



HAL
open science

Azide click chemistry on magnetotactic bacteria: A versatile technique to attach a cargo

Paul Eduardo David Soto Rodriguez, Mila Sirinelli-Kojadinovic, Maximilien Rouzaud, Damien Faivre

► **To cite this version:**

Paul Eduardo David Soto Rodriguez, Mila Sirinelli-Kojadinovic, Maximilien Rouzaud, Damien Faivre. Azide click chemistry on magnetotactic bacteria: A versatile technique to attach a cargo. *Materials Today Bio*, 2023, 19, pp.100587. 10.1016/j.mtbio.2023.100587 . cea-04448209

HAL Id: cea-04448209

<https://cea.hal.science/cea-04448209v1>

Submitted on 9 Feb 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 Title:

2 **Azide click chemistry on magnetotactic bacteria: a versatile technique to attach a cargo**

3 Authors:

4 Paul Eduardo David Soto Rodriguez¹, Mila Sirinelli-Kojadinovic¹, Maximilien Rouzaud¹ and Damien
5 Faivre^{1*}

6

7 ¹Aix Marseille University, CEA, CNRS, BIAM, 13108 Saint Paul-Lez-Durance, France

8 *Corresponding author

9 Email: Damien.FAIVRE@cea.fr

10 **ABSTRACT:**

11 Adding biomolecules to living organisms and cells is the basis for creating living materials or
12 biohybrids for robotic systems. Bioorthogonal chemistry allows covalently modifying biomolecules
13 with functional groups not natively present under biological conditions and is therefore applicable
14 to microorganisms and cells. Click chemistry is a bioorthogonal chemistry approach that allows the
15 study and manipulation of living entities. Incorporating the bioorthogonal click-chemistry handle,
16 azide groups, into living microorganisms has been achieved by metabolic labeling, i.e., by culturing
17 cells or organisms in a modified culture media having a specific natural molecular building block
18 (e.g., amino acid, nucleotide, carbohydrate) modified with a tagged chemical analog. Here we
19 explore the effect of the azide group incorporation into the magnetotactic bacteria
20 *Magnetospirillum gryphiswaldense* (MSR-1) by adding a modified amino acid, 3-Azido-D-Alanine,
21 during their cultivation. We show the existence of a concentration limit to effectively incorporate
22 the azide group while maintaining the magnetic properties of the cells. We explore the use of this
23 modification to explore the combination with versatile single-cell tagging methods.

24 **Keywords: (max6)**

25 *Click chemistry, magnetotactic bacteria, single-cell tagging*

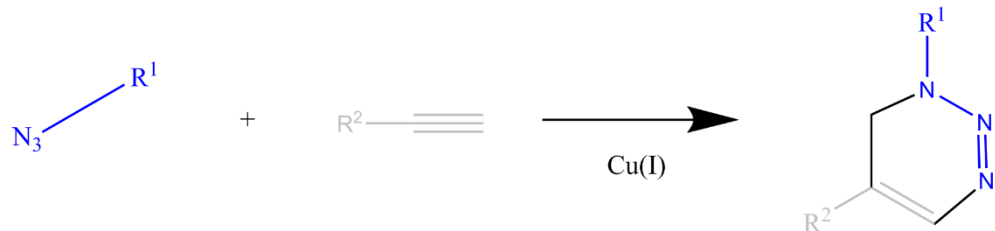
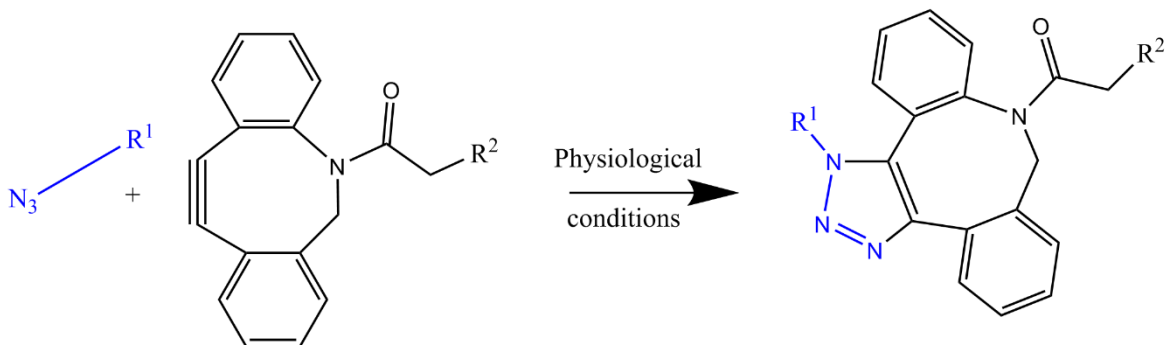
26 **Introduction**

27 Theranostics is one of the main application field of nano and micro-robotics [1]. Many designs
28 developed for theranostics are based on mimicking microorganisms, particularly introducing
29 motility and sensing functionalities [2]. Magnetic properties often provide or at least improve
30 theranostic applications. Therefore, the attention on magnetic nanoparticles is rising in this area [3].
31 Another rising research field is the so-called bacteria bots, which studies the direct modification of
32 bacterial surfaces to attach a cargo that can be used for detection and treatment of a given illness
33 [4]. Different bacterial strains and cargo designs have been proposed [5–8]. The primary desired
34 characteristics are that bacteria should allow easy attachment of biomolecules, be able to control
35 and provide easy characterization remotely. Many bacteria have been genetically engineered or
36 chemically modified to fulfill these properties, including nanoliposomes attachment through amine

37 coupling by activation of carboxylic acids with EDC/NHS chemistry [9], by NHS-ester amine coupling
38 [10] or biotin-streptavidin reactions [11].

39 Magnetotactic bacteria (MTB) mineralize and organize intracellularly magnetic particles called
40 magnetosomes. The latter allow them to swim along the geomagnetic field lines [12] and therefore
41 enable magnetic control of MTB motility by external magnetic fields. In addition, MTB use a
42 magnetically-biased aerotaxis that allows them to find their preferred living conditions [13]. This
43 particular behavior has been used to develop active systems envisioned to deliver drugs to cancer
44 cells [9,14]. Moreover, the magnetic properties of magnetosomes are used as a contrast agent in
45 biomedical imaging applications. For example, for magnetic resonance imaging (MRI) when isolated
46 [15], or as tracer particles in magnetic particle imaging (MPI) isolated [16] or even within the cell
47 [17].

