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1 **Juxtaposed membranes underpin cellular adhesion and display unilateral cell division**
2 **of multicellular magnetotactic prokaryotes**

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25 Running head: Diversity and architecture of MMPs

26 **Summary**

27 Multicellular magnetotactic prokaryotes (MMPs) exhibit peculiar coordination of swimming
28 along geomagnetic field lines. Approximate 40-80 cells assemble, with a helical geometry or
29 axisymmetry, into spherical or ellipsoidal MMPs, respectively. To contribute to a
30 comprehensive understanding of bacterial multicellularity here we took multiple microscopic
31 approaches to study the diversity, assembly, reproduction and motility of ellipsoidal MMPs.
32 Using correlative fluorescence *in situ* hybridization and scanning electron microscopy
33 analysis, we found an unexpected diversity in populations of ellipsoidal MMPs in the
34 Mediterranean Sea. The high-pressure freezing/freeze substitution fixation technique allowed
35 us to show, for the first time, that cells adhere via juxtaposed membranes and are held
36 together by a rimming lattice. Fluorescence confocal microscopy and ultra-thin section
37 images revealed not only the one-layer hollow three-dimensional architecture, but also
38 periphery–core unilateral constriction of constituent cells and unidirectional binary fission of
39 the ellipsoidal MMPs. This finding suggests the evolution toward MMPs multicellularity via
40 the mechanism of incomplete separation of offspring. Remarkably, thousands of flagellar at
41 the periphery surface of cells underpin the coordinated swimming of MMPs in response to
42 mechanical, chemical, magnetic and optical stimuli, including a magnetotactic photokinesis
43 behavior. Together these results unveil the unique structure and function property of
44 ellipsoidal MMPs.

45 **Originality-Significance Statement**

46 The hollow polar organization, unidirectional division of reproduction and mechano-
47 magneto-photosensitive and coordinated swimming, justify multicellular magnetotactic
48 prokaryotes as an attractive model organism in the study of evolution of multicellularity and
49 mechanism of magnetoreception.

50 **Introduction**

51 Multicellularity evolved independently and repeatedly through two major mechanisms,
52 incomplete separation of daughter cells after division and motile cell aggregation (Bonner,
53 1999). Morphological development results in the unbranched filaments with differentiated
54 cells of cyanobacteria, the branched syncytial filaments of actinomycetes, and the fruiting
55 bodies of myxobacteria (Claessen et al., 2014). These multicellular bacteria reproduce,
56 besides binary cell fission, by sporulation or fragmentation of filaments to form propagules.
57 The major modes of multicellular motility appear as the motile filaments of cyanobacteria
58 and the social foraging for food by swarming myxobacteria (Claessen et al., 2014). Other
59 types of multicellular prokaryotes with distinct features of multicellularity remain poorly
60 investigated.

61 During the evolution various organisms have developed magnetoreception capacity to exploit
62 geomagnetic field as an orientation cue and maps while migrating or homing (Johnsen and
63 Lohmann, 2005). Several hypotheses have been proposed to account for the mechanism of

64 the magnetoreception. Increasing evidence supports a potential compass function of
65 magnetite particles in magnetotactic bacteria (MTB). MTB consist of a heterogeneous group
66 of Gram-negative bacteria that are capable of producing single domain magnetite (Fe_3O_4) or
67 greigite (Fe_3S_4) crystals in intracellular organelles termed magnetosomes (Bazylinski and
68 Frankel, 2004). The magnetosomes enable bacteria to align to and swim along geomagnetic
69 field lines, a behavior is referred to as magnetotaxis. The hypothetic function of magnetotaxis
70 is to simplify a three-dimensional search to a one-dimensional movement for cells to
71 efficiently find and maintain at the preferable oxic-anoxic transition zone in chemically
72 stratified sediments (Bazylinski and Frankel, 2004). Besides the most abundant unicellular
73 MTB, a group of multicellular magnetotactic prokaryotes (MMPs) has attracted particularly
74 our interest because they have opted both multicellular and magnetotactic properties during
75 their evolution.

76 Multicellular magnetotactic prokaryotes were first discovered in the Rodrigo de Freitas
77 lagoon in Brazil by Farina et al. (Farina et al., 1983). Typically, 15–45 bacterial cells arrange
78 with a helical geometry in a spherical or mulberry-like multicellular entity (Keim et al., 2006).
79 This type of MMPs has been observed worldwide (Rodgers et al., 1990; Simmons and
80 Edwards, 2007; Winklhofer et al., 2007; Wenter et al., 2009; Shapiro et al., 2011; Zhou et al.,
81 2013; Zhang et al., 2014a). Besides the spherical MMPs (sMMPs), we have identified
82 another morphotype, the ellipsoidal or pineapple-like MMPs (eMMPs) in the Mediterranean

83 Sea (Lefèvre et al., 2007; Chen et al., 2016), the China Sea, and the Pacific Ocean (Zhou et
84 al., 2012; Chen et al., 2015; Du et al., 2015; Dong et al., 2016; Teng et al., 2018).
85 Approximately 60 cells aggregate into four to six interlacing circles to form a ~ 5–10 μM
86 ellipsoidal entity. Both morphotypes synthesize magnetite or greigite, or both, magnetosomes.
87 The common features for the two morphotype MMPs are that the periphery surface of their
88 cells is covered by thousands of flagella whilst the inner surface surrounds a core lumen.
89 Both display two kinds of typical swimming behaviors of MMPs when inspected in droplets.
90 North-seeking MMPs swim and accumulate at the north edge of droplets. Randomly, they
91 escape from the north edge and swim southward with acceleration. At variable distances,
92 MMPs decelerate, stop the motility, and swim with acceleration northward back to the north
93 edge (Rodgers et al., 1990; Simmons et al., 2004; Zhou et al., 2012; Zhou et al., 2013; Zhang
94 et al., 2014a; Chen et al., 2015; Chen et al., 2016). South-seeking MMPs exhibit a similar
95 escape motion, but in the opposite direction (Lins de Barros et al., 1990; Lins et al., 1999;
96 Keim et al., 2004a; Greenberg et al., 2005). This behavior is referred to as escape motility or
97 ping-pong motion. In addition, illumination with blue, violet, or UV light triggers the MMPs
98 to swim away from the droplet edges opposite to the magnetotaxis direction, which is
99 referred to as a photophobic response or is sometimes miscalled negative phototaxis (Shapiro
100 et al., 2011; Zhou et al., 2012; Zhou et al., 2013; Zhang et al., 2014a; Chen et al., 2015; Chen
101 et al., 2016)

102 To gain more insight into the bacterial multicellularity, we performed multiple microscopy
103 analyses of ellipsoidal MMPs. The results obtained contributed to better understanding about
104 diversity, architecture, reproduction and motility of this peculiar multicellular prokaryote
105 model organism.

