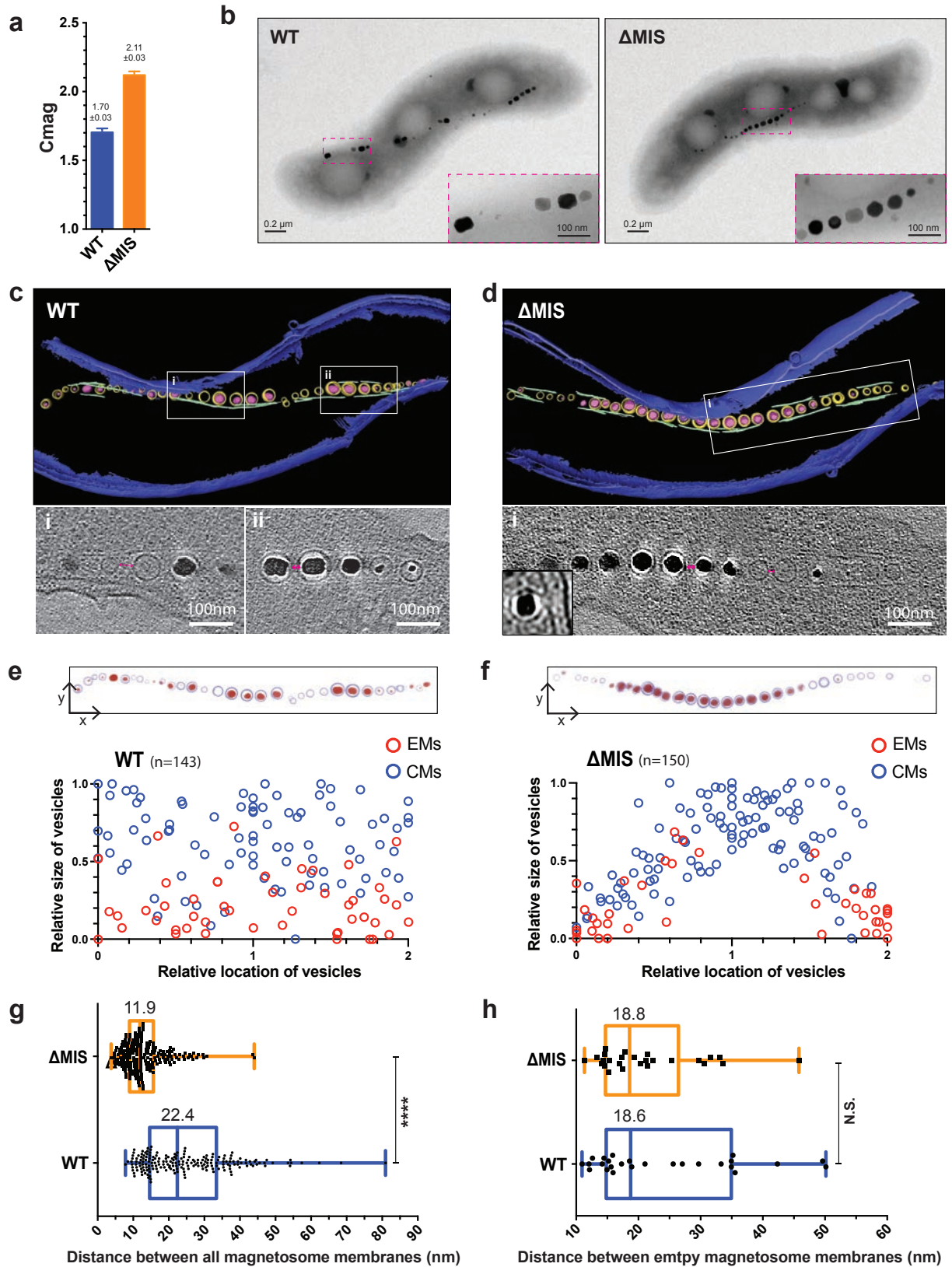


Fig. 1: MIS genes contribute to the magnetosome chain assembly. (a) Magnetic response (Cmag) of WT and Δ MIS cultures grown under microaerobic conditions. Each measurement represents the average and standard deviation from three independent growth cultures. (b) TEM micrographs of WT and Δ MIS cells. 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 Δ 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 Δ 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 ($****P < 10^{-4}$).