48 Click chemistry, topic for which the 2022 Nobel prize awards were given [18], refers to a set of fast
49 reactions, easy to use, simple to purify, versatile, regioselective, and with high product yields [18].
50 Click chemistry permits different biomolecules' easy and fast attachment to particles. The group of
51 reactions mainly used is the Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes. To
52 date, it has not been hardly exploited for its use in magnetotactic bacteria. Indeed, the application
53 of alkyne-azide click chemistry is not straightforward as the needed functional group azide is not
54 naturally present in bacteria. Moreover, as shown in scheme 1 A, the conventional azide-alkyne
55 binding is done with the help of copper(I) as a catalyst [19], which, in general, is harmful to bacteria
56 at the concentration used [20]. However, there are methods to overcome these drawback. For
57 example, azide groups can be incorporated into living animals by genetic engineering [21,22] or
58 metabolic labeling and can therefore enable copper-free click chemistry to label biomolecules in
59 mice [23]. In addition, copper-free strain-promoted azide-alkyne cycloaddition has already been
60 developed and used as an alternative in living cells and was recently awarded with a Nobel prize
61 2022. For this method, a strained molecule (Dibenzocyclooctyne, DBCO) is used that can react with
62 the azide without the need of the copper catalyst as show in Scheme 1B.

A**B**

63

64 Scheme 1: Click chemistry. A) Copper(I)-catalyzed azide alkyne cycloaddition (CuAAC) mechanism.
65 B) Copper free click chemistry with the strained Dibenzo cyclooctene (DBCO) molecule.

66 In the present work, we describe azide group incorporation by bacteria. This will pave the way for
67 future click chemistry on bacterial surface, providing the necessary engineering tools to modify
68 bacteria as needed with the reported advantage of MTB in terms of control and theranostics.

69 More precisely, we propose to couple MTB and copper-free strain-promoted azide-alkyne
70 cycloaddition to join the advantages of these two separate entities. We thus feed MTB with a
71 modified amino acid, 3-Azido-D-alanine, to enable click chemistry on their surface. The unnatural D-
72 amino acids are incorporated into cell walls; the bioorthogonal (azide) group is cut and integrated
73 into the surface [24]. We investigate bacterial physiological properties to determine how the
74 incorporation of the modified amino acid impacts (cell growth, biomineralization capabilities and
75 hence magnetic properties). We show that amino acid concentration must be controlled to avoid
76 stressing bacteria. Finally, we show successful click chemistry by adding different fluorescent
77 chemical compounds but also 10 μm-beads as cargos. These results will enable the facile chemical
78 engineering of MTB for a given application.

79

80 Materials and methods**81 Materials**

82 DBCO-PEG4-FLUOR 545; DBCO-Cy5; DBCO-dPEG[®]12-carboxyfluorescein were purchased from
 83 **Sigma Aldrich**, while the 3-Azido-D-Alanine Hydrochloride was purchased from **BASECLICK**.
 84 Micromer[®]-DBCO 10 μm was purchased from **micromod Partikeltechnologie GmbH**.

85 **Methods**

86 *Bacterial cultivation and incorporation of the modified amino acid*

87 *Magnetospirillum gryphiswaldense* (MSR-1) cells were grown at 28°C under microoxic conditions) in
 88 Hungate tubes containing 12 mL of modified Flask Standard Medium (FSM) containing 50 μM ferric
 89 iron citrate [25]. Bacterial cultures were started at low cell density, i.e., at OD (600 nm) ≅ 0.009 to
 90 0.02, from pre-cultures in mid-stationary phase and were grown for three days. To effectively
 91 incorporate the amino acid and use the cells for further modification, the modified amino acid was
 92 added directly after bacterial inoculation. Three concentrations of 3-Azido-D-alanine were tested:
 93 0.009 mM, 1.080 mM, and 1.800 mM .

94 *Methods used to assert the effect of azide incorporation on the MTB magnetic properties*

95 A customized magnetic microscope was used [26]. Briefly, the microscope has a triaxial Helmholtz
 96 coil set, a controller (C-SpinCoil-XYZ, Micro Magnetics Inc.) and an Andor Zyla 5.5 high-speed
 97 camera. The 3D-axis Helmholtz coils generate D.C. magnetic fields with a precision of 5% of the
 98 Earth's magnetic field (2.5 μT, manufacturer's specifications). The magnetic field was switched by
 99 applying -3.5 and +3.5 mT for the so-called U-turns, programmed at different time steps. The
 100 magnetic field values were fixed for 2 seconds before the switching value, and the switching was
 101 repeated over a fixed time-lapse, ensuring the observation of at least three U-turns in the field of
 102 view. The trajectories and U-turns were extracted and smoothed by a tracking script written in
 103 python and based on the OpenCV Object Tracking Algorithms with the CSRT tracker [27]. A
 104 convolution-based smoothing approach smooth the data. For the U-turn data, 12 cells from each
 105 condition were analyzed. A 40X objective (Nikon Apochromatic Lambda S 40X WI, N.A. 1.15, water
 106 immersion) was used for both trajectory and U-turn measurements. The mathematical relation to
 107 calculate the magnetic moment from the U-turn is the one used by C.J. Pierce et al. [28] valid for
 108 low field as those used in this study with the drag coefficient modification using the prolate ellipsoid
 109 approximation as done by P. Leao et al. considering length and width of the cels [30] :

$$110 \quad \tau_{Uturn} = \frac{f_r}{M_{cell} \cdot H} = \frac{16 \cdot \pi \cdot \eta \cdot A^3}{3 \cdot M_{cell} \cdot H} .$$

111 Here, τ_{Uturn} corresponds to the time the MTB takes to make a U-turn upon switching the magnetic
 112 field (H); η corresponds to the media viscosity; M_{cell} to the magnetic moment of MSR-1 cell; A is
 113 defined as:

$$114 \quad A = \frac{c^3}{\frac{1}{2} \left[\left(\frac{a+c}{a-c} \right) - \left(\frac{a \cdot c}{b^2} \right) \right]}$$

115 With a and b being the major and minor semi-axis respectively of the prolate ellipsoid and $c =$
 116 $\sqrt{(a^2 - b^2)}$; The MSR-1 U-turn time (τ_{Uturn}) was determined by tracking the U-turn trajectory and
 117 then plotting its first derivate, corresponding to the instantaneous velocity. Note that the change in
 118 sign corresponds to the change in direction. The value of τ_{Uturn} is obtained by taking the time

119 difference between the two steps as described in the section results and discussion, such that M_{cell}
120 can then be calculated from equation (1).

121

122 M_{cell} . All data fitting was done by the intrinsic fitting functions of OriginPro[31] .

123 *Fluorescent tagging efficiency*

124 A semi-quantification analysis was done with a custom python script. The script extracts the
125 fluorescence labeled MTB by converting the images to HSV and extracting the colored (fluorescent)
126 layer to a new image and counting the amount of labeled MTB. The total amount (tagged and non-
127 tagged) of MTB in the images are obtained by converting the image to gray scale and improving the
128 image contrast with an adaptive histogram approach it then calculates the outline with a canny edge
129 detector algorithm and finally the total outlines obtained were then counted. A relative error was
130 then defined as total amount of MTB minus number of tagged MTB divided by the total amount of
131 MTB and converted to percentage by multiplying by 100. This relative error was then considered as
132 a parameter to compare the tagging efficiency.