106 **Results**

107 *Diversity of multicellular magnetotactic prokaryotes*

108 In general, single phylotype of MMPs was identified at a given sampling site (Abreu et al.,
109 2007; Wenter et al., 2009; Zhou et al., 2012; Zhou et al., 2013; Kolinko et al., 2014; Zhang et
110 al., 2014a; Chen et al., 2015; Chen et al., 2016). An exception has been reported by Simmons
111 et al. (Simmons and Edwards, 2007), they found five phlotypes of spherical MMPs in
112 samples collected from Little Sippewissette salt marsh (Falmouth, MA, USA). The low
113 natural abundance and similar morphology of MMPs prevented analysis of their diversity.
114 Recently, Li et al. (Li et al., 2017) applied a coordinated FISH-SEM analysis procedure to
115 efficiently identify the phlotypes of magnetotactic bacteria with simultaneous
116 characterization of their magnetic crystals. Here, we used this procedure to assess the
117 diversity of ellipsoidal MMPs of the Mediterranean Sea (Fig. 1A). We collected
118 magnetotactic bacteria (MTB) by racetrack magnetic enrichment and obtained five 16S
119 rRNA gene sequences that potentially belonged to MMPs. One of them (SF-1) was identical
120 to the previously identified ellipsoidal MMPs collected from this site (Chen et al., 2016), and

121 the others shared higher than 90% identity with SF-1. Using correlative FISH-SEM analysis,
122 we confirmed the authenticity of the ellipsoidal MMPs SF-2 to SF-5 and showed species-
123 dependent cell morphology and magnetosome arrangement in the MMPs (Fig. 1B, C, D, and
124 E). These results unambiguously show the diversity of ellipsoidal MMPs at this sampling site.
125 In parallel, we also used magnetic micromanipulation to sort MMPs collected from Lake
126 Yuehu and Six-Fours-les-Plages (see Experimental Procedures), and amplified and
127 sequenced their 16S rRNA genes as previously reported (Kolinko et al., 2014; Zhang et al.,
128 2014a; Chen et al., 2015; Chen et al., 2016). In total, we obtained 11 new 16S rRNA gene
129 sequences, identifying five species belonging to three genera of spherical MMPs and six
130 species belonging to three genera of ellipsoidal MMPs (Fig. 2A), using a 97% and 95%
131 sequence identity as the threshold for classifying species and genera, respectively (Tindall et
132 al., 2010). Our study has therefore extended the total number of identified spherical and
133 ellipsoidal MMPs to eleven species belonging to six genera and nine species belonging to six
134 genera, respectively. They formed branches of a MMP clade that were affiliated with
135 *Deltaproteobacteria* but were distinguished from the multicellular myxobacteria (Fig. 2B).
136 The worldwide distribution and the great diversity of species justify the consideration of
137 MMPs as a distinct taxonomic group of multicellular prokaryotes.

138 *Cellular arrangement within ellipsoidal MMPs*

139 Previously we have reported the architecture of ellipsoidal MMPs using fluorescence
140 microscopy, transmission electron microscope (TEM), scan electron microscope (SEM), and
141 focused ion beam SEM (FIB-SEM) (Zhou et al., 2012; Chen et al., 2015; Chen et al., 2016;
142 Leao et al., 2017). Approximately 40–80 cells arrange in interlacing circles with a pineapple-
143 like ellipsoidal morphology. Partly because of the MMP size, the resolution that can be
144 achieved by these microscopy techniques is limited. In addition, knowledge about overall
145 three-dimensional arrangement of cells in ellipsoidal MMPs needs to be improved. HPF/FS
146 fixation is an optimal method to preserve the structures present in the living cell up to a
147 thickness of 200 μm . Here, we used HPF/FS, for the first time, in combination with TEM
148 and fluorescence confocal microscopy to study the ultrastructure of MMPs. As previously
149 reported (Leao et al., 2017) but with more detail, we observed the conspicuous periphery-
150 core architecture (Fig. 3A, B, C, E and G), an axisymmetrical arrangement of cells along the
151 eMMP longitudinal axis (Fig. 3A), a “open space” at one end (Fig. 3A, top) and a core lumen
152 in the center of ellipsoidal MMPs (Fig. 3G and K). We also found at surface of the MMPs,
153 flagella (Fig. 3A and I), and, in the cytoplasm, magnetite magnetosomes (Fig. 3A, B, D, F, G
154 and H), lipid granules, (Fig. 3A, D, G and I), and periphery bars at the external side of the
155 cells (green arrows in Fig. 3J and SI, Fig. S2).

156 Besides these structures, ultrathin sections of HPF/FS samples unveiled an unreported
157 electron dense dashed line surrounded the outermost surface (Fig. 3A, G, H, and J, yellow
158 arrows). The dashes were approximately 11.6 x 20.7 nm (average of 193 points from two
159 MMPs) and aligned with an interval distance of 10.3 nm (average of 183 points from two
160 MMPs) along the perimeter of a matrix outside the outer membrane of the MMPs (Fig. 3G,
161 H, and J). The dashed lines on two successive ultrathin sections arranged in a similar pattern
162 (SI, Fig. S1). Therefore, it is likely that the regular distributed dashes are a cut-view of a
163 lattice that holds the cells together (Fig. 3G, H, and J). Underneath the matrix layer, there are
164 the well-preserved three-layer structures of the outer membrane, peptidoglycan, and the inner
165 membrane (Fig. 3G, H, I and J). The high quality of preservation unveiled unambiguously
166 that juxtaposed membranes tightly connect cells together to assemble into a multicellular
167 entity (Fig. 3D and G, red asterisks). Close to the periphery side of the inner membranes, a
168 fence-like structure (f) was observed in some cells (cell 4 in Fig. 3G and J), which recalls the
169 structure of photosynthetic membrane lamellae in prokaryotic cells. The laser confocal
170 analysis and reconstitution of ultrathin section images clearly revealed that ellipsoidal MMPs
171 consist of only one layer of cells that are all connected to the core lumen (Fig. 3C, E, SI
172 Movie 1). Taken together, juxtaposed membranes adhere cells together surrounding the core
173 lumen to achieve a one-layer hollow structure that is held by a well-patterned lattice at the
174 surface of ellipsoidal MMPs.

175 ***Reproduction of ellipsoidal MMPs***

176 Ellipsoidal MMPs consist of cells that are arranged axisymmetrically along the long body
177 axis and radially on the short axis dissection. We investigated how they reproduce to
178 maintain the inherent periphery–core architecture and a magnetic polarity. Ultrathin sections
179 of HPF/FS- or chemically fixed MMPs revealed that all dividing cells exhibit a unilateral
180 indentation of the cell wall from the periphery towards the core lumen (Fig. 4B and C, red
181 asterisks and yellow triangles). The high-quality preservation of the membrane structures by
182 HPF/FS exposes the process of constituent cell division. As shown on two successive
183 ultrathin slices of an HPF/FS-fixed eMMP (Fig. 4D and E1), outer membranes bend inward
184 from the peripheral surface and the two sides then join together, doubling the thickness, and
185 invaginate into the cytoplasm. The constriction furrow penetrates unilaterally into the
186 cytoplasm without initiating indentation of the membranes at the core lumen side, even when
187 the constriction furrows into three-fifths of the depth of the cytoplasm (Fig. 4D, E1, E2, and
188 E3). We analyzed ultrathin sections with the core lumen of eleven MMPs and found
189 approximately 50% of cells having a constriction furrow. In all cases, we observed the
190 indentation occurring unilaterally only from the periphery toward the lumen. The inward
191 bending and doubling thickness of the outer membrane at the onset of cytokinesis displayed
192 the same structure as the outer membrane between two neighboring cells (Fig. 4D and E
193 compared to F, red asterisks). As shown in Fig. 4D, the outer membranes connect the cells

194 together. Therefore, the juxtaposed membrane implies a characteristic adhesion of MMP
195 cells. Moreover, the surface lattice and matrix seem to prevent the cells from separation from
196 the aggregates after the division. These observations strongly suggest the evolution of MMP
197 toward multicellularity via the mechanism of incomplete separation of offspring (Bonner,
198 1999).