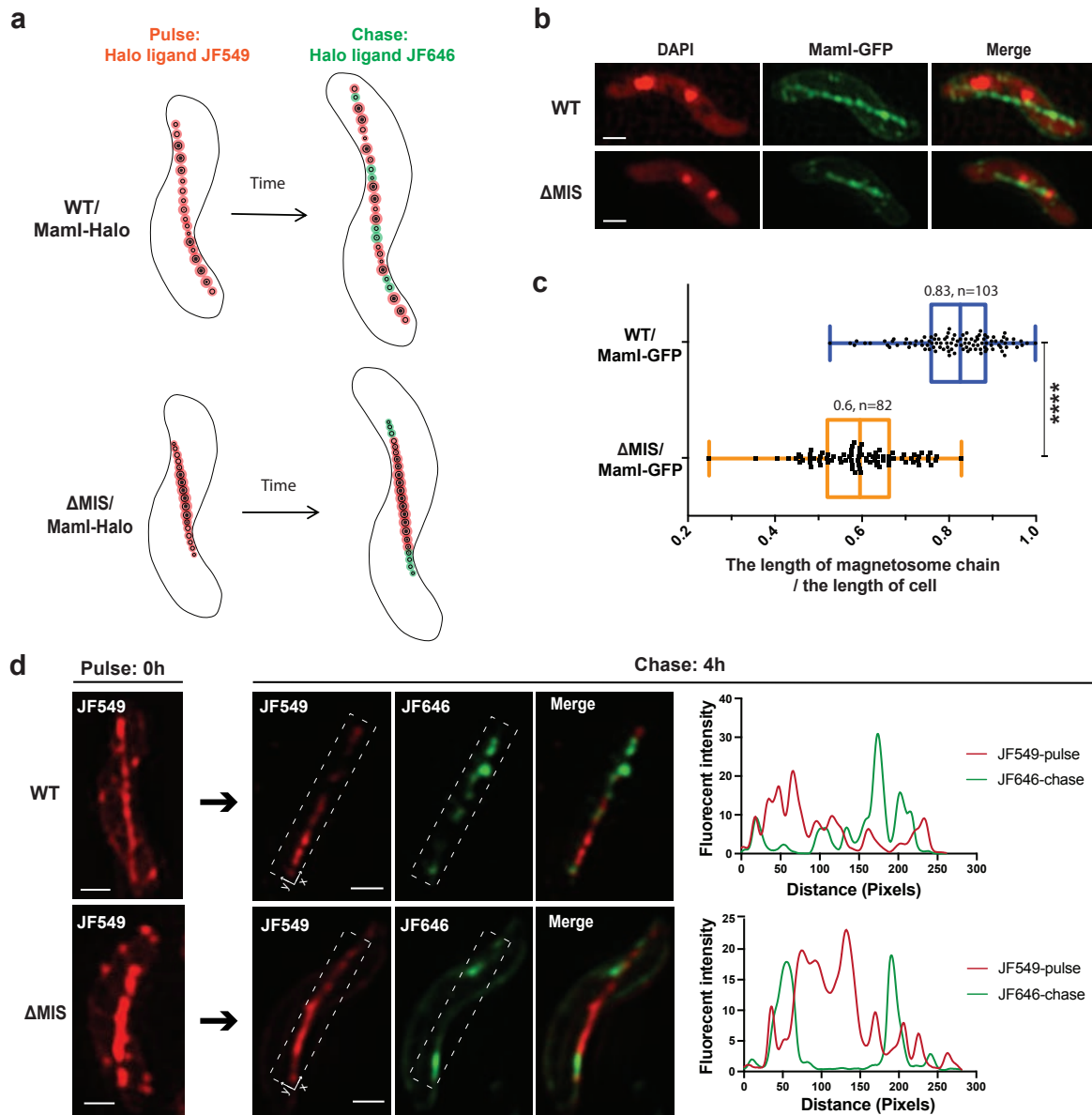


Fig. 2: MIS genes determine the location of newly formed magnetosomes in the chain. (a) Model of the pulse-chase experiment shows how the newly-formed EMs are added to the magnetosome chains in WT and Δ MIS. (b) SIM micrographs of WT and Δ MIS cells expressing Mami-GFP under standard growth conditions. 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 Δ 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 Δ 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 μm in (b) and (d).