133 *Click chemistry*

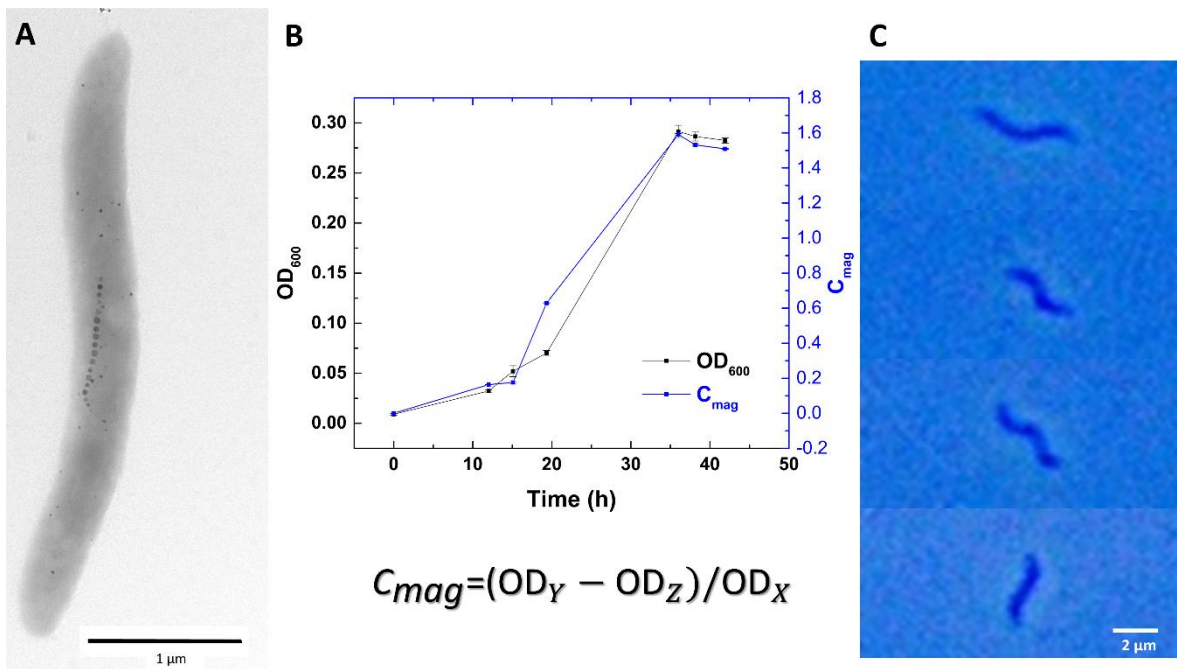
134 DBCO-PEG4-FLUOR 545 a.k.a. DBCO-PEG4-TAMRA (DBCO-TAMRA), was used to show the presence
135 of the azide group after incorporating the modified amino acid. MSR-1 cells grown with the amino
136 acid during 3 days (O.D (600 nm)= 0.2) were re-suspended in PBS (0.1 mM; pH 7.4) and mixed with
137 100 μ M of DBCO-TAMRA for 1 hour while shaking at 700 rpm under dark conditions and at room
138 temperature. The resulting solution was washed 3 times with PBS and centrifuged at 3500 g. For
139 imaging, MSR-1 was left in PBS. For motility measurements, bacteria were re-suspended in FSM.

140 The attachment of the 10 μ m, Micromer®-DBCO beads was done by adding 2 mg/mL of beads to a
141 2 mL solution of MTB (MTB-GFP) in PBS with an OD around 5.0 and left for 1 hour while shaking at
142 700 rpm under dark conditions and at room temperature. The resulting solution was washed 3
143 times with PBS and centrifuged at 3500 g. For imaging, MSR-1 was left in PBS. For motility
144 measurements, they were re-suspended in FSM.

145 **Results and Discussion**

146 We used the strain MSR-1 as a model MTB, as this species is one of the most studied MTB. Figure 1
147 shows a fully grown MTB imaged with TEM with the typical black points indicating magnetosome
148 particles (Figure 1A) as well as a growth curve (Figure 1B) and the semi-quantitative characterization
149 of the magnetic properties by C_{mag} [32]. These C_{mag} values are obtained from the UV-VIS
150 measurements and are directly proportional to cells' magnetization. C_{mag} is a relative value and
151 depends on bacterial concentration and cellular length. To obtain a C_{mag} value from UV-VIS
152 measurements, a spectrophotometer equipped with two sets of Helmholtz coils, which are
153 perpendicular one to another, is used. The Optical Density (OD) at 600 nm of a given bacterial
154 suspension is first measured without applying any magnetic field. This measure is called OD_x. Next,
155 bacterial suspension's OD is measured when a magnetic field is applied in a y direction (OD_y). Finally,
156 bacterial suspension's OD is measured when a magnetic field is applied in a z direction (OD_z) which
157 is perpendicular to the y direction. C_{mag} is calculated using the following formula $\frac{OD_y - OD_z}{OD_x}$.