199 Further fluorescence confocal microscopy inspection of cells shows membranes that
200 separated neighboring cells on the surface stack images (Fig. 4G1, yellow arrows). However,
201 on the deeper stack images in some cases, the membranes disappeared progressively first at
202 the center, leading to ‘H-shaped’ cells (Fig. 4G2 to G4), and then at the opposite corners of
203 the dividing cells ([SI Movie S2](#)). These results are consistent with the hypothesis that
204 unilateral constriction of cell envelope occurs from the periphery toward the core lumen
205 parallel to the long axis of MMPs. Moreover, the “H-shaped” cells that were previously
206 observed using FIB-SEM microscopy reflect a stage of cell division instead of a permanent
207 morphology of MMP cells. Notably the HPF/FS slice in Fig. 4H shows the split of an
208 ellipsoidal multicellular magnetotactic prokaryote, which corresponds to one state of the
209 division process revealed by confocal microscopy (Fig. 5I). A collection of confocal analyses
210 corroborated the hypothetical unidirectional split of the eMMP entity using fluorescence
211 staining to visualize the membrane (Fig. 4I, [SI Movie S3](#)) or intracellular lipid granules (Fig.
212 4J, [SI Movie S4](#)).

213 ***Bounce motion, axial magnetotaxis, and magnetotactic-photokinesis of ellipsoidal MMPs***

214 Multicellular magnetotactic prokaryotes possessed multiple magnetosome chains arranged
215 along their long axis (Fig. 5A) and were covered by flagella (Fig. 5B). The HPF/FS thin
216 section images showed that the flagella have a diameter of approximately 22 nm with an
217 interval distance of 68 nm (Fig. 3I). If the flagella are separated by equal longitudinal and
218 latitudinal distances, we can assume a flagella density of 1 flagellum per 8100 nm squares
219 (123 flagella per μm^2), which could lead to approximately ten thousand flagella on a 4
220 x 8 μm MMP. It is similar to the 147 flagella motors per μm^2 estimated for spherical
221 MMPs (Silva et al., 2007). The multiple bundles of magnetosomes and thousands of flagella
222 underpin the canonical magnetotactic behavior of MMPs. Previous studies of MMPs
223 swimming in droplets have shown the typical escape and photophobic motilities. To gain
224 deeper insight into these behaviors we further analyzed MMPs swimming in microfluidic
225 channels (see Experimental Procedures) to mitigate the air-liquid-solid border effect, and
226 irregular light transmission that occurs in droplets.

227 The swim velocity of ellipsoidal MMPs collected from environmental samples when
228 compared one to another, displayed up to a ten-fold variation and the velocities decreased
229 progressively as the observation time increased. We observed eMMP swimming northward
230 with an average velocity of $34.2 \pm 14.7 \mu\text{m/s}$ (n=12). When they performed escape or ping-
231 pong motion eMMPs swam southward at $20.0 \pm 9.3 \mu\text{m/s}$ (n=14) with instantaneous

232 acceleration ranging from 87.6 to 6.3 $\mu\text{m}/\text{s}^2$, which was followed by a return northward
233 swimming at of $13.5 \pm 6.1 \mu\text{m}/\text{s}$ (n=10). Despite the substantial variation of velocity among
234 individuals the pattern of each type of swimming behavior was very well conserved and
235 examination of instantaneous velocity change of a given multicellular magnetotactic
236 prokaryote in response to an environmental stimulus is meaningful. We observed backward
237 motility of eMMPs accumulated at north side in microchannels (Fig. 5C, *SI Movie 5*).
238 Notably the backward motion in microchannels shares a similar pattern with the escape or
239 ping-pong motion of MMPs in droplets; eMMP swim to and accumulate at the north edge in
240 droplets or in microchannels, where some of them randomly make a backward swim with
241 acceleration. The mechanism involved in eMMPs sensing of microchannel walls might be
242 different from that of sensing the surface/border of the droplets although both the wall and
243 the border prevent MMPs from further northward swim. We also observed a conspicuous
244 backward motion when MMPs encountered obstacles (Fig. 5D, *SI Movie 6*). The channel
245 walls and obstacles blocked the forward north-seeking motility and triggered the MMPs to
246 swim backwards in order to circumvent the obstacles. Hence, we propose that the two
247 behaviors are considered a bounce motion. Moreover, at times MMPs randomly changed
248 their swimming direction southward and subsequently changed back to a north-seeking swim
249 (Fig. 5E, *SI Movie 7*), which is typical behavior of axial magnetotaxis (Bazylinski and
250 Frankel, 2004). The stochastic backward motion may play a similar physiological function to

251 the tumbling of *Escherichia coli* that allows bacteria to randomly explore the favorable
252 direction in which to go. It is noteworthy that the backward swims in bounce motion and
253 axial magnetotaxis started with the highest acceleration (red arrows and 'ac' in Fig. 5D and
254 5E) and had higher instantaneous velocity than the forward swim (red curves versus blue
255 curves).

256 To characterize the photosensitive magnetotaxis of MMPs in microchannels, we applied two
257 light-sources, i.e. a transmission light for observation and imaging and a UV (385 nm, 203
258 $\mu\text{W}/\text{cm}^2$) epi-illumination on a defined area for analyzing the photo-effect on swimming
259 behavior. The swimming of MMPs was maintained within the illumination spots via
260 application of an alternate uniform magnetic field (10 Gs, 0.5 Hz) in order to periodically
261 reverse the swimming direction of MMPs before they left the illumination area. As shown in
262 Fig. 5F1, an eMMP initially swam northward at 100 $\mu\text{m}/\text{s}$ in the UV spot (green track). After
263 the reversal of the field direction, the MMP immediately reversed its swimming direction to
264 keep the north-seeking swim (Fig. 5F1 the U-turn of the green dot track, 3F2 the gap of green
265 track at frame 40 and the change of velocity from positive to negative). Approximately 0.2
266 seconds later, the MMP suddenly changed its swimming direction to southward (red arrow
267 and "r") with an acceleration (ac) that doubled the velocity, reaching approximately 200 $\mu\text{m}/\text{s}$
268 (Fig. 5F1, SI Movie 8 and Movie 9). Therefore, the illumination resulted in not only the

269 photophobic response ([SI Movie 10](#)), but also in photokinesis of the magnetotaxis, i.e. a
270 change to the direction and velocity of motility.

