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1 **Flow cytometry as a rapid analytical tool to determine physiological responses to**
2 **changing O₂ and iron concentration by *Magnetospirillum gryphiswaldense* strain**
3 **MSR-1**

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12 **Abstract.**

13 Magnetotactic bacteria (MTB) are a diverse **group** of bacteria that synthesise
14 magnetosomes, magnetic membrane-bound nanoparticles that have a variety of diagnostic,
15 clinical and **biotechnological** applications. We present the development of rapid methods
16 using flow cytometry to characterize several aspects of the physiology of the commonly-used
17 MTB *Magnetospirillum gryphiswaldense* MSR-1. Flow cytometry is an optical technique that
18 rapidly measures characteristics of individual bacteria within a culture, thereby allowing
19 determination of population heterogeneity and also permitting direct analysis of bacteria.
20 Scatter measurements were used to measure and compare bacterial size, shape and
21 morphology. Membrane permeability and polarization were measured using the dyes
22 propidium iodide and **bis-(1,3-dibutylbarbituric acid) trimethine oxonol** to determine the
23 viability and 'health' of bacteria. Dyes were also used to determine **changes in** concentration
24 of intracellular free iron and polyhydroxylakanoate (PHA), a bacterial energy storage
25 polymer. These tools were then used to characterize the responses of MTB to different O₂
26 concentrations and iron-sufficient or iron-limited growth. Rapid analysis of MTB physiology
27 will allow development of bioprocesses for the production of magnetosomes, and will
28 increase understanding of this fascinating and useful **group** of bacteria.

29

30 **INTRODUCTION.** Magnetic nanomaterials are increasingly important products with myriad
31 applications in diverse settings including but not limited to environmental pollution control,
32 information and energy storage (1), catalysis (2), biotechnological (1, 3, 4) and especially

33 biomedical research (1, 5-7). While most are produced by chemical means there is growing
34 interest in harnessing the cellular machinery of certain naturally occurring bacteria (8-12) to
35 generate useful magnetic, and other metallic nanoparticle materials, biologically. In this
36 context, 'magnetosomes', magnetic nanoparticle based organelles naturally contained within
37 magnetotactic bacteria (MTB), are particularly important (8, 13-17). In most MTB,
38 magnetosomes are arranged in one or more highly ordered 'compass needle-like' chains of
39 single-domain permanently ferrimagnetic magnetite (Fe_3O_4) or greigite (Fe_3S_4) crystals (35-
40 120 nm diameter) each wrapped in a phospholipid bilayer membrane containing a unique set
41 of magnetosome specific proteins, i.e. distinct from those of cytoplasmic and outer
42 membranes (8, 13, 15). These internal structures within MTB function as navigational
43 devices essential for magnetotaxis (18). Unique properties of magnetosomes, not normally
44 associated with chemically synthesized magnetic nanoparticles, of narrow size distribution,
45 uniform morphology, high crystal purity, permanent magnetic character, high heating
46 capacity, low aggregation tendency, ready dispersion in aqueous solution, facile
47 functionalization, high biocompatibility, low toxicity and high specific absorption rates (10, 12)
48 make them especially attractive prospects for biotech and healthcare applications, i.e. in
49 immunoassays (19), magnetic affinity cell sorting (20), magnetic resonance imaging (21),
50 drug and gene delivery (22) and cancer therapy (12, 23).

51 It is recognised that future widespread application of magnetosomes will, to a large extent,
52 depend on the development of intensified high yielding manufacturing platforms for
53 magnetosomes (10, 12, 16). Fundamental to this are appropriate means for analysing MTB
54 growth, viability, physiology and biomineralization of magnetic iron minerals, in order to
55 understand and optimise magnetosome formation at any scale, from initial small (millilitre)
56 studies on strain isolation and cultivability in the laboratory, and pilot scale manufacture (10
57 – 100 L), to fully fledged commercial production in cubic metre scale bioreactors. Qualitative
58 evidence of magnetosome production within MTB can be obtained by observing a
59 shimmering effect in cell suspensions mounted on magnetic stirrer plates, and black
60 coloration of cell suspensions and/or colonies on agar plates, while magneto-
61 spectrophotometric assay of cellular magnetism (C_{mag}) of suspended cells provides a rapid
62 indirect measure of cellular magnetosome content (24, 25). Quantitative determination of
63 magnetosome content in cells and during subsequent recovery and purification operations
64 usually involves measurement of iron content by means of atomic absorption spectrometry
65 (dependent on species and cultivation conditions magnetosomes account for 80 to 99.5 % of
66 the total cell-bound iron in magnetic cells (18, 26, 27)), combined with imaging of
67 magnetosomes by transmission electron microscopy. Recent work indicates the importance
68 of monitoring physiological stress indicators to identify optimal conditions for magnetosome

69 formation (28), and the utility of transcriptome analysis for comparing magnetosome forming
70 and non-forming conditions in MTB (29). Other analytical methods especially pertinent to
71 pilot- and large-scale magnetosome production and downstream processing from high
72 biomass MTB fermentations include the tracking of polyhydroxyalkanoate (PHA) granules.
73 Here, PHA formation diverts cellular resources from growth, lowering yields, and high levels
74 of PHA would be likely to be a troublesome contaminant of magnetosome preparations.
75 Current procedures for the determination of PHA content employ lengthy procedures
76 involving solvent extraction, derivatization and gas chromatography (29).

77 With the exception of at line optical density and C_{mag} measurements all of the
78 aforementioned techniques are labour intensive and/or time consuming. The development of
79 analytical methods is essential for the development of robust production processes, itself a
80 requirement for industrialisation implementation. It is desirable that such methods will be
81 rapid, requiring small volumes of samples and provide data of cellular parameters without
82 the need of further growth, thus giving a 'snapshot' of the current physiological state of the
83 cells. The flow cytometry (FCM) methods applied in this study fulfil these requirements. FCM
84 has previously been applied previously for rapid analysis of microbial physiology (30) and
85 expression of auto-fluorescent proteins (31), monitoring recombinant protein production (32)
86 and for investigating population heterogeneities in cultures. In FCM, multiple physical
87 characteristics of single particles suspended in a fluid can be measured concurrently as they
88 flow through a beam of light. FCM is a fast single-cell analysis technique well suited to
89 collection of large datasets (tens of thousands of cells can be analysed) and allows
90 determination of light scatter (relative size and granularity/internal complexity) and
91 fluorescence properties of individual cells and thus determination of population
92 heterogeneity. An important advantage of FCM is that it does not rely upon microbial growth
93 for analysis of cell viability. 'Viable but non-culturable' (VBNC) cells exist within most
94 microbial cell populations (33), but growth-based methods for determining viable cell
95 numbers (total viable counts generating colony forming unit, CFU data) will not detect the
96 VBNC phenotype, thus total viable cell concentrations are underestimated. FCM does not
97 share this limitation. MTB grow very slowly on agar plates, for example, *M. gryphiswaldense*
98 MSR-1 forms colonies after 7 – 10 days (26). Regardless of cell type FCM analysis can be
99 performed in a matter of seconds. Moreover, when combined with carefully selected
100 mixtures of fluorescent probes FCM can be employed to determine the physiological state of
101 single cells.

102 Reports on the application of FCM to MTB are few in number (34, 35) and the full power of
103 the technique has not exploited in any case. FCM has been used for analysing gene

104 expression in *M. gryphiswaldense* MSR-1 (34), and in the development of new expression
105 systems for the same species (35). Green fluorescent protein (GFP) was used as a reporter
106 in both studies, i.e. for magnetosomal localization and expression of GFP tagged
107 magnetosome proteins under magnetite forming conditions (34); and for identification of
108 promoters (fused to GFP) for efficient gene expression (35).

109 In this work, we present a battery of FCM methods tailored *a priori* to the study of *M.*
110 *gryphiswaldense* MSR-1 and other MTB, and applicable to cells grown in liquid cultures and
111 on agar plates. Specifically, we describe methods for determination of cellular concentration,
112 cell size distribution, single-cell physiology and **relative changes over time** of intracellular
113 contents of PHA and the chelatable iron pool.

114 **RESULTS AND DISCUSSION**

115 **Morphological difference between cells grown on plates and in suspension.**

116 FCM analysis was employed to monitor cell size and optical complexity of *M.*
117 *gryphiswaldense* MSR-1 by means of light scattering. In FCM, light scatter is collected at two
118 different angles: in the direction of the laser path (forward light scatter, FSC); and orthogonal
119 to it (side scatter, SSC). For spherical particles (e.g. of latex), FSC correlates with the
120 logarithm of particle diameter (36). For cells and other non-spherical particles, changes in
121 FSC are roughly indicative of changes in cell size. When applied to cells, SSC measures
122 ‘granularity’ (37), a parameter that includes optical complexity caused by particulate material
123 contained within the cell. Figure 1 shows the results of comparative FCM scatter and light
124 microscopic analyses of MSR-1 cells cultured on ACA plates (resuspended in PBS) and in
125 the liquid medium FSM. Clear differences in the heterogeneity of cell populations cultured in
126 FSM (Fig. 1a) *cf.* those grown on ACA (Fig. 1b) can be discerned from the scatter patterns of
127 FSC vs. SSC dot plots (Figs 1a & 1b). Larger cells are represented by high FSC-A values (y-
128 axis) whereas more granular cells are characterized by higher SSC-A (x-axis) values. Cells
129 grown in liquid FSM appear less heterogeneous, smaller and less granular than those grown
130 on ACA plates. Moreover, differences in particle size distribution and cell shape of
131 suspension and plate grown MSR-1 cells are respectively inferred from ‘Count vs FSC-A’
132 histograms and light microscopy, with plate-grown cells appearing more polydisperse in size
133 (Fig 1c) and filamentous (Fig. 1e) compared to liquid-grown cells (Fig. 1d).