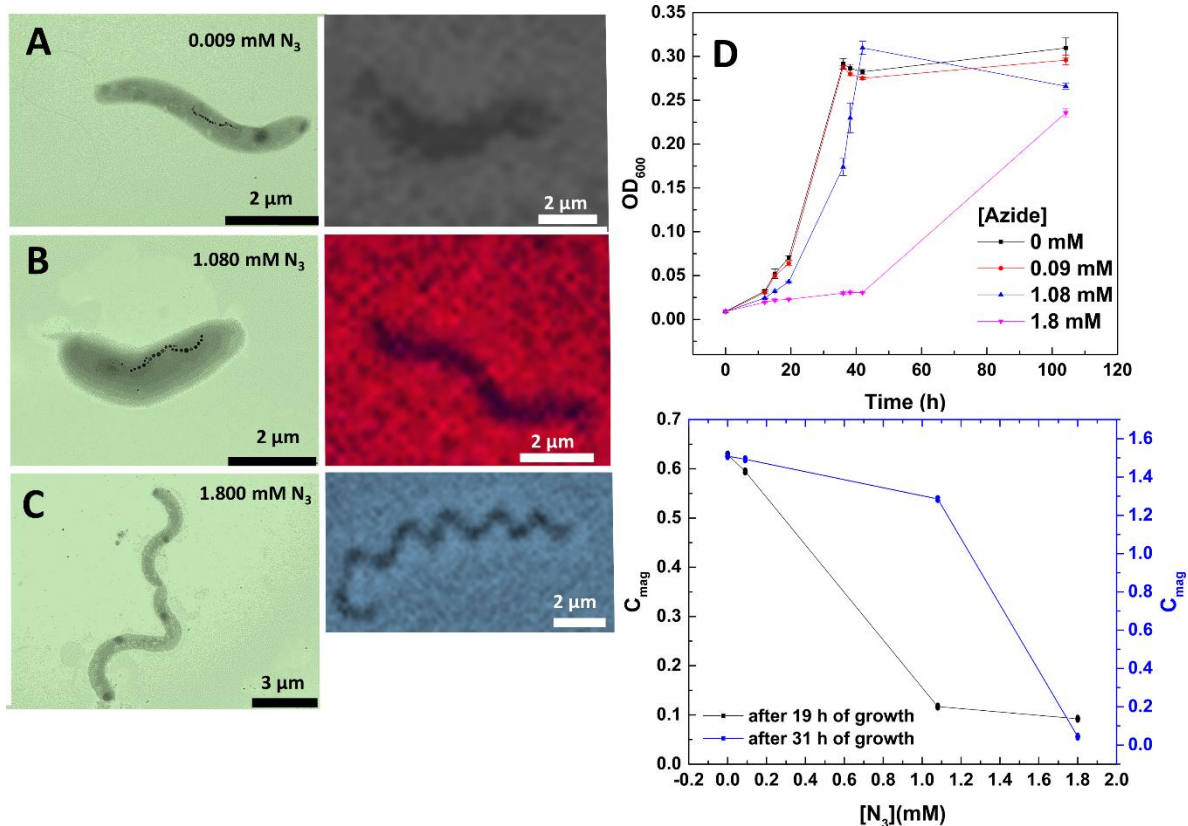
158 Therefore, C_{mag} is an estimation of cellular magnetization (given that the difference $OD_y - OD_z$ will
 159 depend on this parameter) normalized to cell density (OD_x). From the growth curve, the generation
 160 or doubling time is calculated to be around 8 hours for different batches, in accordance with
 161 reported literature data [33,34]. It can be observed that after the complete formation of the
 162 magnetosomes, the C_{mag} stays constant. It is essential to highlight that when one needs to compare
 163 different batches with this method, special care has to be taken to cultures inoculation: the cultures
 164 have to be inoculated at low cellular density (e.g., [OD in the range of 0.009 - 0.02] in this case) to
 165 ensure enough bacterial divisions and therefore incorporation of the modified amino acid 3-azido-
 166 d-alanine hydrochloride in bacterial cell wall. The typical MTB shapes observed from optical
 167 microscopy are presented in Figure 1 and are to be taken as a reference for the following analysis.



168
 169 Figure 1: Basic physiological characterization of MSR-1 cells: A) TEM image of MSR-1 presenting the
 170 classical chain of magnetosomes. B) Combined plot presenting the average growth curve and cell
 171 magnetization (C_{mag}) obtained by UV-vis measurements for triplicate measurements of a cultured
 172 batch of MSR-1. The optical density (OD) was measured at 600 nm (OD_x). $OD_{y,z}$ are the optical
 173 densities in the presence of a y and z magnetic fields. C) Optical image displaying the MTB shapes
 174 observed to be used as reference.

175 We next moved to the first step of our process, corresponding to the incorporation of the azide
 176 groups. MSR-1 cells were grown in 3 different concentrations of the modified amino acid 3-Azido-
 177 D-alanine: 0.009 mM, 1.080 mM, and 1.800 mM. The TEM measurements together with the
 178 corresponding optical image for all three cases are shown in Figures 2A-2C. The TEM hints that the
 179 number of cells with magnetosome chains decreases upon increased modified amino acid and even
 180 more, the long cells do not show any magnetosome production. The growth curve shown at the top
 181 part of Figure 2D, indicates that for both 1.08 and 1.800 mM there is a clear delay in growth. The
 182 respective doubling times were calculated to be 6.69 ± 0.15 hours for 0.009 mM of azide, $7.048 \pm$
 183 0.23 hours for 1.080 mM of azide and 9.08 ± 0.06 hours for 1.800 mM of azide. A non-parametrical
 184 statistical test was done (Kruskal-Wallis test) obtaining a p-value of 0.36 with $\alpha = 0.05$ and

185 therefore the obtained doubling times cannot be considered significantly different. Analysis of C_{mag}
 186 evolution during growth showed that it decreases upon increasing modified amino acid
 187 concentration (Figure 2D). This decrease seems to be stronger during the exponential phase. To
 188 verify if this decrease in C_{mag} could be related to the cells being stressed upon the rise of modified
 189 amino acid concentration, the shape of the cells was analyzed by optical microscopy. Typical forms
 190 found for all 3 cases are presented beside the TEM images respectively, and cell shape changes are
 191 observed. For 1.080 mM many cells stop dividing and are much larger, indicating physiological
 192 stress. It is worth noticing that for all three cases, the cells were still motile, as observed from the
 193 videos in S1, S2, and S3.



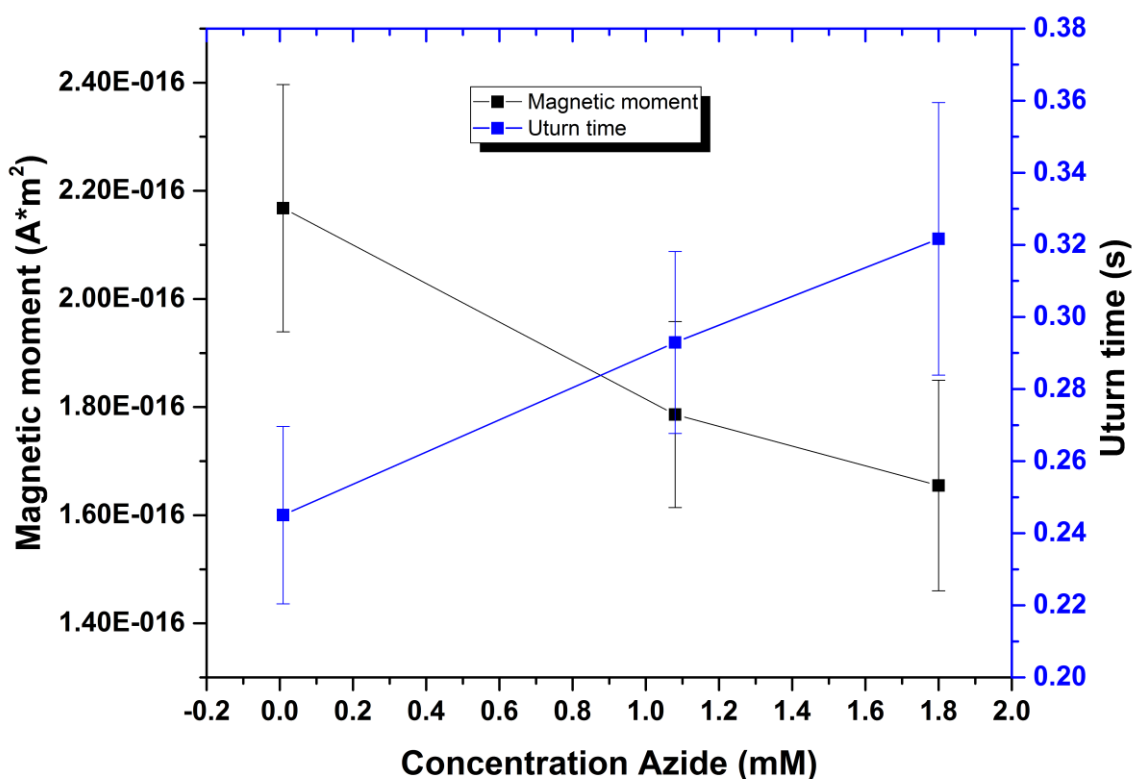
194

195 Figure 2: Characterization of the incorporation of the modified amino acid 3-azido-d-alanine
 196 hydrochloride in MSR-1. A) - E) show the TEM and optical images of MSR-1 grown in the presence
 197 of 0.009 mM, 1.080 mM and 1.800 mM modified amino acid. The plots in D, show the growth curve
 198 (top) for MSR-1 in the presence of the different concentrations of the modified amino acid and C_{mag}
 199 changes (bottom) for all concentrations after 19 and 31 hours of growth. All plots are the average
 200 of triplicate measurements and are plotted with the standard error as error bar.