271 **Discussion**

272 Our structure and behavior analyses unveil hallmark features of multicellularity that
273 distinguishes MMPs from other extensively studied multicellular bacteria. Both myxobacteria
274 and MMPs are affiliated with *Deltaproteobacteria* (Fig. 2B). Lying on the boundary between
275 uni- and multicellular organisms, myxobacteria grow and divide as proper unicellular Gram-
276 negative bacteria. However, they feed socially via collective predation. Moreover, under
277 starvation conditions, hundreds of thousands of cells form compact and symmetric structures,
278 i.e. fruiting bodies that confer on myxobacteria selective advantages for spore dispersion and
279 cooperative-feeding (Kaiser, 2001). On the contrary, MMPs are patterned, permanent
280 multicellular entities. They are constructed via tight cell membrane juxtaposition and an outer
281 matrix envelope. The spherical and ellipsoidal morphotypes of MMPs display different
282 assembly symmetries. The structures of the well-studied spherical MMPs consist of 15–40
283 cells that are arranged with a helical symmetry into a mulberry-like morphology (Farina et
284 al., 1983; Bazyliniski et al., 1990; Farina et al., 1990; Lins de Barros et al., 1990; Mann et al.,
285 1990; Rodgers et al., 1990; Lins et al., 1999; Keim et al., 2004b). Although the short-axis
286 dissection of ellipsoidal MMPs exhibits radial symmetry like spherical MMPs, but only the
287 ellipsoidal MMP cells axisymmetrically assemble into several rows to form a pineapple-like

288 morphology. At present no unicellular stage has been observed for MMPs ((Keim et al.,
289 2004a; Keim et al., 2004b; Abreu et al., 2006; Zhou et al., 2012; Abreu et al., 2013; Zhang et
290 al., 2014a; Chen et al., 2015; Chen et al., 2016), and FISH in this study). Our phylogenetic
291 analysis, based on 16S rRNA gene sequences, revealed that the MMP cluster is located
292 distantly to myxobacteria and other unicellular magnetotactic bacteria (Fig. 2B).
293 Interestingly, the genes involved in magnetosome biosynthesis are well conserved among
294 uni- and multicellular magnetotactic bacteria from the *Deltaproteobacteria* class (Leao et al.,
295 2017). This result cannot distinguish between acquisitions by nonmagnetic multicellular
296 microbes of magnetosome coding genes via horizontal gene transfer from evolution of MMPs
297 from unicellular magnetotactic *Deltaproteobacteria*. Future genomic phylogenetic analyses
298 should shed light on the evolution mechanism of MMPs. The data presented here support the
299 hypothesis that myxobacteria and MMPs gained their multicellularity in separate events.

300 Coordinated swimming behavior is a fundamental feature that emerged during the evolution
301 of multicellularity. The paradigm of bacterial chemotaxis is underpinned by intracellular
302 diffusion of phosphorylated proteins and their binding to flagellar motors to steer the
303 swimming behavior in response to environmental stimuli (Wadhams and Armitage, 2004).
304 MMPs exhibit a highly complex motion. In addition to the escape swim and photophobic
305 response, here we showed the bounce motion, axial magnetotaxis, and magneto-photokinesis
306 behavior of ellipsoidal MMPs. While unicellular magnetotactic bacteria display aero-

307 magnetotaxis and photophobic behavior (Frankel et al., 1997; Zhu et al., 2010), magneto-
308 photokinesis has been observed only for multicellular magnetotactic prokaryotes. MMPs
309 dwell in intertidal sediments as deep as 30 cm and undergo seasonal, and probably also daily,
310 vertical movement in response to nutrient distribution changes (Liu et al., 2018). Shapiro et
311 al. have suggested that photophobic behavior enables MMPs to optimize their location to
312 adapt to circadian variation of chemical gradients and light intensity (Shapiro et al., 2011).
313 Indeed genes involved in controlling circadian rhythm were found in the genomes of
314 spherical (Abreu et al., 2013) and ellipsoidal MMP strain SF-1 ([SI Table S1](#)). The bounce
315 motility and photokinesis capacity may be selective strategies for MMPs to move to
316 favorable places in sediments. Moreover, MMPs swim faster than most unicellular bacteria
317 and have large sizes, which imply an advantage in mitigating predation risk.

318 Bacterial photo-sensing might rely directly on dedicated photoreceptor systems or indirectly
319 on the products of photosynthesis or other illumination by-products, i.e. reactive oxygen
320 species, ATP, change of intracellular redox or force proton motif. The fence-like structure
321 (Fig. 3J), which looks like photosynthetic membrane lamellae, could be an appropriate
322 candidate for accommodating photoreceptors involved in MMPs photo-sensing. Multicellular
323 magnetotactic prokaryotes displayed a helical trajectory of swimming and were illuminated
324 with UV-light perpendicularly to the translation direction. They changed the magnetotaxis
325 direction and velocity suddenly within the illumination area. Therefore, the magneto-

326 photokinesis is unlikely to be a result of the detection of an intracellular spatial light gradient.

327 The sudden change of swim direction under constant illumination would suggest a

328 cumulating effect of periodical exposure of photoreceptive structures to UV-light or

329 production of harmful by-products. Therefore, multicellular magnetotactic prokaryotes

330 reversed their swimming direction to escape from the deleterious light. It remains an enigma

331 how thousands of flagella of 60–80 cells coordinate their rotations to propel the helical

332 translation of MMPs. Therefore, the mechanism that governs the change of magnetotaxis

333 direction is inexplicable. Both HPF/FS section and confocal data revealed that the core lumen

334 connects all cells in a MMP, and the lumen thus might play a crucial role in the

335 communication required to regulate the operation of flagella at the surface.

336 Reproduction of MMPs shows some particularities compared to other multicellular

337 prokaryotes. Until now, no single-cell stage has been observed in the life cycle of MMPs

338 (Keim et al., 2004a; Keim et al., 2004b; Abreu et al., 2006; Abreu et al., 2007; Abreu et al.,

339 2013). Keim et al. have proposed that spherical MMPs reproduce by two major steps:

340 division of individual cells via invaginations of the cell membrane at the external side, and

341 “8”-shaped torsion fission of MMPs into two equal spheres (Keim et al., 2004b). Here, the

342 highly preserved structure of ultrathin sections unambiguously reveals the unilateral

343 constriction of MMP cells (Fig. 4). It has been reported recently that nematode symbiont rod-

344 shaped *Gammaproteobacteria* initiate division by membrane invagination only at the

345 proximal pole that is attached to the host cuticle (Pende et al., 2018). The fission progresses
346 by membrane invagination at the distal end and the two constriction furrows penetrate
347 longitudinally towards the center until there is a complete division. Similarly, electron cryo-
348 tomography analysis of unicellular bacteria reveals that some dividing cells from eight
349 bacterial species initiate indentations of the cell envelope on only one side of the cells at the
350 early-constriction state (Yao et al., 2017). Subsequently, all mid-constriction cells exhibit
351 constriction on both sides of the cell body, and the division furrows converge into a neck-like
352 structure in the late-constriction cells, which indicates bidirectional division of these cells.

353 Cyanelles are primitive photosynthetic organelles of protists *Cyanophora paradoxa*. During
354 their reproduction a shallow furrow starts asymmetrically on one side of the division plane,
355 resulting in kidney-shaped cyanelles. Subsequently the furrow extends, covers, and deepens
356 around the entire circumference of the division plane, leading to dumbbell-shaped cyanelles
357 and a complete division (Chang et al., 2009). The MMP cells are distinguished from other
358 asymmetrically constricted unicellular cells by the apparent absence of bilateral indentations
359 of the membrane even at late stages of the division. The periphery–core architecture of
360 MMPs might prevent the symmetric bilateral constriction. The periphery bars, which are
361 located underneath the outer curved membrane and close to the magnetosome chains in MMP
362 cells (Fig. 3J, [SI Appendix, Fig. S2](#)), might play a role in determining the onset of unilateral
363 cell constriction. Interestingly, the unicellular magnetotactic bacterium *Magnetospirillum*

364 *gryphiswaldense* reproduces via asymmetric, wedge-like constriction of the cell wall at the
365 division site, which leads to snapping of the magnetosome chain into halves in the two
366 offspring with minimal magnetostatic attraction (Katzmann et al., 2011). Unilateral
367 indentation of the MMP cell wall seems to be a similar mechanism of the segregation of
368 magnetosome chains in the multiple daughter cells that remain tightly connected after
369 division.