134 **Determination of cell concentration by FCM.**

135 FCM analysis was also used as a rapid method for determining cell concentrations in shake
136 flask experiments. In auto-calibration mode and operating at a medium flow rate of 35

137 $\mu\text{L}\cdot\text{min}^{-1}$ a strong correlation ($R^2 > 0.95$) between OD_{565} and FCM event counts was
138 observed for MSR-1 cells (Supplemental Fig. S1a) with 1 OD_{565} equivalent to 1.16×10^9
139 cells mL^{-1} . This relationship is strikingly different to Schultheiss and Schüler's (38) correlation
140 of OD_{565} with CFU, i.e. 1 OD_{565} equivalent to 3.3×10^8 CFU mL^{-1} and likely reflects an
141 important advantage of FCM over CFU counting, namely its ability to detect viable but non-
142 culturable cells (VBNC). We also used Syto[®]62, a permeant DNA dye, to stain MSR-1 cells
143 and so distinguish them from noise particles of similar size; with Syto[®]62-stained cells a
144 similar correlation was found between OD_{565} and cell count ($\text{OD}_{565} = 1.03 \times 10^9$ cells mL^{-1} ;
145 Supplemental Fig. S1b).

146 **Use of FCM to determine MSR-1 membrane polarization and cellular death.**

147 Two fluorescent probes were used to monitor the respiratory potential and death of *M.*
148 *gryphiswaldense* MSR-1 cells using FCM. BOX (DiBac₄(3); bis-(1,3-dibutylbarbituric acid)
149 trimethine oxonol) is a green lipophilic fluorescent probe that can only enter cells if their
150 membranes are depolarized (39). Healthy cells possess intact polarized cytoplasmic
151 membranes, which are impermeant to BOX (BOX⁻). In contrast, cells with depolarised
152 cytoplasmic membranes (whether injured, stressed or dead) permit BOX access and
153 fluoresce green (BOX⁺). PI (propidium iodide), a nucleic acid intercalator, is excluded by the
154 intact membrane of viable cells (PI⁻), but is taken up by dead cells which fluoresce red (PI⁺)
155 (39). Staining procedures were optimised using actively growing *M. gryphiswaldense* cells,
156 starving cells and dead cells killed with ethanol. Fig. 2 shows the two-colour fluorescence dot
157 plots of MSR-1 cells co-stained with BOX and PI (fluorescence being detected on FL1-A and
158 FL3-A channels, respectively). The fluorescence patterns from actively growing magnetic
159 cells (Fig. 2a) and non-magnetic cells (Fig. 2c) were strikingly alike; i.e.: 86-90 % of the cell
160 populations were 'healthy', staining negatively with both fluorescent markers (BOX⁻ PI⁻,
161 Quadrant 1); 5-8 % were 'injured', staining positively with BOX, but negatively with PI (BOX⁺
162 PI⁻, Quadrant 2); and 3-4 % were dead (BOX⁺ PI⁺, Quadrant 3). The fluorescence patterns
163 from starving magnetic (Fig. 2b) and non-magnetic (Fig. 2d) MSR-1 cells were comparable
164 with one another, but indicated noticeably fewer healthy populations (31-39 % healthy, ~40%
165 injured, 19-26% dead) than those of actively growing cells (~90 % healthy, 5-8 % injured, <3
166 % dead). The low healthy population in starving cultures suggests the presence of large
167 numbers of VBNC cells. This confirms the observation that the correlation between OD_{565}
168 and $\text{cells}\cdot\text{mL}^{-1}$ as measured by FCM is different to the correlation between OD_{565} and CFU
169 mL^{-1} (38) due to the presence of VBNC cells. Only 5 % of the positive control population, i.e.
170 cells killed with ethanol, were healthy MSR-1 cells (Fig. 2e).

171 **Accumulation of PHA aggregates in MSR-1.**

172 It has been widely reported that limiting nitrogen and O₂ availability under carbon excess
173 results in high-level accumulation of polyhydroxyalkanoates (PHA) in several species of
174 bacteria (40-44). Ban et al. (43) specifically examined the effect of hydrogen metabolism on
175 cell growth and magnetosome synthesis in *M. gryphiswaldense* MSR-1 concluding that in
176 MTBs PHA formation occurs under conditions of excess reducing power. Liu and co-workers
177 (45) succeeded in isolating an MSR-1 mutant capable of higher level magnetosome
178 production and lower PHA accumulation than the wild type, indicating a possible link
179 between the formation of PHA and magnetosomes in this bacterium. In more recent work,
180 genomic excision of the *phbCAB* operon in MSR-1 was shown to abolish PHA granule
181 formation albeit at the expense of much reduced growth (46). Collectively, the above findings
182 hint at the existence of an energy competition between the processes of PHA and
183 magnetosome formation. Here we have used FCM to investigate PHA accumulation within
184 individual bacteria of starved non-magnetic and magnetic MTB cultures. Cells were stained
185 with the lipophilic dye 1,3,5,7,8-pentamethylpyrromethene-difluoroborate complex
186 (pyrromethene-546 or Pyr-546) which on entering bacteria stains PHA green (47). **Previous**
187 **studies have shown that Pyr-546 fluorescence correlates to intracellular PHA content (48)**
188 **and is superior to Nile red as a dye for PHA.** After incubating for various times (10 – 300 s)
189 samples were immediately analysed by FCM. Fig. 3 shows that when used at a
190 concentration of 0.5 µg·mL⁻¹ the timeframe for Pyr-546 penetration and near full staining of
191 intracellular PHA was the time taken to add the dye and analyse the sample (of the order of
192 10–15 s). No further enhancement in fluorescence occurred between 70 and 300 s exposure
193 to Pyr-546 for both non-magnetic (Fig. 3a) and magnetic (Fig. 3b) cells. This said, FCM
194 analysis reveals salient differences in the PHA content of magnetic and non-magnetic MSR-
195 1, for example, revealing the presence of two discrete populations with low (Fig. 3b, labelled
196 '1') and high (Fig. 3b, labelled '2') PHA content in magnetic cells *cf.* just a single 'high PHA'
197 population in non-magnetic cells (Fig. 3a marked '2'). **Fluorescence microscopy images of**
198 **cells containing different quantities of Pyr-546 stained PHA are shown in Fig. 3. Similarly to**
199 **our findings, recent studies performed with *Cupriavidus necator* observed sub-populations**
200 **with more and less PHA (47). Other works with *Pseudomonas putida* have recently observed**
201 **an asymmetric PHA distribution during cell division under carbon limiting conditions**
202 **suggesting that this could be explained by different cellular growth rates, distinct ability to**
203 **degrade PHA or uneven distribution of PHA granules to daughter cells (49).**

204 **Measurement of intracellular chelatable iron.**

205 The intracellular pool of chelatable iron is considered a critical component in the
206 biomineralization of magnetosomes. Recent studies in *M. gryphiswaldense* MSR-1 suggest

207 that at least some of the iron transport for magnetite synthesis occurs through two copies of
208 the ferrous iron transporter FeoB which is common to most bacteria. Strains lacking *feoB1*
209 (*feoB1*) and *feoB2* (*feoB2*) were found to have lower magnetite contents than the wild type.
210 Deletion of the iron response regulator, Fur, which activates *feoB1* and *feoB2* also resulted
211 in reduced magnetosome formation (52). All the above studies compare magnetosome
212 production of wild type and 'deficient' strains, but do not provide dynamic measurements of
213 iron transport in MSR-1. Moreover, it is well known that biomineralization depends not only
214 on iron, but also on O₂ availability (26,53).

215 Typically, magnetosome production is quantified off-line (by measuring the iron content in
216 cells using atomic absorption spectroscopy), and is backed up by visualization of
217 magnetosomes under the transmission electron microscope. In both cases sample
218 preparation and analysis are laborious and time consuming. Therefore there is clearly need
219 of rapid new methods to interrogate and quantify magnetosome production and
220 biomineralization in MTBs, as well as inform the development and optimization of large-scale
221 magnetosome production strategies in bioreactors. It is this context that we developed a
222 FCM based method for detecting chelatable iron in *M. gryphiswaldense* MSR-1 cells using
223 phen green™ SK (PG-SK), whose fluorescence is quenched by metal ions including Fe²⁺
224 and Fe³⁺. PG-SK has been previously used to study iron transporters (54) and efflux
225 systems in *E. coli* as well as applied to studies in human cell lines for iron, copper and silver
226 uptake (55,56).