201

202 We further performed U-turn analyses to assess cell magnetization quantitatively. For this analysis
 203 we exclude all long cells as no magnetosome were observed from the TEM measurements thus no
 204 response with external magnetic fields is expected. We therefore considered the average size found
 205 from 40 TEM images, namely length $L = 3.46 \pm 0.24 \mu$ m and width of $W = 0.54 \pm 0.11 \mu$ m for the U-
 206 turn analysis. Briefly, the trajectory of a motile cell is recorded in the case in which an applied

207 magnetic field switches direction. This switching makes the cells do a U-turn and change direction.
 208 It is possible to calculate cell magnetization from the time it takes the cell to make the U-turn ([35]
 209 and method section). In Figure 3, cell magnetization and U-turn time for all 3 concentrations are
 210 shown, and the apparent decrease of cell magnetization upon modified amino acid concentration
 211 shown in Figure 2D is again observed. The same Figure is shown in Figure S1A together with the
 212 detailed graphs S1B-S1D necessary to calculate the U_{turn} . Figure S1B, shows a typical U-turn
 213 trajectory a single MSR-1 cell performs upon external magnetic field switching. Figure S1C shows
 214 the motion along a single direction (Y), displaying the change in orientation upon the applied
 215 magnetic field; the peak indicates this change in direction. Figure S1D shows the derivative of S1C
 216 and transforms the peak into a small step used to extract the U-turn time more precisely.



217

218 Figure 3: U-turn determination of C_{mag} for MSR-1. U-turn time and cell magnetization for MSR-1
 219 grown in different concentrations of modified amino acid.

220 The presence of the biorthogonal azide group was verified, and for this, the batches grown with no
 221 modified amino acids, i.e., zero (control) and the same three concentrations, i.e. 0.009mM, 1.080
 222 mM, and 1.800 mM of the modified amino acid were mixed with 100 μM of the fluorescent
 223 biomolecule DBCO-TAMRA. The samples were analyzed by confocal imaging, and the images are
 224 shown in Figures 4A-4D. The control shows some clusters of fluorescent biomolecules, but almost
 225 no MTB tagging (Figure 4A). The amount of MSR-1 tagged increases from 0.009 mM to 1.080 mM
 226 but decreases for 1.80 mM. A semi-quantification was done with a costum python script (see

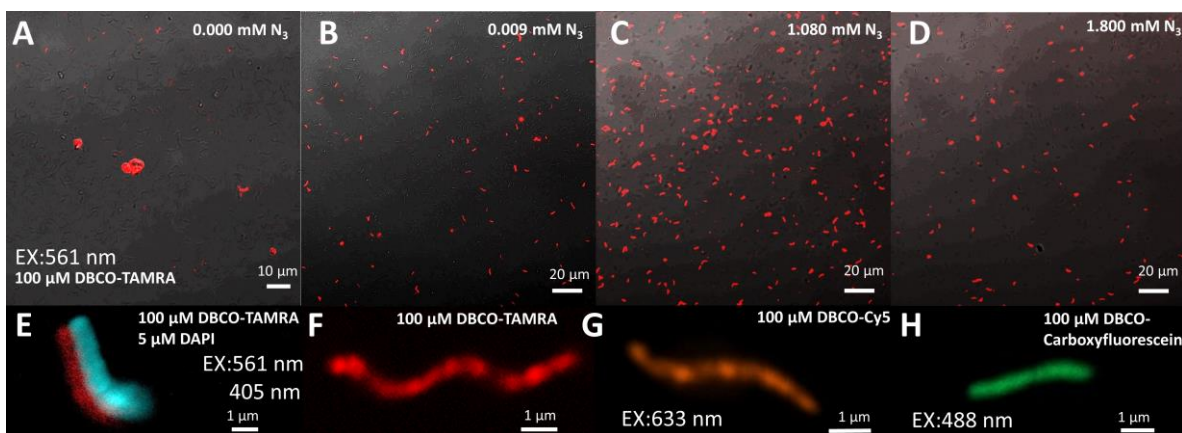
227 method section). The script extracts the total amount of fluorescence labeled MTB and the total
 228 amount of MTB in the images. A relative error is then defined as total amount of MTB minus number
 229 of tagged MTB divided by the total amount of MTB and converted to percentage by multiplying by
 230 100. This relative error is considered as a parameter to compare the tagging efficiency. Please note
 231 that this represents a minimum of tagging efficiency as it includes all MTB present in the images
 232 including those slightly out of focus that could potentially be tagged but not observed. We used 5
 233 images for each azide concentration with over 100 MTB in each image and averaged the relative
 234 error. In table 1, the tagging efficiency for each used azide concentration is indicated.

Azide concentration (mM)	Tagging efficiency (%)
0.009	(5.30± 0.01)
1.080	(24.11± 0.02)
1.800	(17.57± 0.01)

235 Table1: Fluorescent tagging efficiency obtained for each azide concentration used.

236

237 The increased labeled number of MSR-1 cells observed for 1.080 mM suggests that this
 238 concentration is the best tested concentration (among those probed) allowing to have bacterial
 239 azide incorporation while retaining cell magnetization. Therefore, we considered this concentration
 240 and used it to exemplify the versatility of our approach with other tagging methods. Optical images
 241 with different functionalization are shown below: A single cell labeled with DAPI and TAMRA (Figure
 242 4E and movie S3), TAMRA (Figure 4F), Cy5 (Figure 4G) and carboxyfluorescein (Figure 4H).