370 The lack of MMP cultures prevents detailed study of the mechanisms of cell and entity
371 divisions. Whole-genome sequencing and analysis might circumvent this obstacle. Moreover,
372 it is crucial to develop a dedicated microscope device in order to determine precisely the
373 wavelength spectra that trigger photokinesis and to perform real-time inspection of the MMP
374 lifecycle in microchannels. Using multicellular magnetotactic prokaryotes as a novel model
375 of multicellular organisms could provide a different way of thinking about the evolution of
376 multicellularity. The periphery–core architecture restricts the mechanisms that govern cell
377 division, cell-cell adhesion and communication, swimming coordination, and distinguishes
378 MMPs from the filamentous cyanobacteria and actinomycetes.

379 **Experimental Procedures**

380 *Phylogenetic analysis*

381 Sediment samples were collected from Six-Fours-les-Plages (43°07'N, 5°79'E) of the
382 Mediterranean Sea, or Lake Yuehu (37°21'N, 122°34'E) of the China Sea. Magnetotactic

383 bacteria were magnetically enriched using the race-track method (Wolfe et al., 1987). The
384 16S rRNA genes were directly amplified from the collected samples and sequenced, or after
385 further collection of MMPs by magnetic manipulation microsorting, and then amplified and
386 sequenced as previously reported (Chen et al., 2015). The ellipsoidal MMPs were sorted
387 using a TransferMan ONM-2D micromanipulator and an IM-9B CellTram Oil manual
388 hydraulic pressure-control system installed on an IX51 microscope equipped with a 40 × LD
389 objective (all from Olympus). One droplet (20 µl) containing magnetically collected MMPs
390 and several droplets of 20 µl filter-sterilized (0.2 µm pore size) sample water were placed
391 onto a glass slide. The ellipsoidal MMPs were transferred using micromanipulator from
392 magnetic collections successively to the droplets of filter-sterilized sample water. In the
393 transferred washing steps, the MMPs were aspirated and expelled by the glass capillary (at
394 least 10 times) for removal of contaminants and extracellular DNA (Chen et al., 2015).
395 Finally, two to ten purified MMPs were directly transferred to one reaction tube containing 3
396 µl of PBS, which was in REPLI-g Single Cell Kit (cat # 150343; QIAGEN, German). The
397 PBS-suspended sample was frozen and thawed repeatedly, and the WGA reaction was carried
398 out using the REPLI-g Single Cell Kit. Only the samples containing single homogenous 16S
399 rRNA sequence were used in this study.

400 *Characterization of swimming behavior*

401 Swimming behaviors of MMPs were analyzed either in droplets with the optical microscopes
402 Olympus BX51 or Nikon TiE (Chen et al., 2015), or in microfluidic channels with a
403 Magnetodrome device (2D and 3D μ -Slide Chemotaxis channels, Ibidi, GmbH, Germany; or
404 home-made channels as described in (Zhang et al., 2014b)). To characterize photokinesis
405 behavior, we modified the Zeiss Axio Observer A1 microscope on the Magnetodrome device
406 (Zhang et al., 2014b) by replacing the HBO 100 light source with Zeiss Colibri 7. A UV light
407 (385 nm) at $203 \mu\text{W}/\text{cm}^2$ was used to trigger photophobic and photokinesis behavior. The
408 swimming of MMPs was maintained within the UV spot by application of an alternate
409 uniform magnetic field (10 Gs, 0.5 Hz) in order to periodically reverse the swimming
410 direction of MMP before they left the illumination area. The motility was analyzed using
411 MTrackJ Plugins of ImageJ (Meijering et al., 2012).

412 *Microscopy analyses*

413 Routine optical microscopy observation was performed with Zeiss AxioStar Plus, Zeiss Axio
414 Vert 200M, and Olympus BX51. To perform laser confocal analysis, MMPs were fixed with
415 1% paraformaldehyde for 1.5 hours at room temperature, stained with $15 \mu\text{g}/\text{ml}$ Nil red (for
416 lipids), $7.5 \mu\text{g}/\text{ml}$ FM4-64 (for membranes), and $2 \mu\text{g}/\text{ml}$ DAPI (for chromosomal DNA) and
417 observed with the Olympus FV1000 microscope with laser 405 nm excitation and 425–475
418 nm emission for DAPI and 543 nm excitation and 555–655 nm emission for Nil Red and

419 FM4-64. Images were collected at a series of focal levels at 0.17 μm intervals. Three-
420 dimensional image reconstitution was performed using ImageJ and Amira software.

421 Correlative FISH-SEM analyses were carried out on coverslips as described by Li et al. (Li et
422 al., 2017). Briefly, we designed four probes that specifically target to the 16S rRNA gene
423 sequences of ellipsoidal MMPs (*SI Appendix*, Table S2) and evaluated their specificity using
424 the online probe evaluation tool Ribosomal Database Project (Cole et al., 2009; Cole et al.,
425 2014). The MMP-specific probes were synthesized and fluorescently labeled with the
426 hydrophilic sulfoindocyanine dye Cy3 at the 5' end. The universal bacterial probe EUB338
427 (Table S1) was fluorescently labeled with fluorescein phosphoramidite 200 FAM at the 5'
428 end and used for FISH as a positive-control probe for bacteria (Amann et al., 1990). FISH
429 observation was performed with a Nikon TiE microscope by following the protocols reported
430 by Simmons and Edwards (Simmons and Edwards, 2007) and Li et al. (Li et al., 2017). The
431 same coverslips were treated for SEM observation using a Zeiss Ultra-55 field-emission gun
432 SEM (Carl Zeiss, Germany) operating at 5 kV (Li et al., 2017). General SEM morphology
433 analyses of MMPs were carried out using a KVKV-2800B microscope as described by Chen
434 et al. (Chen et al., 2015).

435 *Transmission electron microscopy*

436 Magnetotactic bacteria were pelleted, high-pressure frozen, freeze substituted, embedded in
437 Epon resin (Medium Grade), as reported in (McDonald, 2014). Because only low amount of

438 MMPs could be collected from fresh environmental samples each time, cells of marine
439 unicellular photobacterium cultures were added during HPF/FS preparation. Ultrathin
440 sections (60–90 nm) were cut with an ultracryomicrotome (EM UC7 Leica) and stained with
441 uranyl acetate and lead citrate (Reynolds, 1963). The samples were analyzed using a Tecnai
442 200KV electron microscope (FEI) and digital acquisitions were made using a numeric
443 camera (Eagle, FEI). Alternatively, the grids were analyzed using STEM-HAADF for Z-
444 contrast imaging, using a JEM-2100F microscope (JEOL Ltd., Tokyo, Japan) operating at 200
445 kV equipped with a field emission gun, an ultra-high resolution pole piece and a Gatan
446 energy filter (GIF2001) system (Gatan, Inc., Pleasanton, CA, USA) to determine element
447 composition. High resolution TEM images were analyzed with Fast Fourier Transform (FFT)
448 procedure using ImageJ 1.52o software (<http://imagej.nih.gov/ij/>).