227 Non-magnetic MSR-1 cells grown aerobically in FSM-Fe⁻ (without iron) were stained with
228 PG-SK. The staining procedure was partially optimized with respect to staining time (600 –
229 900 s) and PG-SK concentration (0.2 – 10 μM) at three different temperatures (22, 27 and
230 30 °C). The highest fluorescence was observed at a PG-SK concentration of 5 μM after 10
231 minutes of incubation (Fig. 4a). Doubling the PG-SK concentration did not enhance the
232 fluorescence intensity of stained MSR-1 cells further (Fig. 4a), and longer staining times
233 were not needed (Fig 4b). Peak fluorescence intensity was similar at all staining
234 temperatures employed (Fig. 4c).

235 **Physiological changes of cells cultured with limited O₂ availability.**

236 The effect of O₂ limitation on growth and cellular magnetism of MSR-1 cells was investigated
237 indirectly by varying the volume of headspace provided (i.e. 20 %, 40 %, 60 %, and 80 %) in
238 tightly sealed 50 mL Falcon tubes. In all experiments the initial OD₅₆₅ was 0.086 ± 0.004.
239 After 48 h in culture, OD₅₆₅ and C_{mag} values were recorded (Fig. 5a). Two clear and opposite
240 trends were observed; whereas biomass production paralleled the increase in headspace

241 volume, and therefore O₂ availability, conversely, the magnetism of *M. gryphiswaldense*
242 MSR-1 cells dropped dramatically from strongly magnetic ($C_{\text{mag}} = 2$) at 20 % (v/v) headspace
243 to very weakly magnetic $C_{\text{mag}} = 1.1$ at 60 % (v/v) headspace. These results are in agreement
244 with findings from previous studies (26). Fig. 5b shows corresponding FCM analyses for
245 relative quantification of intracellular iron and PHA content as a function of headspace
246 volume. The highest intracellular PHA accumulation was observed in cells cultured in tubes
247 with the lowest O₂ availability (i.e. lowest headspace volume of 20 %). Increased PHA
248 formation during O₂ limitation has previously been reported (reviewed by 41). Conversely,
249 cells cultured at high O₂ concentrations (80% headspace volume) had the lowest PG-SK
250 fluorescence among the tested conditions and thus the highest free iron concentration.
251 Microarray data showed that iron transporter *feoB1* is upregulated aerobically (29),
252 suggesting that iron transport into cells is highest aerobically.

253 Staining cells grown with different headspace volumes with PI and BOX (Fig. 5c) revealed
254 that overall cell health was highest with 20 % headspace and lowest with 80% headspace
255 volume. PI was also used for analysis of physiology of MSR-1 cells from ACA agar plates;
256 this indicated that 15 – 20 % of cells on plates are dead (PI⁺). This highlights the difficulty in
257 transferring MSR-1 cultures from single colonies to liquid cultures and emphasises the need
258 to use a large amount of cells for setting up liquid cultures.

259 **Physiological characterization of MSR-1 in shake flask experiments with free air** 260 **exchange.**

261 To gain new insight into *M. gryphiswaldense* MSR-1 physiology during the shift from O₂-
262 limited to aerobic conditions, we transferred cells grown under O₂-limited conditions to O₂-
263 rich conditions with or without the supplementation of iron. Magnetic cells grown in FSM
264 batch medium and using a pH-stat feeding strategy in an O₂-limited bioreactor were
265 aseptically transferred to non-baffled shake flasks containing fresh media, either FSM or
266 FSM without Fe (FSM-Fe⁻), and grown in O₂ rich conditions (free air exchange) at 30 °C on
267 an orbital shaker (150 rpm). OD₅₆₅ and C_{mag} were monitored immediately before ($t = 0$ h) and
268 24 or 48 h after transfer and intracellular free iron and cellular PHA content were measured
269 and compared to the pre-transfer culture using FCM (Fig. 6). After O₂-limited growth in the
270 bioreactor, and at the point of transfer to shake flasks ($t = 0$ h), MSR-1 cells were moderately
271 magnetic ($C_{\text{mag}} = 1.71$). Strong Pyr-546 fluorescence (Fig. 6b, 0 h) and electron and
272 fluorescence microscopy (Supplemental Fig. S2) confirmed that cells contained large
273 quantities of PHA. After transfer to aerobic conditions, cells grew better in the presence of
274 iron (FSM) compared to the absence (FSM-Fe⁻). After 24 h, C_{mag} rose slightly from 1.71 to
275 1.84 (although variation was high at 24 h), but then dropped to 1.46 at 48 h. Cultures grown

276 in FSM had > 3 fold decreased Pyr-546 fluorescence at 24 h and 48 h, **indicating a decrease**
277 **in PHA content**, suggesting that growth utilised PHA as a substrate; FSC and SSC values
278 also dropped (Fig. 6c), indicative of decreasing cell size and **potentially corroborating** loss of
279 PHA granules. The impact of PHA utilisation on cell morphology has previously been
280 reported in *C. necator* (57) and *Pseudomonas oleovorans* (58). **In addition, in a parallel**
281 **experiment, TEM analysis and fluorescence microscopy with Pyr-546 stained cells allow**
282 **comparison of PHA detection methods (Supplemental Fig. S3).**

283 After transfer of magnetic cells to culture medium lacking iron citrate (FSM-Fe⁻), very little
284 growth ensued (OD₅₆₅ ~ 0.3; Fig. 6a). C_{mag} fell steadily (reaching 1.2 after 48 h; Fig. 6a). Pyr-
285 546 fluorescence dropped to 65 % of its pre-transfer value (Fig. 6b), reflecting low PHA
286 utilisation, whereas FSC and SSC increased, indicating an increase in cell size and
287 granularity (Fig. 6c).

288 PG-SK fluorescence dropped over time for cultures grown in FSM but not in the absence of
289 iron; as expected, this reflects an increase in chelatable iron concentration in cells grown in
290 the presence of iron, but not in the absence. Headspace volume experiments revealed an
291 increase in intracellular iron concentration under more aerobic conditions in the presence of
292 iron (Fig. 4). As with the regulation of intracellular iron concentrations in response to O₂, high
293 extracellular iron concentrations were shown to increase the expression of the *feo* iron
294 transporters (59).

295 In summary, FCM analysis of viability, intracellular chelatable iron and PHA, employing PI /
296 BOX, PG-SK and Pyr-546 dyes respectively provides valuable insight on the effects of O₂
297 and iron levels on the growth, magnetosome and PHA production of MTBs. The data are
298 rapidly obtained, does not require growth of MTBs on agar plates, and when used together
299 with similarly fast measurements of optical density and C_{mag} can be useful in the design of
300 growth strategies for production of magnetosome rich cells.

301

302 **METHODS**

303 **Strains, growth media and culture conditions.**

304 *Magnetospirillum gryphiswaldense* MSR-1 was obtained from Leibniz-DSMZ (Deutsche
305 Sammlung van Mikroorganismen und Zellkulturen GmbH) and used in all experiments.
306 Unless otherwise indicated all chemicals were from Sigma-Aldrich Chemical Company Ltd
307 (Gillingham, Dorset, UK). *M. gryphiswaldense* MSR-1 cells were grown on solid activated

308 charcoal agar (ACA) plates and in liquid media. ACA plates contained 3 g L⁻¹ activated
309 charcoal. 15 g L⁻¹ agar (Formedium, Hunstanton, Norfolk, UK), 0.1 g L⁻¹ yeast extract, 3 g L⁻¹
310 soybean peptone, 3 g L⁻¹ sodium pyruvate, 0.34 g L⁻¹ NaNO₃, 0.1 g L⁻¹ KH₂PO₄, 0.15 g L⁻¹
311 MgSO₄·7H₂O and 2.38 g L⁻¹ 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)
312 buffer in MiliQ water. The pH was adjusted to 7.0 with sodium hydroxide (Heyen and
313 Schüler, 2003) before autoclaving. After autoclaving iron(III) citrate (final concentration of
314 500 µM) and 1.4-dithiothreitol (DTT; final concentration of 1 mM) were aseptically added to
315 the plate mix before pouring (38). Set ACA plates were incubated at 30°C in 12-plates
316 anaerobic jars with one Anaerocult[®]C sachet (Merck Chemicals Ltd, Beeston Notts, UK) to
317 achieve microaerobic conditions. Liquid cultures of *M. gryphiswaldense* MSR-1 were
318 routinely grown in a shaking incubator (30° C, 150 rpm) using a flask standard medium
319 (FSM) composed of 0.1 g L⁻¹ yeast extract, 3 g L⁻¹ soybean peptone, 3.5 g L⁻¹ potassium L-
320 lactate, 100 µM iron(III) citrate, 0.34 g L⁻¹ NaNO₃, 0.1 g L⁻¹ KH₂PO₄, 0.15 g L⁻¹ MgSO₄·7H₂O,
321 5 mL L⁻¹ of EDTA-chelated trace element mixture (60) and 2.38 g L⁻¹ HEPES buffer in
322 deionized water; the whole adjusted to pH 7.0 prior to sterilization in an autoclave. Cells
323 were grown at 30°C in a shaking incubator at 150 rpm. O₂-limiting cultures were grown in
324 tightly sealed screw cap 50 mL Falcon tubes with variable headspace volumes (10 – 40 mL),
325 whereas aerobic cultivations were performed with 50 mL of media in 250 mL shake flasks
326 allowing free air exchange. Non-magnetic cells were cultured in FSM without iron (FSM-Fe⁻)
327 for a minimum of three sequential sub-cultures in an attempt to eliminate all trace of the
328 metal. Magnetic cells were obtained from cultures grown in bioreactor experiments under
329 controlled conditions using a growth strategy adapted from previous works (26, 28, 61).