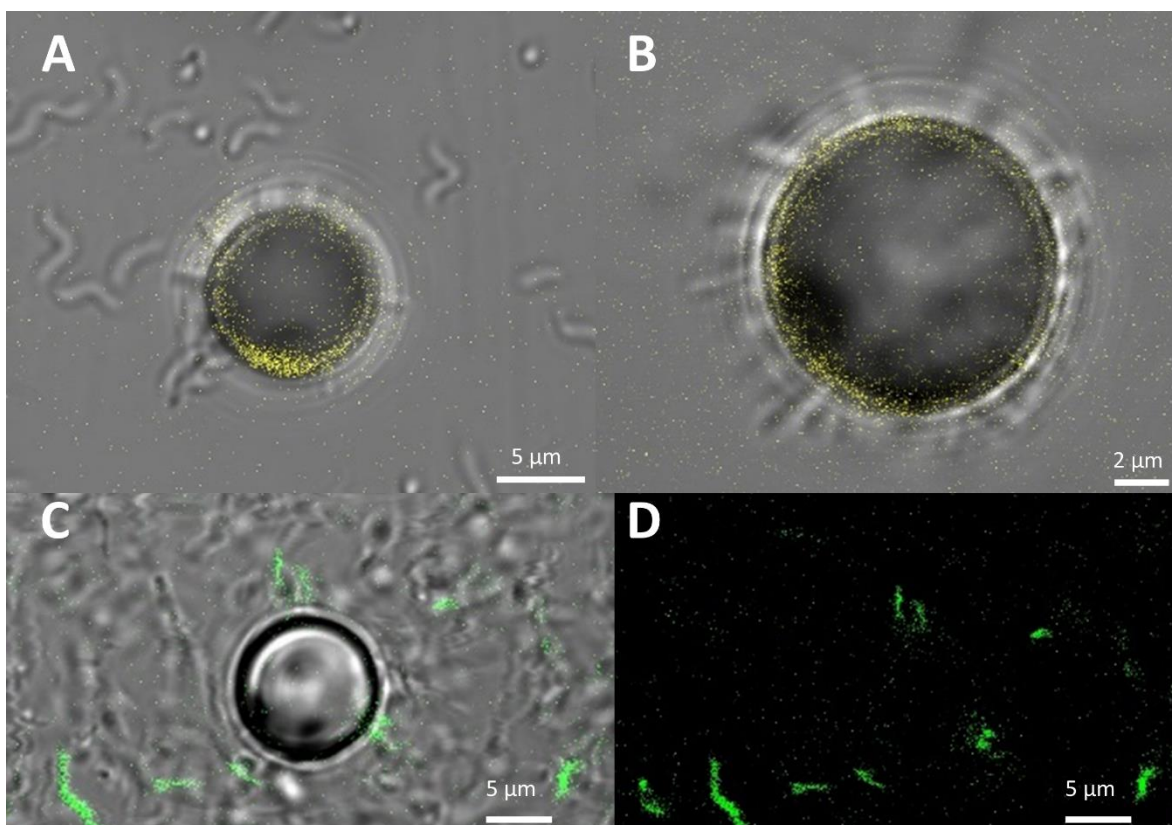


243

244 Figure 4: Fluorescent chemical compounds attachment by copper-free click-chemistry. A-D) 100 μM
 245 of DBCO-TAMRA attachment for MSR-1 grown in 4 different modified amino acid concentrations 0,
 246 0.009, 1.080 and 1.800 mM, respectively. E) DNA staining by DAPI in conjunction with DBCO-TAMRA
 247 with double excitation source 405 nm for DAPI and 561 nm for TAMRA. F-H) A single-cell image for
 248 MSR-1 with DBCO-PEG4-FLUOR 545; DBCO-Cy5 and DBCO-dPEG[®]12-carboxyfluorescein.

249 We analyzed the attachment to relatively large, 10 μm, Micromer[®]-DBCO beads. In figure 5 A) an
 250 optical image is presented showing the effective attachment of N₃ modified MSR-1 to the
 251 Micromer[®]-DBCO beads in different configurations and in B) the N₃ modified MSR-1 are shown to
 252 attach homogenously over the surface. Figure 5 C) shows a combined optical and fluorescence

253 image of the same Micromer®-DBCO beads in the presence of N₃ modified GFP tagged MSR-1, The
254 blurred background are swimming MSR-1. Figure 5 D) shows the GFP fluorescence solely
255 demonstrating the attachment on the beads surface. We checked if the beads with attached
256 bacteria presented any motion or magnetic response and verified that there was none. Movie S5
257 indicates that the beads with the attached MSR-1 do not present any directional motion while movie
258 S6 shows that even with high number of attached MSR-1 there still is no motion. In Movie 7 we used
259 a custom magnetic microscope [26] to apply a 3mT rotating magnetic field and show the lack of
260 response form the bead together with the rotating MSR-1.



261

262 Figure 5: A) and B) show the optical image of the 10µm Micromer® beads with N₃ modified MSR-1
263 attached to the surface. The Micromer® beads show fluorescence at an excitation wavelength of 405
264 nm. Similarly C) shows combined fluorescence (excitation wavelength of 488 nm) and bright field
265 MSR1-GGFP-N₃ attached to the beads and D) only the GFP Fluorescence from MSR-1.

266 Compared with previous works, while assuming that the available surface of the bacteria is the same
267 between each culture batch of a given strain, the immobilization success rate depends on two
268 things: 1) the yield of bonding and 2) the yield of healthy bacteria. The click chemistry is bio-
269 orthogonal and compatible with different biomolecules. Nevertheless, as shown here, the azide
270 group incorporation through metabolic labeling can influence the physiological properties of MTB,
271 specifically magnetosome production. It remains to be seen if a similar effect occurs when bacteria
272 are genetically engineered. For example, overproduction of magnetosomes has been observed
273 previously by genomic amplification [36].

274 For nanoparticles, several studies have shown a higher immobilization yield through click chemistry
275 compared to carbodiimide (EDC/NHS chemistry) [37,38]. Carbodiimide was previously used to
276 attach nanoliposomes on MTB (specifically to the strain *Manetococcus marinus* (MC-1) [9]). The
277 chemical conjugation of liposomes functionalized with reactive groups (–COOH) allows the covalent
278 binding to primary amino groups (–NH₂) intrinsic to bacterial cell membrane proteins. Another
279 approach that bonds cargo to the primary groups of a MTB, more specifically to *Magnetospirillum*
280 *magneticum* (AMB-1), was presented [11]. The authors used an MTB/PEG–biotin complex,
281 corresponding to a covalent bond formation between bacteria and biotin–PEG–NHS polymer. In that
282 case, the main mechanism also includes a nucleophilic attack as in the case of EDC/NHS where NHS
283 esters are formed and attached to the primary amino groups leaving the biotin–PEG–NHS on the
284 bacteria surface and allowing further functionalization by streptavidin–biotin interaction. A third
285 approach introduced immobilized Indocyanine green nanoparticles (INPs) functionalized with
286 maleimide groups on AMB-1 [10]. The INPs were chemically conjugated to activated sulfhydryl on
287 the surface of AMB-1 bacteria by Michael addition reaction. Overall, just looking at an individual
288 cell, the amount of cargo that can be immobilized will then depend on the available functional
289 groups, amino, activated sulfhydryl (activated disulfide surface proteins) or in our case the amount
290 of azide groups and of course the affinity and selectivity of the bonding at hand. Click chemistry has
291 the advantage of selectivity and therefore of yield when compared to the other immobilization
292 techniques. Another advantage of the present work is that we do not need to activate surface
293 groups. Still in all cases, many steps are needed to add or have available chemical groups and attach
294 the cargo. The click chemistry has the advantage, compared to other covalent immobilization
295 techniques used, of the bio-orthogonality and selectivity of the reactions. However, it still presents
296 the disadvantage that to achieve the immobilization of specific groups on the biomolecule and
297 bacterial surface, several reaction steps must be carried out on the respective units to prepare them
298 for the click reaction. The problem of having several reaction steps could be partially overcome by,
299 for example, bacterial genetic engineering. Nevertheless, as demonstrated in this work, care must
300 be taken to find the right conditions to maintain the MTB's general physiological properties. In this
301 work we also show the effective attachment to 10 µm Micromer®-DBCO beads. However, this
302 attachment prevents bacterial motion and magnetic response. We attribute this lack of response to
303 the size of the beads as they precipitate and get fixed to the glass slide adding additional resistance
304 to external forces. We envision that smaller particles should allow directional motion stirred by the
305 MTB as shown in previous work [9].