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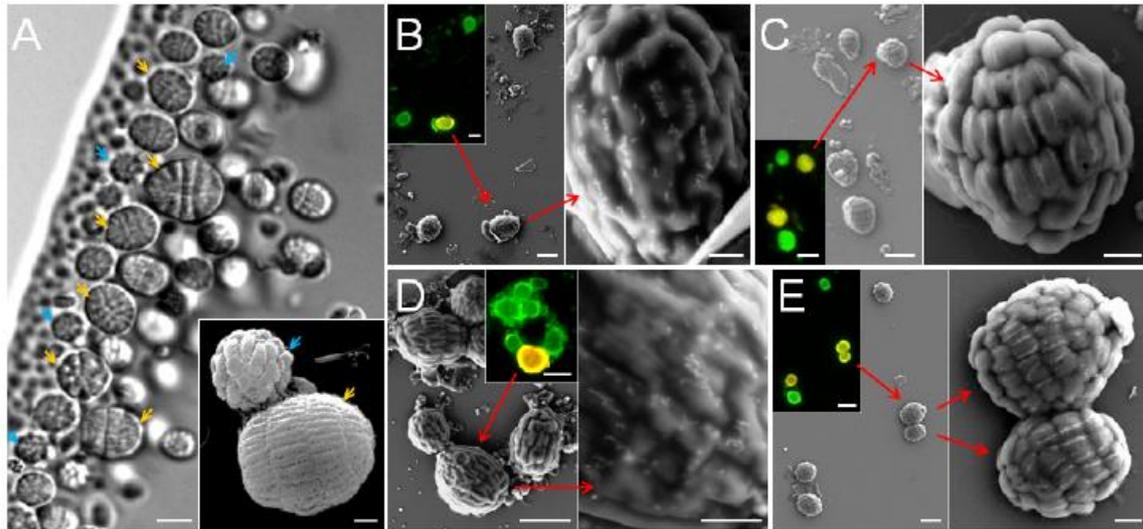
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632 Figure legends

633 Figure 1. Identification of Mediterranean MMPs by correlative microscopy.



634

635 (A) Differential interference contrast (DIC) and scanning electron microscopy (inset)

636 micrographs of magnetically collected MTB accumulated at the north edge of a droplet

637 (Experimental Procedures). Blue and yellow arrows indicate spherical and ellipsoidal MMPs,

638 respectively. (B) to (E) Correlative FISH- (insets) SEM analysis of ellipsoidal MMPs. Green

639 color indicates bacteria hybridized only with the 5'-FAM-labeled universal bacterial probe

640 EUB338 and yellow color shows the MMPs hybridized with both the green universal probe

641 and the 5'-Cy3-labeled specific probes (SF-5, SF-3, SF-4, SF-2 in B, C, D, and E,

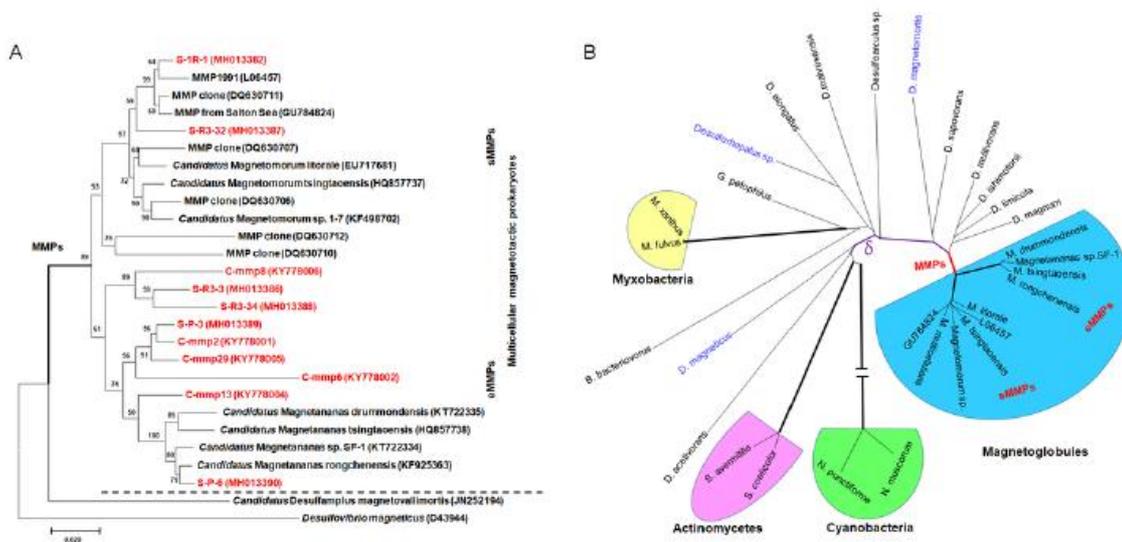
642 respectively). Red arrows correlate the MMPs on SEM micrographs with those specifically

643 hybridized in FISH insets. The images on the right panel of every set are the amplification of

644 the images from the left panel. Scale bars: 5 μ m in A, in FISH insets, and on left SEM panels

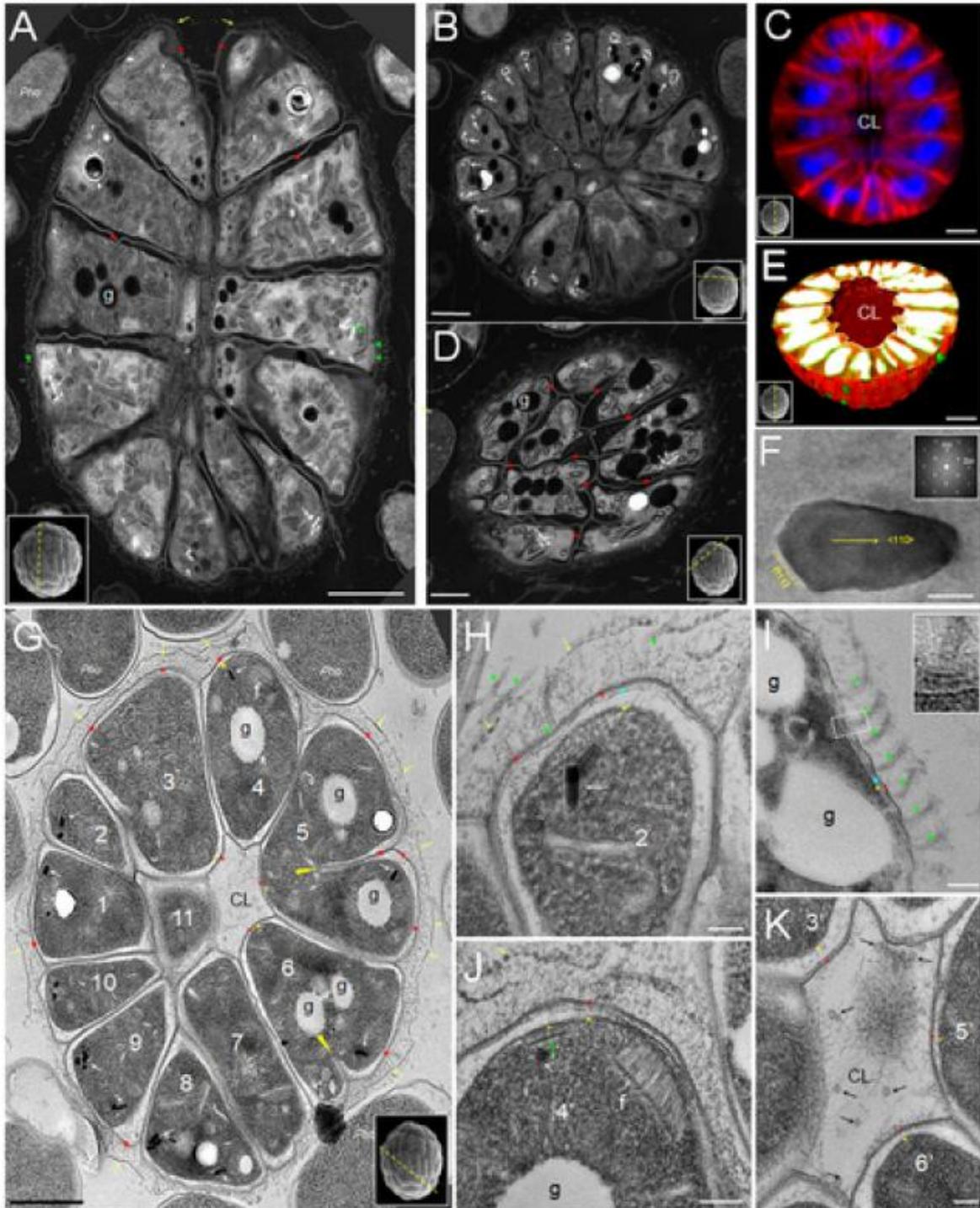
645 of B to E; 2 μ m in inset of A; 1 μ m on the right SEM panels of B to E.