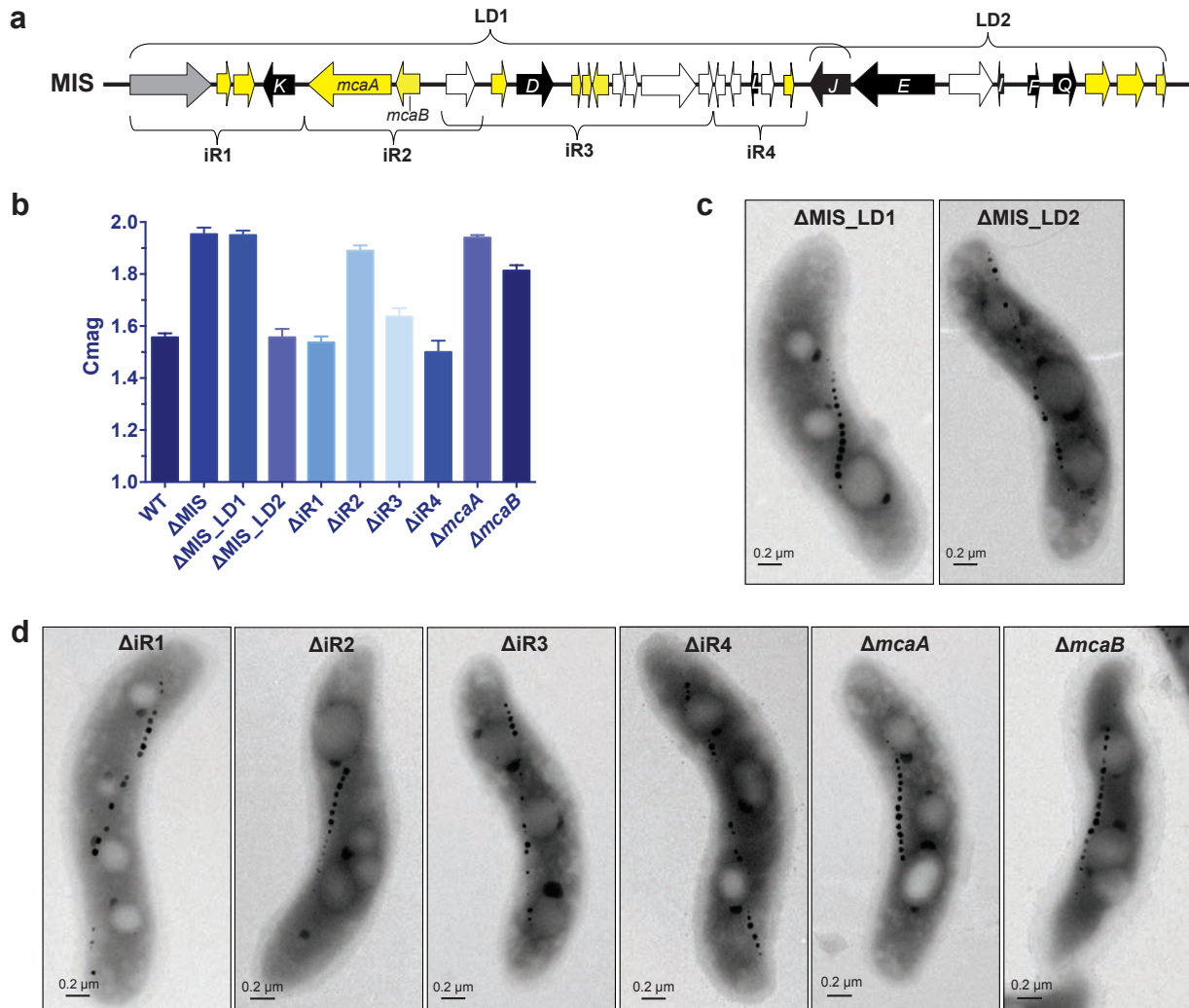


Fig. 3: Comprehensive genetic dissection of the MIS identified *mcaA* and *mcaB* as chain assembly factors. (a) Schematic depicting the MIS region of AMB-1, including predicted magnetosome gene homologs (black), a phage-associated protein (grey), transposases (white), and hypothetical genes (yellow). (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 Δ MIS_LD1 and Δ MIS_LD2 cells. (d) TEM micrographs of Δ iR1- Δ iR4, Δ *mcaA*, and Δ *mcaB* cells.