330 **Flow cytometry (FCM).**

331 Bacterial samples taken directly from agar plates or liquid cultures were resuspended in
332 phosphate-buffered saline (PBS) and then analysed directly in a BD Accuri C6 flow
333 cytometer (Becton, Dickinson and Company, Oxford, UK) for cell size and granularity, or
334 after staining with various fluorescent dyes (see Supplemental Table 1) using protocols
335 developed and detailed in the Results and Discussion. During FCM on fluorescently labelled
336 cells, samples were excited using a 488 nm solid-state laser and fluorescence was detected
337 using three different filters, i.e.: a 533/30 BP filter (FL1-A) for bis (1.3-dibutylbarbituric acid)
338 trimethine oxonol (referred to here as bis-oxonol or BOX), pyromethene-546 (Pyr-546) and
339 phen green[™] SK (PG-SK); a 670 LP filter (FL3-A) for propidium iodide (PI); and a 675/25
340 BP filter (FL4-A) for Syto[®]62.

341 **Analytical methods.**

342 Culture optical densities were recorded at a wavelength of 565 nm (OD_{565}) in an Evolution
343 300 UV-Vis spectrophotometer (Thermo Fisher Scientific, Hemel Hempstead, Herts, UK)
344 controlled by Thermo Scientific™ VISION pro ™ software.

345 Cellular magnetic response (C_{mag}) values of cultures were determined immediately after
346 OD_{565} measurements using a purpose-built magnetic measurement system mounted within
347 the spectrophotometer. In common with previous magneto-spectrophotometry apparatus
348 (24, 25) our system features two pairs of Helmholtz coils arranged around the cuvette
349 holder, one pair perpendicular to the light beam and the other in line with it. Each pair of coils
350 is energized in turn, and the OD_{565} is measured in each condition. Magnetic cells will align
351 with the magnetic field and thus orient in line with or perpendicular to the light beam; the
352 optical density will therefore change between the two conditions. Non-magnetic cells do not
353 align with the magnetic field, thus the optical density does not change on switching the field
354 orientation. C_{mag} values for culture samples are calculated by dividing the OD_{565} values of
355 suspensions of cells aligned parallel to the light beam by those obtained when the same
356 cells are perpendicularly aligned. C_{mag} values greater than unity reflect the presence of
357 magnetic cells.

358 Cellular morphology was routinely examined by light microscopy using an Olympus BX50
359 optical microscope (Olympus Corporation, Tokyo, Japan). Images were captured using a
360 MotiCam 1 (800×600 pixel) camera (Microscope Systems Limited, Glasgow, UK) and
361 processed with Motic Images Plus 2.0 software (Motic Europe S.L.U., Barcelona, Spain).

362 Cells stained with fluorescent probes were observed and imaged using a Zeiss AxioLab
363 microscope (Carl Zeiss Ltd., Cambridge, UK) fitted with a Zeiss AxioCam ICm1 camera, and
364 the images were processed in auto-exposure mode with the aid of Zeiss ZEN Lite 2012
365 software. Samples were excited with a Zeiss VHW 50f-2b ultraviolet light source and a 520
366 LP filter was employed for detection of fluorescence from Syto® 9 and pyromethene-546
367 (Pyr-546).

368 **Data availability.**

369 The datasets generated during and analysed during the current study are available from the
370 corresponding author on reasonable request.

371 **REFERENCES**

- 372 1. Frey, N. A., Peng, S., Cheng, K. & Sun, S. Magnetic nanoparticles: synthesis,
373 functionalization, and applications in bioimaging and magnetic energy storage. *Chem. Soc.*
374 *Rev.* **38**, 2532–2542 (2009).
- 375 2. Schätz, A., Reiser, O. & Stark, W. J. Nanoparticles as semi-heterogeneous catalyst
376 supports. *Chem. Eur. J.* **16**, 8950–8967 (2010).
- 377 3. Franzreb, M., Siemann-Herzberg, M., Hobley, T.J. & Thomas, O. R. T. Protein
378 purification using magnetic adsorbent particles. *Appl. Microb. Biotechnol.* **70**, 505–516
379 (2006).
- 380 4. Fischer, I., Hsu, C-C., Gärtner, M., Müller, C., Overton T. W., *et al.* Continuous
381 protein purification using functionalized magnetic nanoparticles in aqueous micellar two-
382 phase systems. *J. Chromatogr. A.* **1305**, 7–16 (2013).
- 383 5. Pankhurst, Q. A., Thanh, N. T. K., Jones, S. K. & Dobson, J. Progress in applications
384 of magnetic nanoparticles in biomedicine. *J. Phys. D: Appl. Phys.* **42**, 224001 (2009).
- 385 6. Hao, R., Xing, R., Xu, Z., Hou, Y., Gao, S. & Sun, S. Synthesis, functionalization, and
386 biomedical applications of multifunctional magnetic nanoparticles. *Adv. Mater.* **22**, 2729–
387 2742 (2010).
- 388 7. Wilhelm, S., Tavares, A. J., Dai, Q., Ohta, S., Audet, J. *et al.* Analysis of nanoparticle
389 delivery to tumours. *Nat. Rev. Mat.* **1**, 16014 (2016).
- 390 8. Lang, C. & Schüler, D. Biogenic nanoparticles: production, characterization, and
391 application of bacterial magnetosome. *J. Phys. Condens. Matter* **18**, S2815–S2828 (2006).
- 392 9. Arakaki, A., Nakazawa, H., Nemoto, M., Mori, T. & Matsunaga, T. Formation of
393 magnetite by bacteria and its application. *J. R. Soc. Interface* **5**:977–999 (2008).
- 394 10. Yan, L., Zhang, S., Chen, P., Liu, H., Yin, H., Li, H. Magnetotactic bacteria,
395 magnetosomes and their application. *Microb. Res.* **167**, 507–519 (2012).
- 396 11. Alphandéry, E. Applications of magnetosomes synthesized by magnetotactic bacteria
397 in medicine. *Front. Bioeng. Biotechnol.* **2**, 5 (2014).
- 398 12. Jacob, J. J. & Suthindhiran, K. Magnetotactic bacteria and magnetosomes – Scope
399 and challenges. *Mater. Sci. Eng. C.* **68**, 919–928 (2016).
- 400 13. Bazylinski, D.A. & Frankel, R.B. Magnetosome formation in prokaryotes. *Nat. Rev.*
401 *Microbiol.* **2**, 217–230 (2004).
- 402 14. Pollithy, A., Romer, T., Lang, C., Müller, F. D., Helma, J. *et al.* Magnetosome
403 expression of functional camelid antibody fragments (nanobodies) in *Magnetospirillum*
404 *gryphiswaldense*. *Appl. Environ. Microbiol.* **77**, 6165–6171 (2011).
- 405 15. Pósfai, M., Lefèvre, C. T., Truitsyn, D., Bazylinski, D. A. & Frankel, R.B.
406 Phylogenetic significance of composition and crystal morphology of magnetosome minerals.
407 *Front. Microbiol.* **4**, 344 (2013).