646 Figure 2. Diversity and phylogenetic tree of MMPs.



647
 648 (A) Phylogenetic tree of currently identified MMPs. The species in red are newly identified
 649 in this study. The unicellular magnetotactic bacteria *Desulfamplus magnetomortis* and
 650 *Desulfovibrio magneticus* were used as outgroup references. The trees were constructed
 651 based on neighbor-joining analysis. Bootstrap values at the nodes are percentages of 1,000
 652 replicates. The scale bar indicates 2% sequence divergence. The GenBank accession numbers
 653 of the sequences are given in parentheses. (B) Phylogenetic tree shows the approximate
 654 evolutionary position of MMPs with respect to myxobacteria (highlighted in yellow),
 655 actinomycetes (in pink), and cyanobacteria (in green). The branch length of cyanobacteria is
 656 truncated. All orders of *Deltaproteobacteria* are presented, including the unicellular
 657 magnetotactic bacteria (in blue) and the previously published MMPs.
 658

659 Figure 3. Ultrastructure of ellipsoidal MMPs.



660

661 STEM-HAADF (Scan-TEM high-angle annular dark-field) mode (A, B and D) and TEM-

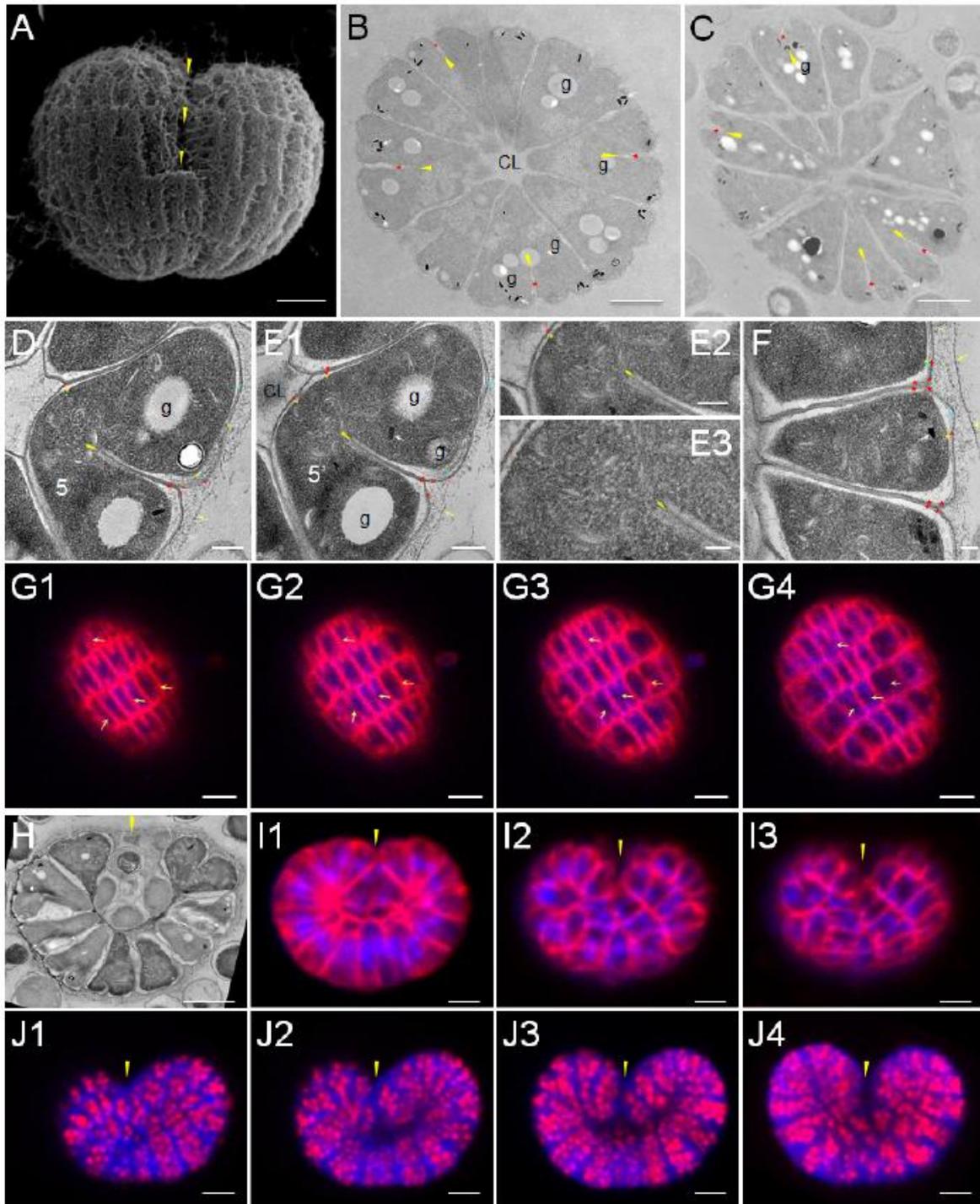
662 bright field (BF) mode (G to K) micrographs of ultrathin sections of HPF/FS-fixed ellipsoidal

663 MMPs. (C) and (E) are, respectively, a center stack or reconstitution of laser confocal images

664 of ellipsoidal MMPs; the membrane is stained by FM4-64 (red) and DNA by DAPI (blue) in
665 (C) or artificial colors in (E). (F) shows a high-resolution image of a representative bullet-
666 shaped magnetosome of ellipsoidal MMPs. From the corresponding fast Fourier transform
667 (inset), it can be deduced that the magnetite elongation direction is $\langle 110 \rangle$ and that a
668 prominent $\{111\}$ basal plane is also visible. (G) shows cells surrounding a core lumen (CL).
669 The yellow arrows visualize the dashed line on the surface of the MMP and the red and
670 yellow asterisks indicate the outer and the inner membrane, respectively. White arrows point
671 to the magnetosomes and the (f) is a fence-like structure. The yellow triangles indicate the
672 extremity of the growing septa in cells 5 and 6. (H) is a magnified image of cell 2 showing
673 the periphery structure that consists of flagella (green triangle), the dashed line, the outer and
674 inner membranes, the peptidoglycan layer (blue asterisks), and magnetosomes. (I) shows
675 flagella (green triangles) and a magnified image (inset) with likely the hook and basal body
676 of a flagellum. (J) is a magnified image of cell 4 on a slice that is successive of the slice in
677 (H). It visualizes the fence-like structure (f) and a periphery bar (green arrow). (K) is a
678 magnified view of the core lumen (CL) on another successive slice of (G) with vesicles
679 (black arrows) and filaments. The cell numbers correspond to the same numbers in panel (G).
680 “g” are Nil red-stainable lipid granules. “pho” indicate the photobacterium cells (see
681 Experimental Procedures). Scale bars: 1 μm in A, C, and G, 500 nm in B, D, and E, 100 nm

682 in H–K images. The insets in A to D and G illustrate the estimated cut direction of the
683 corresponding sections.