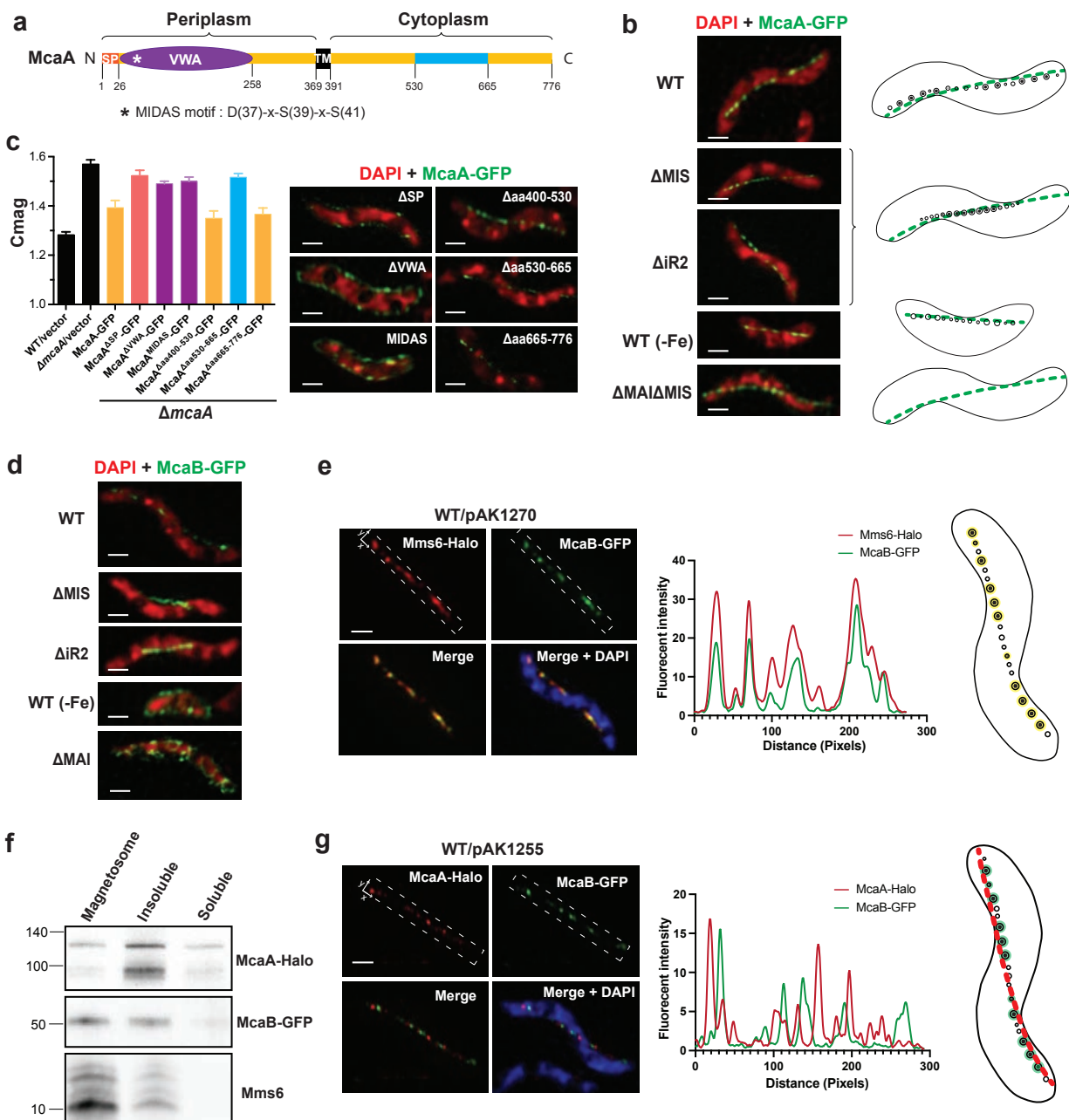


Fig. 4: Localization of McaA and McaB. (a) Predicted secondary structure and topology of McaA. SP, signal peptide. VWA, von Willebrand factor type A domain. TM, transmembrane domain. (b) SIM micrographs of cells expressing McaA-GFP (left) and models of magnetosome production (right) in WT and different 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 μm .