306 The copper-free strain-promoted azide-alkyne cycloaddition is one of the most widely employed in
307 bio-orthogonal chemistry and, as indicated previously, has been employed by other bacteria to
308 attach cargo [25]. However, in the mentioned work, the physiological properties of the bacterium
309 *E. coli strain Seattle 1946* used were not studied. The binding efficiency was assessed on fixed
310 bacteria, and the motility after modification was assumed to have negligible changes compared to
311 the non-modified bacteria referring to a previous work that indicated a 65 % decrease in speed at
312 the highest bacteria motility of a bacterium with cargo compared to a free bacterium [39]. It was
313 not experimentally tested, nor was the shape of the bacterium analyzed upon different azide
314 concentrations to see if any physiological stress builds up. Although in our work, we also do not
315 observe a clear change in speed, the MTB growth is clearly affected showing difference in shape and
316 even effects upon the biosynthesis of magnetosomes. Fixating bacteria and inhibiting their growth
317 are most likely the reasons, as the physiological stress build-up was not observed previously.

318 **Conclusion**

319 Copper-free click chemistry was successfully proven by mixing DBCO- TAMRA with MTB where a
320 modified amino acid was integrated into the periplasm. The best conditions for the azide
321 incorporation were then used to show its compatibility with other tagging methods such as DAPI
322 and biomolecules by single cell confocal imaging. In addition, we exemplarily have attached 10 μm
323 DBCO-Polystyrene beads. However, due to their dimension, the beads sediment to the glass and get
324 fixed. Despite the fixation, many MTB are capable of attaching to the beads using the protocol we
325 have developed. We observe that to maintain bacterial mobility once attached, smaller particles
326 should be used. These findings open the possibilities of designing MTB functionalized through click
327 chemistry with any biomolecules and are extremely important for future MTB bots designs or MTB
328 applied to bio-sensing, energy or environmental applications where functionalized MTB are
329 envisioned for increased performance [40].

330

331 **Acknowledgement**

332 The project leading to this publication has received funding from the Excellence Initiative of Aix-
333 Marseille Univeristy – A*Midex, a French “Investissements d’Avenir” programme.

334

335 **Supporting information**

336 Movie S1 shows motile MSR-1 grown with 0.005 mM of modified amino azide with a +/-3.5mT
337 magnetic field applied along the vertical, y, direction, switching every 2 sec once the field is
338 activated.

339 Movie S2 shows motile MSR-1 grown with 0.06 mM of modified amino azide with a +/-3.5mT
340 magnetic field applied along the vertical, y, direction, switching every 2 sec once the field is
341 activated.

342 Movie S3 shows motile MSR-1 grown with 0.1 mM of modified amino azide with a +/-3.5mT
343 magnetic field applied along the vertical, y, direction, switching every 2 sec once the field is
344 activated.

345 Movie S4 shows overlaying optical and confocal imaging of the tagged MTB with DAPI and TAMRA.

346 Movie S5 shows 10 μm Micromer[®] beads with N₃ modified MSR-1 attached to the surface with
347 different configuration

348 Movie S6 shows 10 μm Micromer[®] beads with N₃ modified MSR-1 homogenously attached to the
349 surface

350 Movie S7 shows 10 μm Micromer[®] beads with N₃ modified MSR-1 attached to the surface with an
351 3mT rotating magnetic field.