684 Figure 4. Division of constituent cells and reproduction of MMPs.

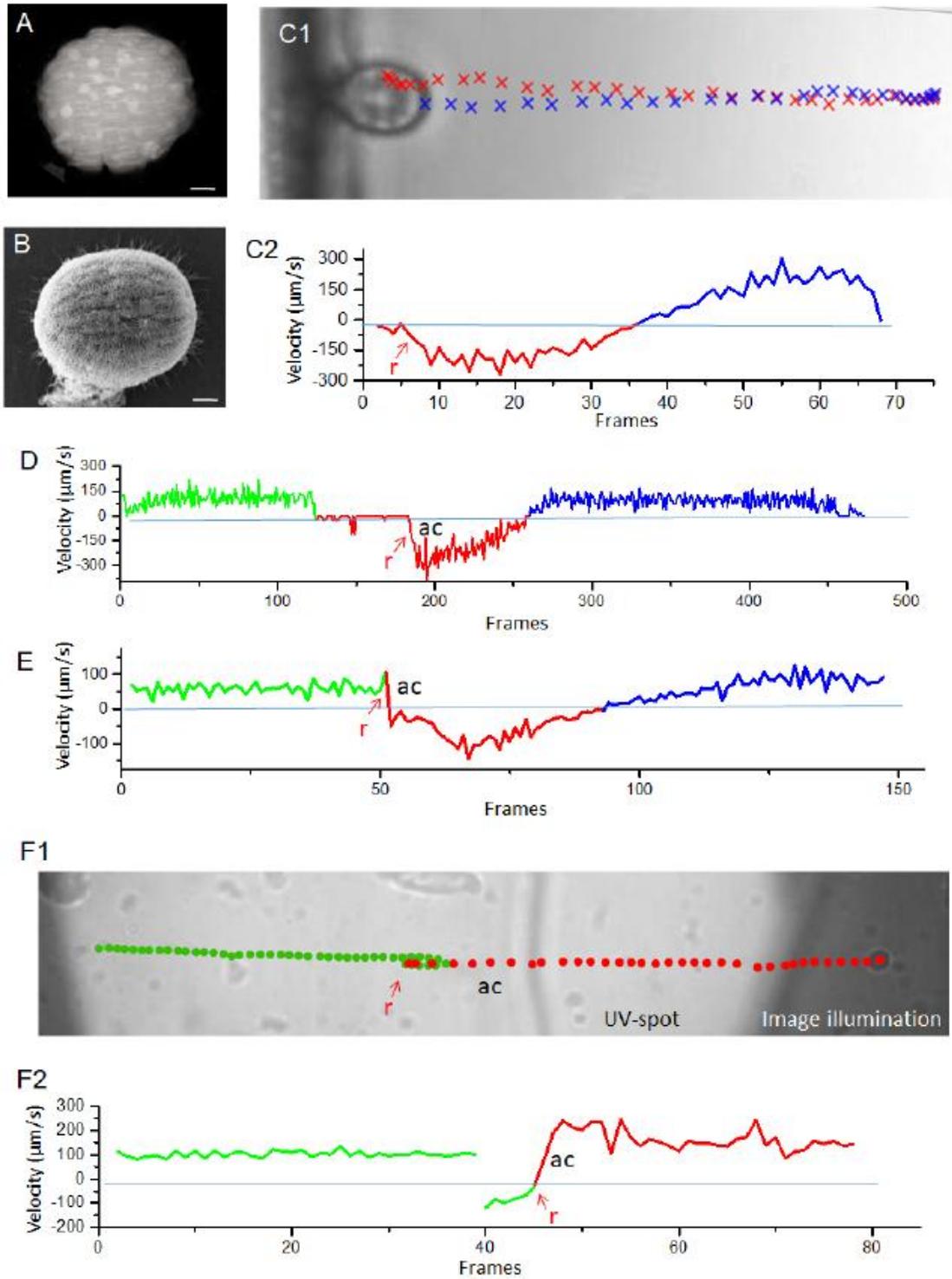


685

686 (A) is a SEM micrograph of a dividing MMP. Chemically (B) and HPF/FS- (C) fixed

687 ultrathin sections show the unilateral indentation of cell outer membranes (red asterisks and
688 yellow triangles). (D) and (E) are two successive slices each of approximately 75 nm. The
689 cell on the two slices is indicated with 5 and 5', that correspond to the same cell number 5 in
690 Fig. 3G. (E2) and (E3) are magnified images of (E1) to show the end (yellow triangles) of the
691 outer membrane (red asterisks) indentation. The yellow arrows (in D, E and F) visualize the
692 dashed line on the surface of the MMP. The red, yellow and blue asterisks indicate the outer
693 and inner membranes, and the peptidoglycan layer. White arrows point to the magnetosomes.
694 (G), (I), and (J) are fluorescence confocal microscope images that show indentation of cell
695 membranes of individual cells (yellow arrows in G) and division of a MMP (yellow
696 triangles). (H) is a HPF/FS slice. DAPI staining (G, I, and J) was used to visualize
697 chromosomes (blue spots), FM-4-64 (G and I, red) to visualize membranes and Nil red (J,
698 red) to visualize lipids. Scale bars: 1 μ m in A, B, C, G, H, I, and J and 100 nm in other
699 images.
700

701 Figure 5. Bounce motion and photokinesis of ellipsoidal MMPs.



702

703 (A) Micrograph of scanning transmission electron microscope in high-angle annular dark-

704 field mode (STEM-HAADF) shows the aligned magnetosome chains along the long axis of

705 the ellipsoidal MMP. (B) SEM micrograph reveals flagella on the surface of MMPs. (C)
706 showcases the escape backward (red track) and return north-seeking forward (blue track)
707 ping-pong swimming behavior (C1) and corresponding velocity (C2, positive is north-
708 seeking). (D) and (E) show representative swimming behaviors when MMPs confronted
709 obstacles or displayed axial magnetotaxis, respectively. Reversal of swimming direction to
710 south-seeking is indicated by arrows “r” and red tracks. (F) shows a north-seeking MMP
711 (green track) changing its swimming direction in response to the reversal of the alternate
712 uniform magnetic field (10 Gs, 0.5 Hz). After 0.2 second it abruptly reversed its swimming
713 direction to south-seeking (arrow “r” and red track) with acceleration (ac) and continued a
714 south-seeking motion out of the UV spot. The movies were recorded at 60 frames per second
715 (fps) for D and E and 30 fps for F.