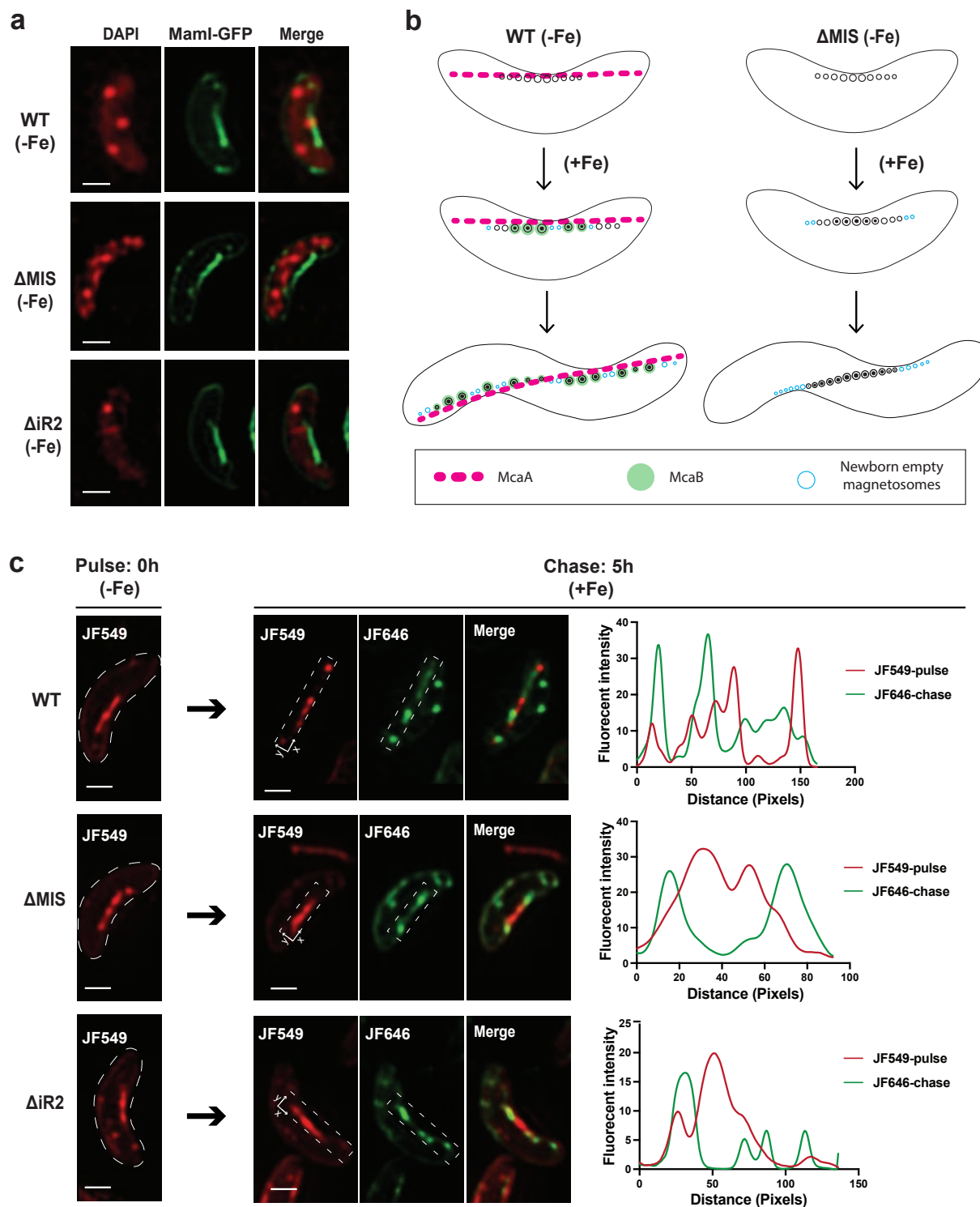


Fig. 5: Addition of newly formed magnetosomes to the chain from iron starvation to normal iron growth conditions. (a) Representative SIM micrographs of WT, Δ MIS, and Δ iR2 cells expressing MamI-GFP under iron starvation growth conditions. The DAPI staining is shown in

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 Δ 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^{20, 22}. 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 μ m in (a) and (c).