- 408 16. Uebe, R. & Schüler, D. Magnetosome biogenesis in magnetotactic bacteria. *Nat.*
409 *Rev. Microbiol.* **14**, 621–637 (2016).
- 410 17. Barber-Zucker, S. & Zarivach, R. A look into the biochemistry of magnetosome
411 biosynthesis in magnetotactic bacteria. *ACS Chem. Biol.* **12**, 3–22 (2017).
- 412 18. Frankel, R.B. Magnetic guidance of organisms. *Annu. Rev. Biophys. Bioeng.* **13**:85–
413 103 (1984).
- 414 19. Wacker, R., Ceyhan, B., Alhorn, P., Schueler, D., Lang, C. & Niemeyer, C. M.
415 Magneto immuno-PCR: a novel immunoassay based on biogenic magnetosome
416 nanoparticles. *Biochem. Biophys. Res. Commun.* **357**, 391–396 (2007).
- 417 20. Yoshino, T., Hirabe, H., Takahashi, M., Kuhara, M., Takeyama, H. & Matsunaga, T.
418 Magnetic cell separation using nano-sized bacterial magnetic particles with reconstructed
419 magnetosome membrane. *Biotechnol. Bioeng.* **101**, 470–477 (2008).
- 420 21. Boucher, M., Geffroy, F., Prévéral, S., Bellanger, L., Selingue, E. *et al.* Genetically
421 tailored magnetosomes used as MRI probe for molecular imaging of brain tumor.
422 *Biomaterials* **121**, 167–178 (2017).
- 423 22. Tang, Y.S., Wang, D., Zhou, C., Ma, W., Zhang, Y.Q. *et al.* Bacterial magnetic
424 particles as a novel and efficient gene vaccine delivery system. *Gene ther.* 19(12):1187–
425 1195 (2012).
- 426 23. Felfoul, O., Mohammadi, M., Taherkhani, S., De Lanauze, D., Xu, Y. Z. *et al.*
427 Magneto-aerotactic bacteria deliver drug-containing nanoliposomes to tumour hypoxic
428 regions. *Nat. Nanotechnol.* **11**, 941–947 (2016).
- 429 24. Zhao, L., Wu, D., Wu, L. F. & Song, T. A simple and accurate method for
430 quantification of magnetosomes in magnetotactic bacteria by common spectrophotometer. *J.*
431 *Biochem. Biophys. Methods* **70**, 377–383 (2007).
- 432 25. Lefèvre, C. T., Song, T., Yonnet, J-P. & Wu, L-F. Characterization of bacterial
433 magnetotactic behaviors by using a magnetospectrophotometry assay. *Appl. Environ.*
434 *Microbiol.* **75**:3835–3841 (2009).
- 435 26. Heyen, U. & Schüler, D. Growth and magnetosome formation by microaerophilic
436 *Magnetospirillum* strains in an oxygen-controlled fermentor. *Appl. Microbiol. Biotechnol.* **61**,
437 536–544 (2003).
- 438 27. Grünberg, K., Müller, E. C., Otto, A., Reszka, R., Linder, D. *et al.* Biochemical and
439 Proteomic Analysis of the Magnetosome Membrane in *Magnetospirillum gryphiswaldense*.
440 *Appl. Environ. Microbiol.* **70**, 1040–1050 (2004).
- 441 28. Yang, J., Li, S., Huang, X., Tang, T., Jiang, W., Zhang, T. & Li, Y. A key time point for
442 cell growth and magnetosome synthesis of *Magnetospirillum gryphiswaldense* based on
443 real-time analysis of physiological factors. *Front. Microbiol.* **4**, 1–7 (2013).

- 444 29. Wang, X., Wang, Q., Zhang, Y., Wang, Y., Zhou, Y., *et al.* Transcriptome analysis
445 reveals physiological characteristics required for magnetosome formation in
446 *Magnetospirillum gryphiswaldense* MSR-1. *Environ. Microbiol. Rep.* **8**, 371–381 (2016).
- 447 30. Geng, J., Beloin, C., Ghigo, J-M., Henry, N., Stewart, P., *et al.* Bacteria hold their
448 breath upon surface contact as shown in a strain of *Escherichia coli*, using dispersed
449 surfaces and flow cytometry analysis. *PLoS One* **9**, e102049 (2014).
- 450 31. Lagendijk, E.L., Validov, S., Lamers, G. E. M., De Weert, S. & Bloemberg, G. V.
451 Genetic tools for tagging Gram-negative bacteria with mCherry for visualization in vitro and
452 in natural habitats, biofilm and pathogenicity studies. *FEMS Microbiol. Lett.* **305**, 81–90
453 (2010).
- 454 32. Sevastyanovich, Y., Alfasi, S., Overton, T., Hall, R., Jones, J. *et al.* Exploitation of
455 GFP fusion proteins and stress avoidance as a generic strategy for the production of high-
456 quality recombinant proteins. *FEMS Microbiol. Lett.* **299**, 86–94 (2009).
- 457 33. Khan, M. M. T., Pyle, B. H. & Camper, A.K. Specific and rapid enumeration of viable
458 but nonculturable and viable-culturable Gram-negative bacteria by using flow cytometry.
459 *Appl. Environ. Microbiol.* **76**, 5088–5096 (2010).
- 460 34. Lang, C. & Schuler, D. Expression of green fluorescent protein fused to
461 magnetosome proteins in microaerophilic magnetotactic bacteria. *Appl. Environ. Microbiol.*
462 **74**, 4944–4953 (2008).
- 463 35. Lang, C., Pollithy, A. & Schüler, D. Identification of promoters for efficient gene
464 expression in *Magnetospirillum gryphiswaldense*. *Appl. Environ. Microbiol.* **75**, 4206–4210
465 (2009).
- 466 36. Day, J.B., Kell, D.B. & Griffith, G.W. Differentiation of *Phytophthora infestans*
467 sporangia from other airborne biological particles by flow cytometry. *Appl. Environ. Microbiol.*
468 **68**, 37–45 (2002).
- 469 37. Lee, Y-H., Chen, S-Y., Wiesner, R.J., Huang, Y-F. Simple flow cytometric method
470 used to assess lipid accumulation in fat cells. *J. Lipid Res.* **45**, 1162–7 (2004).
- 471 38. Schultheiss, D. & Schüler, D. Development of a genetic system for *Magnetospirillum*
472 *gryphiswaldense*. *Arch. Microbiol.* **179**, 89–94 (2003).
- 473 39. Nebe-von-Caron, G., Stephens, P., Hewitt, C., Powell, J. & Badley, R. Analysis of
474 bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. *J.*
475 *Microbiol. Methods* **42**, 97–114 (2000).
- 476 40. Ward, A.C., Rowley, B.I. & Dawes, E.A. Effect of Oxygen and Nitrogen Limitation on
477 Poly-hydroxybutyrate Biosynthesis in Ammonium-grown *Azotobacter beijerinckii*. *J. Gen.*
478 *Microbiol.* **102**, 61–68 (1977).
- 479 41. Kessler, B. & Witholt, B. Factors involved in the regulatory network of
480 polyhydroxyalkanoate metabolism. *J. Biotechnol.* **86**, 97–104 (2001).

- 481 42. García-Torreiro, M., Lu-Chau, T. A. & Lema, J. M. Effect of nitrogen and/or oxygen
482 concentration on poly(3-hydroxybutyrate) accumulation by *Halomonas boliviensis*.
483 *Bioprocess Biosyst. Eng.* **39**, 1365–1374 (2016).
- 484 43. Ban, J., Jiang, W., Li, Y., Zhang, Y. P. & Li, J. L. Functional analysis of hydrogenases
485 and their effects on cell growth and magnetosome synthesis in *Magnetospirillum*
486 *gryphiswaldense*. *Chinese Sci. Bull.* **55**:1271–1277 (2010).
- 487 44. Schultheiss, D., Handrick, R., Jendrossek, D., Hanzlik, M. & Schüler, D. The
488 presumptive magnetosome protein Mms16 is a poly(3-hydroxybutyrate) granule-bound
489 protein (phasin) in *Magnetospirillum gryphiswaldense*. *J. Bacteriol.* **187**, 2416–2425 (2005).
- 490 45. Liu, J., Ding, Y., Jiang, W., Tian, J., Li, Y. & Li, J. A mutation upstream of an ATPase
491 gene significantly increases magnetosome production in *Magnetospirillum gryphiswaldense*.
492 *Appl. Microbiol. Biotechnol.* **81**, 551–558 (2008).
- 493 46. Raschdorf, O., Plitzko, J. M., Schuler, D. & Muller, F. D. A tailored *galK*
494 counterselection system for efficient markerless gene deletion and chromosomal tagging in
495 *Magnetospirillum gryphiswaldense*. *Appl. Environ. Microbiol.* **80**, 4323–4330 (2014).
- 496 47. Vizcaino-Caston, I., Kelly, C. A., Fitzgerald, A. V. L., Leeke, G. A., Jenkins, M.,
497 Overton, T. W. Development of a rapid method to isolate polyhydroxyalkanoates from
498 bacteria for screening studies *J. Biosci. Bioeng.* **121**, 101–104 (2016).
- 499 48. Kacmar, J., Carlson, R., Balogh, S.J., Srienc, F. Staining and quantification of poly-3-
500 hydroxybutyrate in *Saccharomyces cerevisiae* and *Cupriavidus necator* cell populations
501 using automated flow cytometry. *Cytometry A.* **69**, 27–35 (2006).
- 502 49. Karmann, S.; Panke, S.; Zinn, M. The Bistable Behaviour of *Pseudomonas putida*
503 **KT2440** during PHA Depolymerization under Carbon Limitation. *Bioengineering.* **4**, 58
504 (2017).
- 505 50. Rong, C., Huang, Y., Zhang, W., Jiang, W., Li, Y. & Li, J. Ferrous iron transport
506 protein B gene (*feoB1*) plays an accessory role in magnetosome formation in
507 *Magnetospirillum gryphiswaldense* strain MSR-1. *Res. Microbiol.* **159**, 530–536 (2008).
- 508 51. Rong, C., Zhang, C., Zhang, Y., Qi, L., Yang, J., et al. FeoB2 Functions in
509 magnetosome formation and oxidative stress protection in *Magnetospirillum*
510 *gryphiswaldense* strain MSR-1. *J. Bacteriol.* **194**, 3972–3976 (2012).
- 511 52. Uebe, R., Voigt, B., Schweder, T., Albrecht, D., Katzmann, E., et al. Deletion of a *fur*-
512 like gene affects iron homeostasis and magnetosome formation in *Magnetospirillum*
513 *gryphiswaldense*. *J. Bacteriol.* **192**, 4192–4204 (2010).
- 514 53. Schüler, D. & Baeuerlein, E. Dynamics of iron uptake and Fe₃O₄ biomineralization
515 during aerobic and microaerobic growth of *Magnetospirillum gryphiswaldense*. *J. Bacteriol.*
516 **180**, 159–162 (1998).