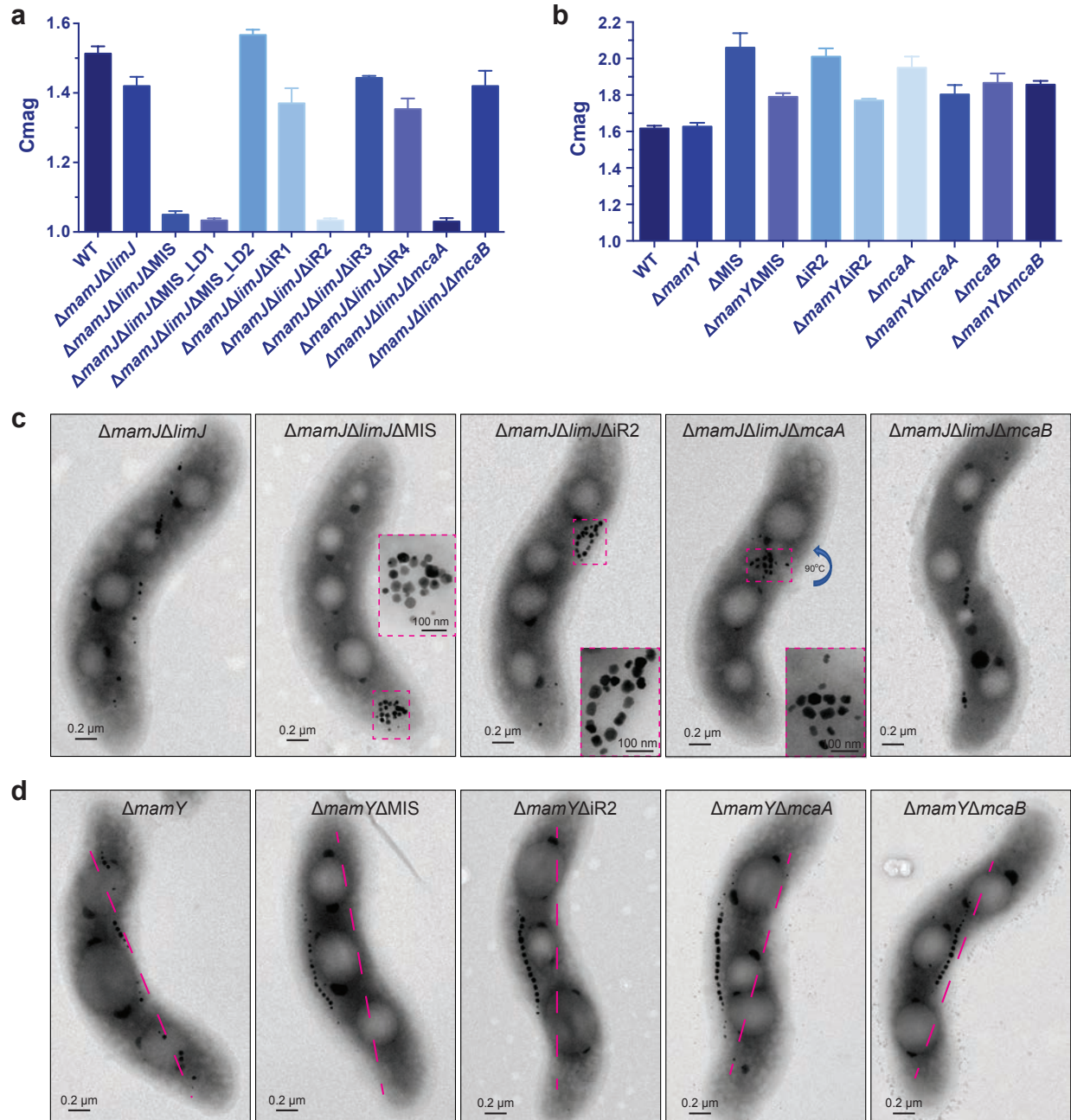


Fig. 6: McaA prevents magnetosome chain aggregation and directs the chain to the positively curved regions of the cytoplasmic membrane. (a) and (b) C_{mag} of WT and different mutants in the $\Delta mamJ\Delta limJ$ (a) and $\Delta mamY$ (b) backgrounds. Each measurement represents the average and standard 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.

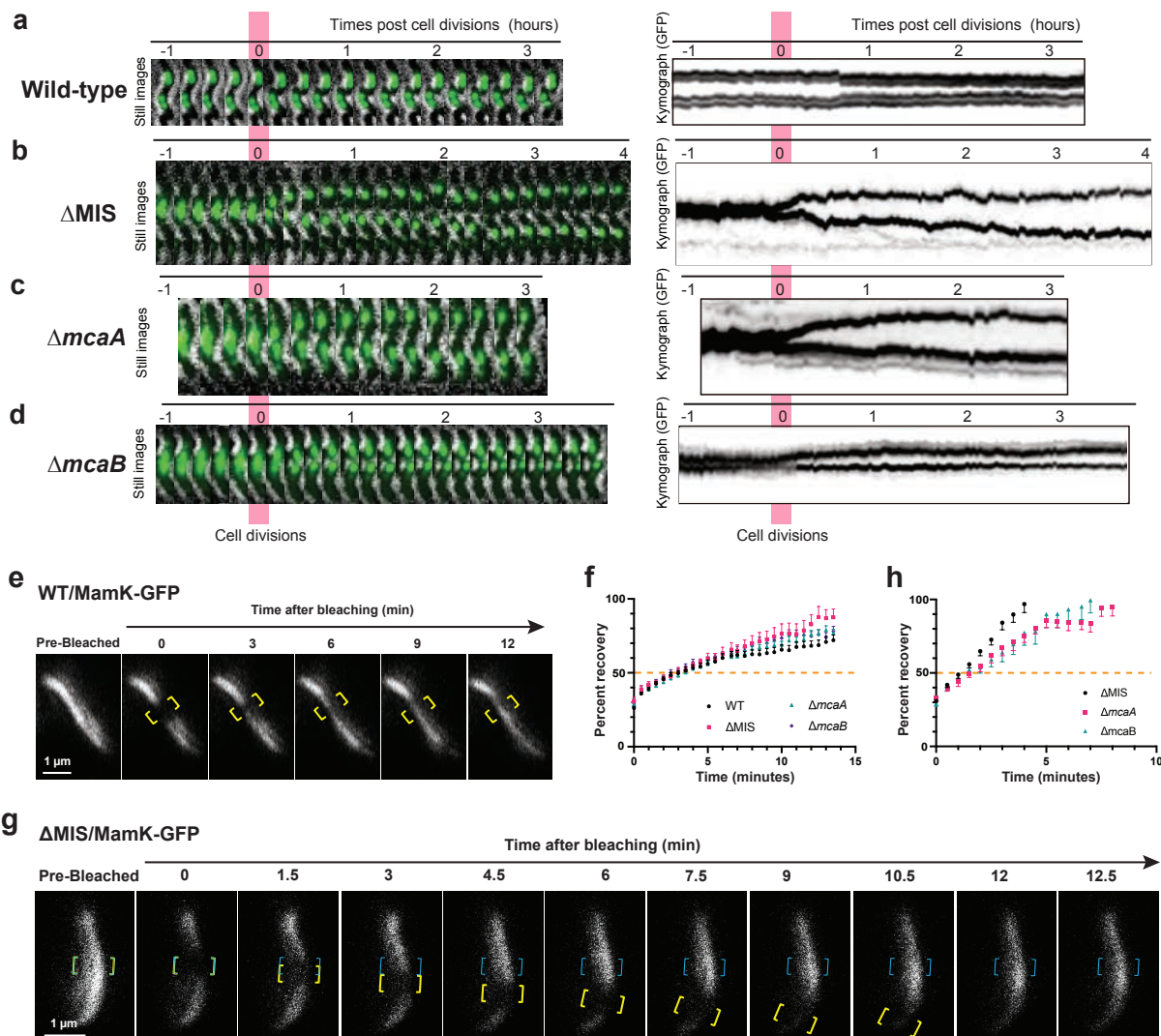


Fig. 7: McaAB control the segregation of magnetosomes and dynamics of MamK filaments.