- 517 54. Große, C., Scherer, J., Koch, D., Otto, M., Taudte, N. & Grass, G. A new ferrous iron-
518 uptake transporter, EfeU (YcdN), from *Escherichia coli*. *Mol. Microbiol.* **62**, 120–131 (2006).
- 519 55. Xu, G., Ahn, J., Chang, S., Eguchi, M., Ogier, A., *et al.* Lipocalin-2 induces
520 cardiomyocyte apoptosis by increasing intracellular iron accumulation. *J. Biol. Chem.* **287**,
521 4808–4817 (2012).
- 522 56. Zhao, G., Di, D., Wang, B., Zhang, P., Xu, Y. Iron regulates the expression of
523 ferroportin 1 in the cultured hFOB 1.19 osteoblast cell line. *Exp. Ther. Med.* **8**, 826–830
524 (2014).
- 525 57. Tian, J., Sinskey, A. J. & Stubbe, J. Kinetic studies of polyhydroxybutyrate granule
526 formation in *Wautersia eutropha* H16 by transmission electron microscopy. *J. Bacteriol.* **187**,
527 3814–3824 (2005).
- 528 58. Ruiz, J. A., López, N. I., Fernández, R. O. & Méndez, B. S. Polyhydroxyalkanoate
529 degradation is associated with nucleotide accumulation and enhances stress resistance and
530 survival of *Pseudomonas oleovorans* in natural water microcosms. *Appl. Environ. Microbiol.*
531 **67**, 225–230 (2001).
- 532 59. Wang, Q., Liu, J. X., Zhang, W. J., Zhang, T. W., Yang, J. & Li, Y. Expression
533 patterns of key iron and oxygen metabolism genes during magnetosome formation in
534 *Magnetospirillum gryphiswaldense* MSR-1. *FEMS Microbiol. Lett.* **347**, 163–72 (2013).
- 535 60. Widdel, F. & Bak, F. Gram-negative mesophilic sulfate-reducing bacteria in *The*
536 *Prokaryotes* (eds. Balows, A., Troper, H., Dworkin, M., Harder, W. & Schleifer, K.) 3352–
537 3378 (Springer, 1992)
- 538 61. Sun, J-B., Zhao, F., Tang, T., Jiang, W., Tian, J., *et al.* High-yield growth and
539 magnetosome formation by *Magnetospirillum gryphiswaldense* MSR-1 in an oxygen-
540 controlled fermentor supplied solely with air. *Appl. Microbiol. Biotechnol.* **79**, 389–397
541 (2008).

542

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546 **Author Contributions**

547 AFC and HL completed the practical work. ORTT and TWO supervised the work. AFC,
548 ORTT and TWO wrote the manuscript. All authors reviewed the manuscript.

549 **Additional Information**

550 Competing Financial Interests: The authors declare no competing financial interests.

551

552 **Figure legends**

553 **Figure 1. Analysis of *M. gryphiswaldense* MSR-1 using FSM and light microscopy.**

554 Scatter plots (Forward scatter, FSC-A vs. Side scatter, SSC-A) of cells cultured (a) in liquid
555 FSM and (b) on ACA plates; (c) comparison of individual particle count vs FSC-A plots for
556 liquid (red trace) and plate (blue trace) grown cells; light microscope images of (d) liquid and
557 (e) plate grown cells.

558 **Figure 2. Viability analysis of MSR-1 cells using FCM.** MSR-1 cells were co-stained with

559 BOX (fluorescence measured on FL1-A channel, y axis) and PI (fluorescence measured on
560 FL3-A channel, x axis). Key: (a) actively growing magnetic cells; (b) starving magnetic cells;
561 (c) actively growing non-magnetic cells; (d) starving non-magnetic cells; and (e) cells
562 incubated with absolute ethanol for 10 minutes, centrifuged and then resuspended in
563 phosphate buffered saline. The numbers of cells in each of the four quadrants of all plots are
564 indicated in red font and are expressed as percentages of the total population.

565 **Figure 3. Analysis of PHA content using FCM.** Fluorescence intensity histograms of

566 starved (a) non-magnetic and (b) magnetic cells after staining with Pyr-546 ($0.5 \mu\text{g}\cdot\text{mL}^{-1}$) for
567 various times. The numbers '1' & '2' marked on the inserts and the fluorescence
568 micrographs correspond to non-magnetic and magnetic cells, respectively to identify those
569 with low and high PHA content. The scale bars indicate a length of 5 μm .

570 **Figure 4. Analysis of intracellular iron by FCM.** Fluorescence intensity histograms of non-

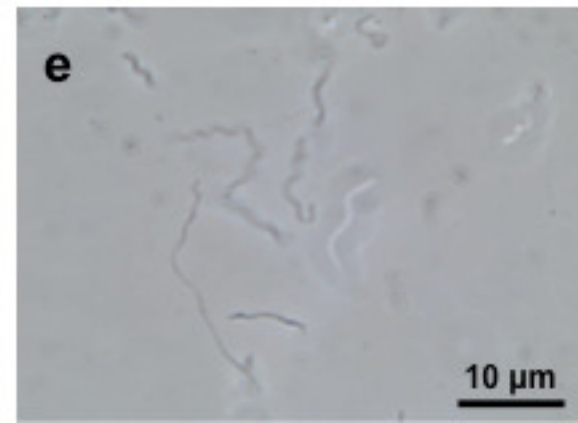
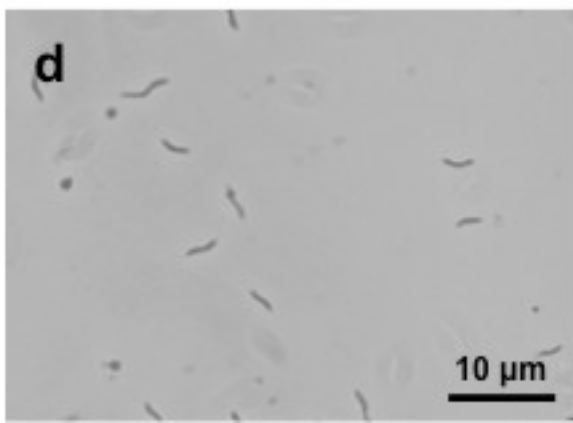
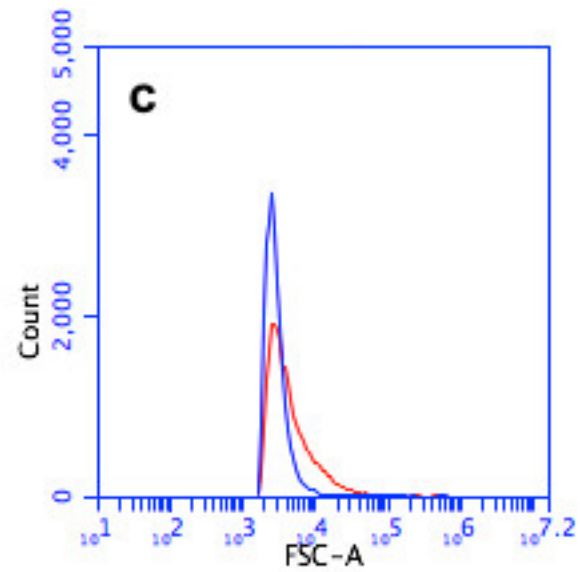
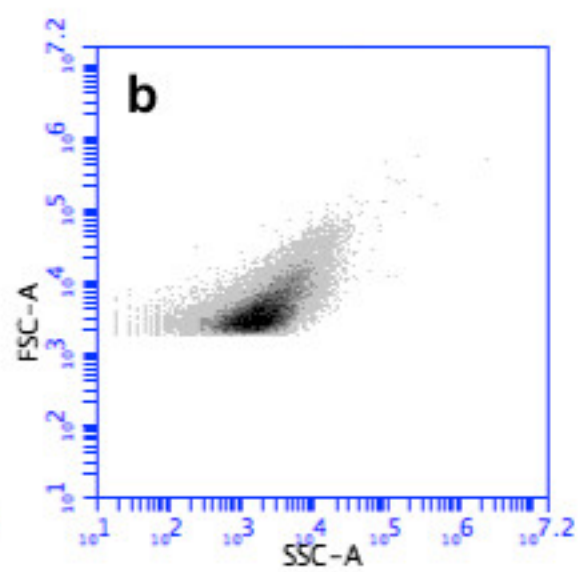
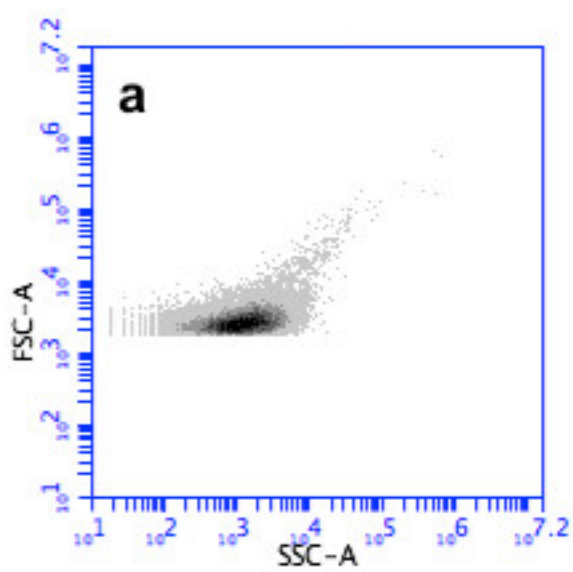
571 magnetic MSR-1 cells growth in FSM-Fe⁻ after staining: (a) with various concentrations of
572 PG-SK for 600 s at 30°C; (b) for various times with 10 μM PG-SK at 30°C; and (c) at various
573 temperatures using 10 μM PG-SK for 600 s.