(a) to (d) Effects of *mcaA* and *mcaB* deletions on magnetosomes segregation. Live-cell time-lapse imaging of magnetosome segregation in wild-type (a), Δ MIS (b), Δ *mcaA* (c), and Δ *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 Δ 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.

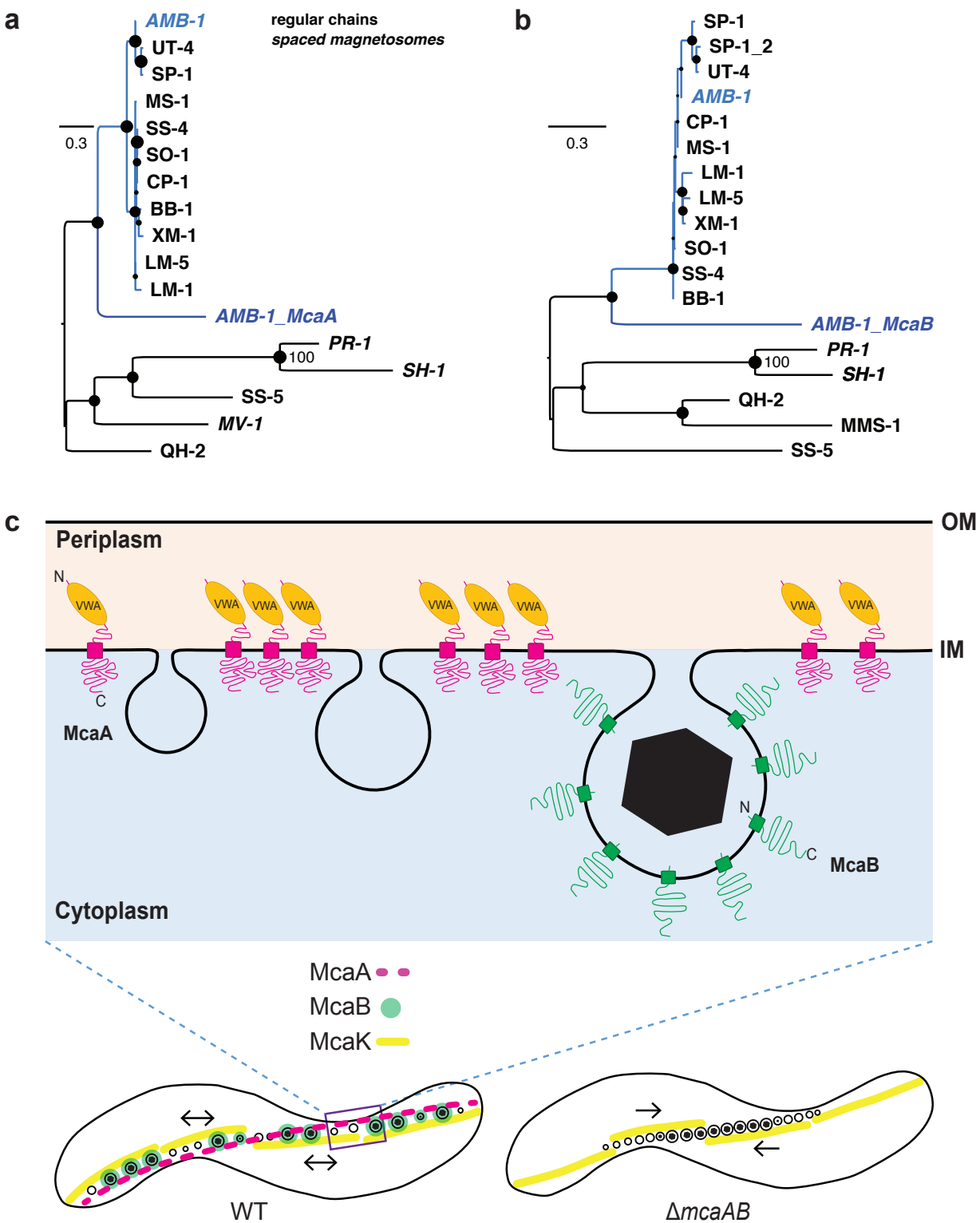


Fig. 8: Phylogenetic analysis and model of McaAB-mediated magnetosome chain assembly.
(a) and (b) Maximum likelihood trees showing the ancestry of McaA (a) and McaB (b) proteins in

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.