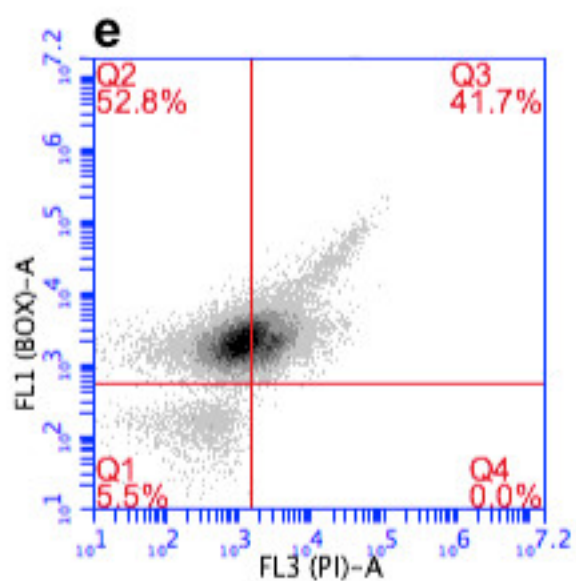
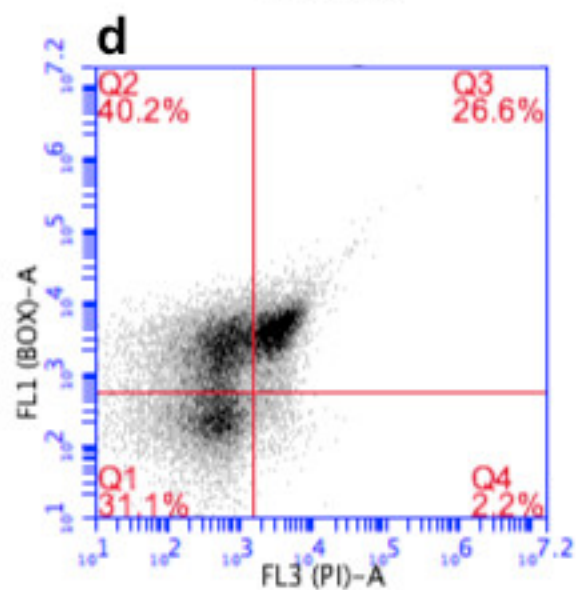
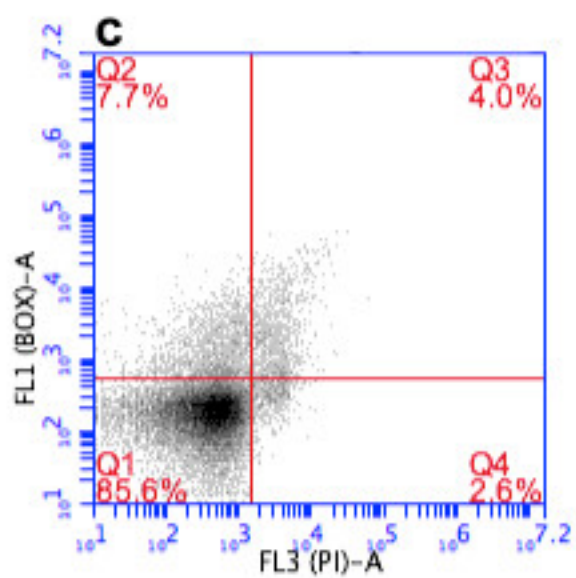
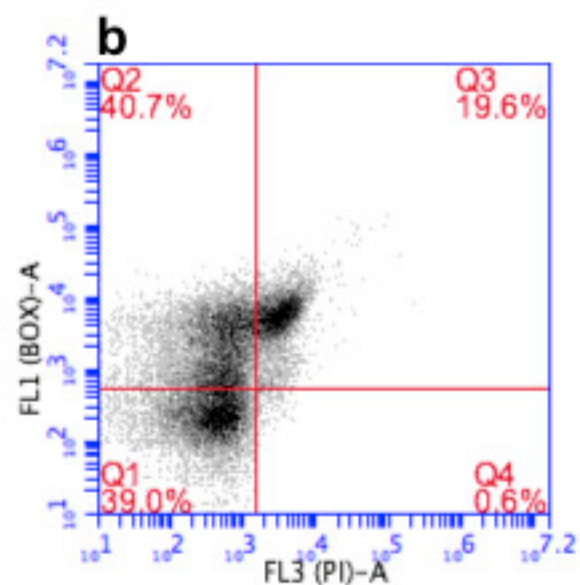
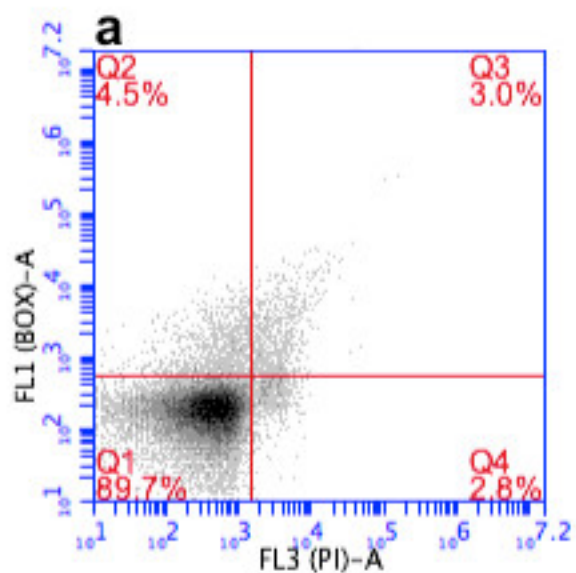
574 **Figure 5. Effect of O₂ limitation on physiology.** MSR-1 cultures were grown in tubes with

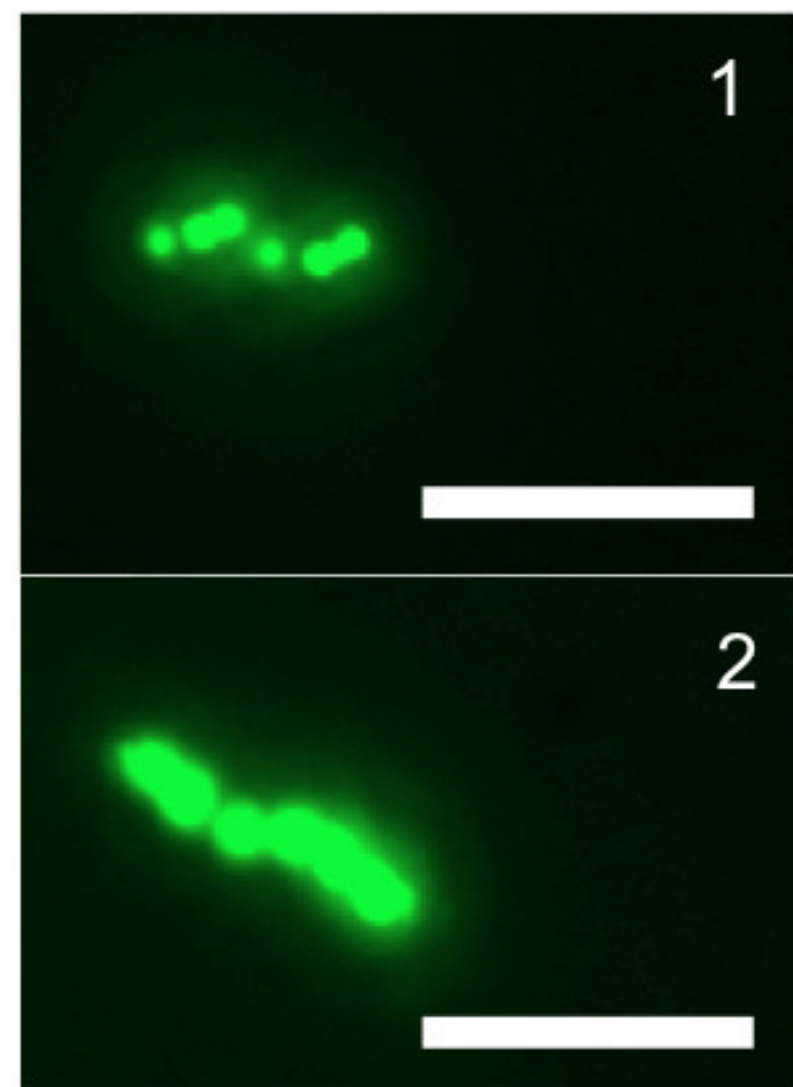
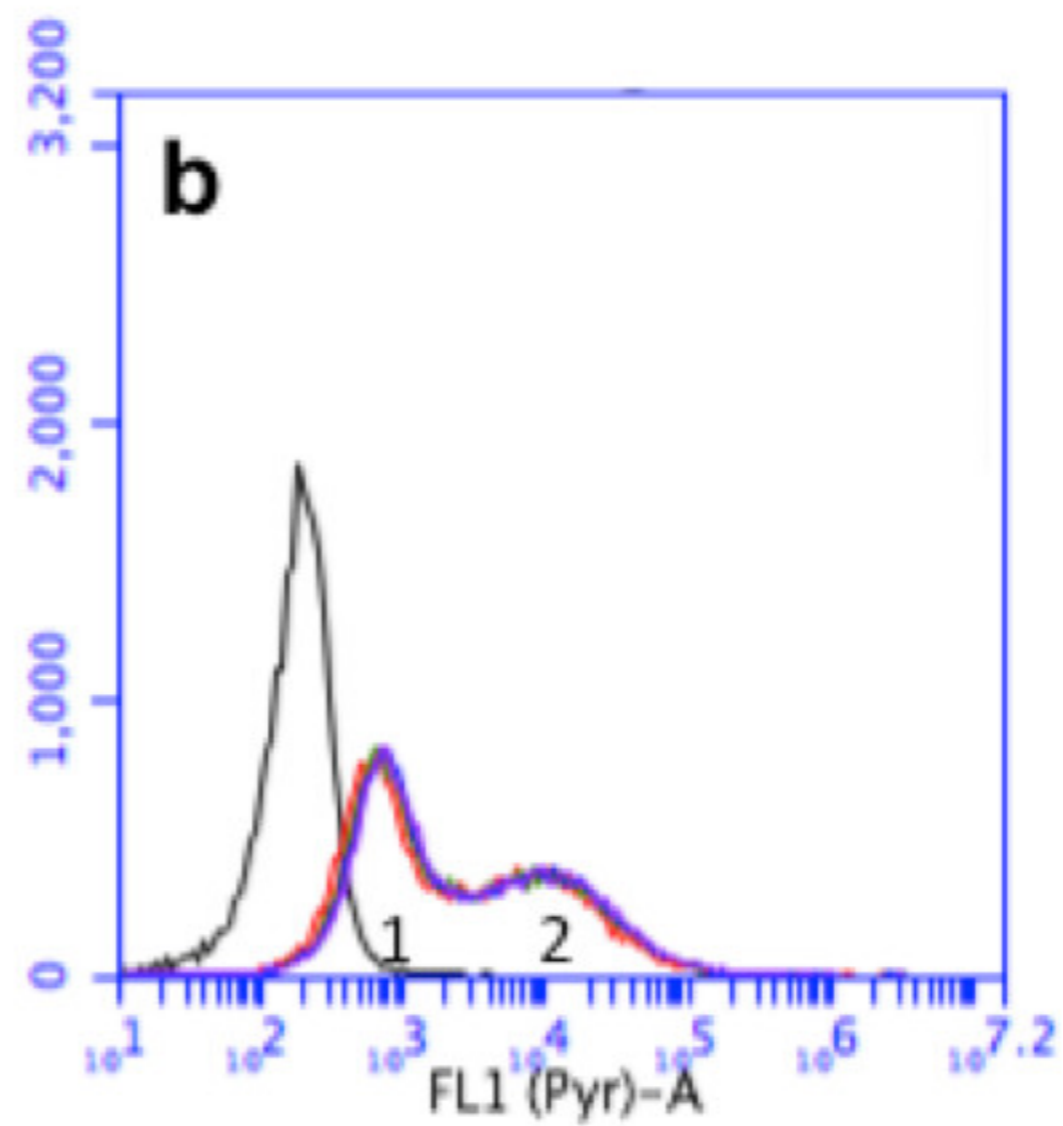
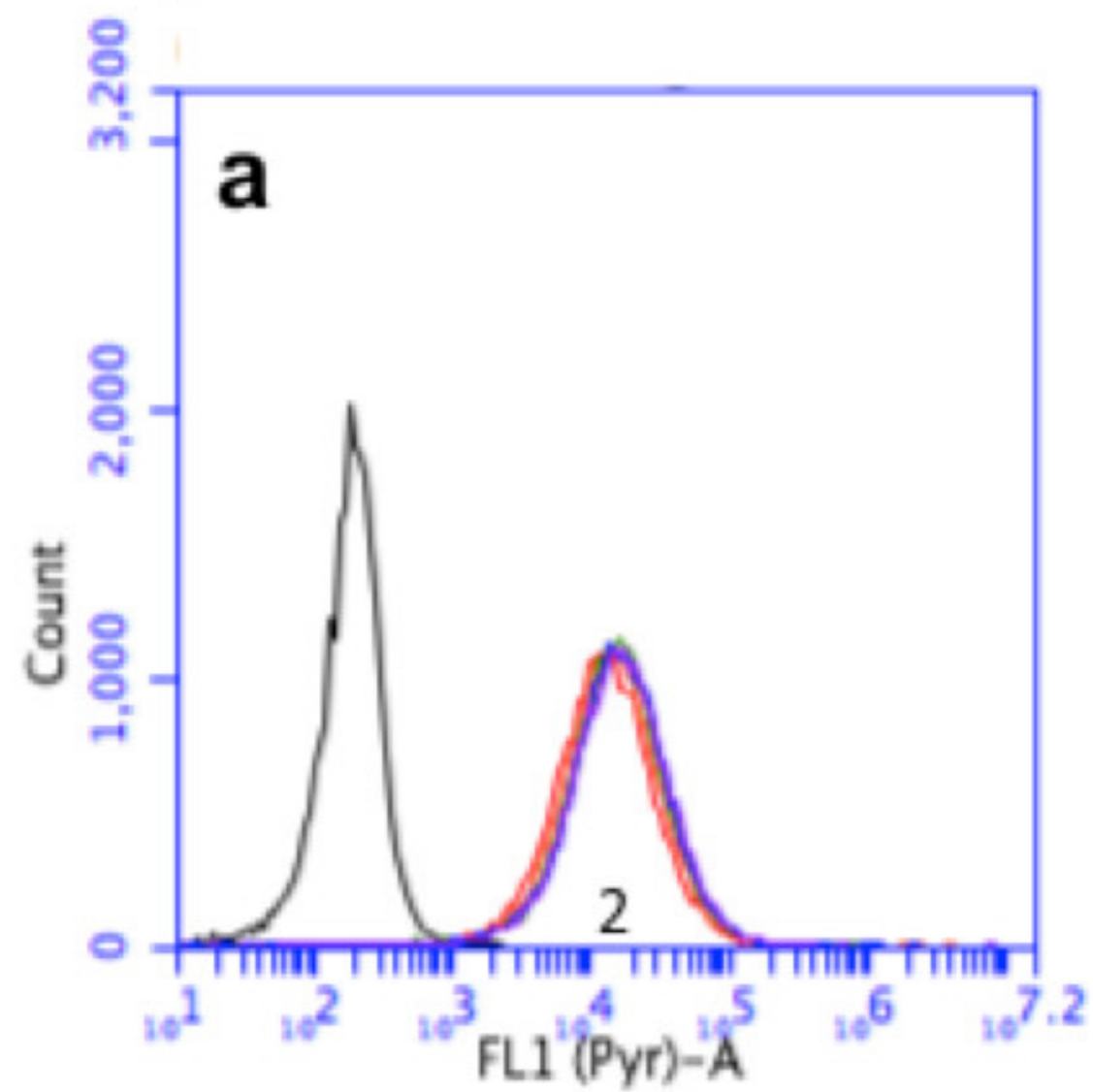
575 different headspace volumes for 48 h. (a) OD₅₆₅ (pale grey bars) and cellular magnetism
576 (C_{mag} ; black bars). Error bars are standard deviation. (b) Fluorescence of cells stained with
577 PG-SK (dark grey bars) and Pyr-546 (white bars) as measured using FCM. Error bars are
578 covariance. (c) Viability as determined using FCM and staining with PI and BOX; percentage
579 of healthy (black bars), injured (pale grey bars) and dead (dark grey bars) cells are shown.

580 **Experiments were performed in triplicate.**

581 **Figure 6. Physiology of magnetic cells during shift to aerobic conditions.** MSR-1 cells
582 grown under O_2 -limited conditions in a bioreactor were transferred to O_2 -sufficient conditions
583 with either iron-containing (FSM) or iron-lacking (FSM- Fe^-) media. (a) OD_{565} (pale grey bars)
584 and C_{mag} value (black bars). Error bars are standard deviation; **cells were taken from a single**
585 **bioreactor into three replicate flasks for each condition.** (b) Mean fluorescence intensity of
586 cells stained with $0.5 \mu g \cdot mL^{-1}$ pyrrromethene-546 (Pyr546) (white bars) or $5 \mu M$ **phen green**TM
587 SK (PG-SK) (dark grey bars). Error bars are covariance. (c) Forward scatter (FSC, white
588 bars) and side scatter (SSC, grey bars) of cells as determined by FCM. Error bars are
589 covariance. 25 000 events were analysed per sample by FCM.
590







No stain 0 min 1 min 2 min 3 min 5 min