352 Figure S1 presents the graphs showing related to the Uturn measurement and cell magnetization
353 determination.

355 **References**

- 356 [1] F. Soto, R. Chrostowski, *Frontiers of Medical Micro/Nanorobotics: in vivo Applications and*
357 *Commercialization Perspectives Toward Clinical Uses*, *Front. Bioeng. Biotechnol.* 6 (2018) 170.
358 <https://doi.org/10.3389/fbioe.2018.00170>.
- 359 [2] H. Zhou, C.C. Mayorga-Martinez, S. Pané, L. Zhang, M. Pumera, *Magnetically Driven Micro and*
360 *Nanorobots*, *Chem. Rev.* 121 (2021) 4999–5041.
361 <https://doi.org/10.1021/acs.chemrev.0c01234>.
- 362 [3] A. Coene, J. Leliaert, *Magnetic nanoparticles in theranostic applications*, *J. of Appl. Phys.* 131
363 (2022) 160902. <https://doi.org/10.1063/5.0085202>.
- 364 [4] S. Martel, *Swimming microorganisms acting as nanorobots versus artificial nanorobotic*
365 *agents: A perspective view from an historical retrospective on the future of medical*
366 *nanorobotics in the largest known three-dimensional biomicrofluidic networks*,
367 *Biomicrofluidics.* 10 (2016) 021301. <https://doi.org/10.1063/1.4945734>.
- 368 [5] A. Sahari, M.A. Traore, A.M. Stevens, B.E. Scharf, B. Behkam, *Toward Development of an*
369 *Autonomous Network of Bacteria-Based Delivery Systems (BacteriaBots): Spatiotemporally*
370 *High-Throughput Characterization of Bacterial Quorum-Sensing Response*, *Anal. Chem.* 86
371 (2014) 11489–11493. <https://doi.org/10.1021/ac5021003>.
- 372 [6] B. Mostaghaci, O. Yasa, J. Zhuang, M. Sitti, *Bioadhesive Bacterial Microswimmers for Targeted*
373 *Drug Delivery in the Urinary and Gastrointestinal Tracts*, *Adv. Sci.* 4 (2017) 1700058.
374 <https://doi.org/10.1002/advs.201700058>.
- 375 [7] O. Schauer, B. Mostaghaci, R. Colin, D. Hürtgen, D. Kraus, M. Sitti, V. Sourjik, *Motility and*
376 *chemotaxis of bacteria-driven microswimmers fabricated using antigen 43-mediated biotin*
377 *display*, *Sci Rep.* 8 (2018) 9801. <https://doi.org/10.1038/s41598-018-28102-9>.
- 378 [8] J. Bastos-Arrieta, A. Revilla-Guarinos, W.E. Uspal, J. Simmchen, *Bacterial Biohybrid*
379 *Microswimmers*, *Front. Robot. AI.* 5 (2018) 97. <https://doi.org/10.3389/frobt.2018.00097>.
- 380 [9] O. Felfoul, M. Mohammadi, S. Taherkhani, D. de Lanauze, Y. Zhong Xu, D. Loghin, S. Essa, S.
381 Jancik, D. Houle, M. Lafleur, L. Gaboury, M. Tabrizian, N. Kaou, M. Atkin, T. Vuong, G. Batist, N.
382 Beauchemin, D. Radzioch, S. Martel, *Magneto-aerotactic bacteria deliver drug-containing*
383 *nanoliposomes to tumour hypoxic regions*, *Nat. Nanotechnol.* 11 (2016) 941–947.
384 <https://doi.org/10.1038/nnano.2016.137>.
- 385 [10] J. Xing, T. Yin, S. Li, T. Xu, A. Ma, Z. Chen, Y. Luo, Z. Lai, Y. Lv, H. Pan, R. Liang, X. Wu, M. Zheng,
386 L. Cai, *Sequential Magneto-Actuated and Optics-Triggered Biomicrobots for Targeted Cancer*
387 *Therapy*, *Adv. Funct. Mater.* (2020) 2008262. <https://doi.org/10.1002/adfm.202008262>.
- 388 [11] R. Chaturvedi, Y. Kang, Y. Eom, S.R. Torati, C. Kim, *Functionalization of Biotinylated*
389 *Polyethylene Glycol on Live Magnetotactic Bacteria Carriers for Improved Stealth Properties*,
390 *Biology.* 10 (2021) 993. <https://doi.org/10.3390/biology10100993>.
- 391 [12] C.T. Lefevre, D.A. Bazylinski, *Ecology, Diversity, and Evolution of Magnetotactic Bacteria*,
392 *Microbiol. Mol. Bio. Rev.* 77 (2013) 497–526. <https://doi.org/10.1128/MMBR.00021-13>.
- 393 [13] C.T. Lefèvre, M. Bennet, L. Landau, P. Vach, D. Pignol, D.A. Bazylinski, R.B. Frankel, S. Klumpp,
394 D. Faivre, *Diversity of Magneto-Aerotactic Behaviors and Oxygen Sensing Mechanisms in*
395 *Cultured Magnetotactic Bacteria*, *Biophys. J.* 107 (2014) 527–538.
396 <https://doi.org/10.1016/j.bpj.2014.05.043>.
- 397 [14] D. Kuzajewska, A. Wszolek, W. Żwieręto, L. Kirczuk, A. Maruszevska, *Magnetotactic Bacteria*
398 *and Magnetosomes as Smart Drug Delivery Systems: A New Weapon on the Battlefield with*
399 *Cancer?*, *Biology.* 9 (2020) 102. <https://doi.org/10.3390/biology9050102>.

- 400 [15] S. Mériaux, M. Boucher, B. Marty, Y. Lalatonne, S. Prévéral, L. Motte, C.T. Lefèvre, F. Geffroy,
401 F. Lethimonnier, M. Péan, D. Garcia, G. Adryanczyk-Perrier, D. Pignol, N. Ginet,
402 Magnetosomes, Biogenic Magnetic Nanomaterials for Brain Molecular Imaging with 17.2 T
403 MRI Scanner, *Adv. Healthcare Mater.* 4 (2015) 1076–1083.
404 <https://doi.org/10.1002/adhm.201400756>.
- 405 [16] A. Kraupner, D. Eberbeck, D. Heinke, R. Uebe, D. Schüler, A. Briel, Bacterial magnetosomes –
406 nature’s powerful contribution to MPI tracer research, *Nanoscale.* 9 (2017) 5788–5793.
407 <https://doi.org/10.1039/C7NR01530E>.
- 408 [17] A.V. Makela, M.A. Schott, C.S. Madsen, E.M. Greeson, C.H. Contag, Magnetic Particle Imaging
409 of Magnetotactic Bacteria as Living Contrast Agents Is Improved by Altering Magnetosome
410 Arrangement, *Nano Lett.* 22 (2022) 4630–4639.
411 <https://doi.org/10.1021/acs.nanolett.1c05042>.
- 412 [18] N.K. Devaraj, M.G. Finn, Introduction: Click Chemistry, *Chem. Rev.* 121 (2021) 6697–6698.
413 <https://doi.org/10.1021/acs.chemrev.1c00469>.
- 414 [19] B.T. Worrell, J.A. Malik, V.V. Fokin, Direct Evidence of a Dinuclear Copper Intermediate in
415 Cu(I)-Catalyzed Azide-Alkyne Cycloadditions, *Science.* 340 (2013) 457–460.
416 <https://doi.org/10.1126/science.1229506>.
- 417 [20] L. Li, Z. Zhang, Development and Applications of the Copper-Catalyzed Azide-Alkyne
418 Cycloaddition (CuAAC) as a Bioorthogonal Reaction, *Molecules.* 21 (2016) 1393.
419 <https://doi.org/10.3390/molecules21101393>.
- 420 [21] J.W. Chin, S.W. Santoro, A.B. Martin, D.S. King, L. Wang, P.G. Schultz, Addition of *p*-Azido- L -
421 phenylalanine to the Genetic Code of *Escherichia coli*, *J. Am. Chem. Soc.* 124 (2002) 9026–
422 9027. <https://doi.org/10.1021/ja027007w>.
- 423 [22] S. Reddington, P. Watson, P. Rizkallah, E. Tippmann, D.D. Jones, Genetically encoding phenyl
424 azide chemistry: new uses and ideas for classical biochemistry, *Biochem. Soc. Trans.* 41 (2013)
425 1177–1182. <https://doi.org/10.1042/BST20130094>.
- 426 [23] P.V. Chang, J.A. Prescher, E.M. Sletten, J.M. Baskin, I.A. Miller, N.J. Agard, A. Lo, C.R. Bertozzi,
427 Copper-free click chemistry in living animals, *Proc. Natl. Acad. Sci. U.S.A.* 107 (2010) 1821–
428 1826. <https://doi.org/10.1073/pnas.0911116107>.
- 429 [24] V.M. Moreno, E. Álvarez, I. Izquierdo-Barba, A. Baeza, J. Serrano-López, M. Vallet-Regí,
430 Bacteria as Nanoparticles Carrier for Enhancing Penetration in a Tumoral Matrix Model, *Adv.*
431 *Mater. Interfaces.* 7 (2020) 1901942. <https://doi.org/10.1002/admi.201901942>.
- 432 [25] U. Heyen, D. Schüler, Growth and magnetosome formation by microaerophilic
433 Magnetospirillum strains in an oxygen-controlled fermentor, *Appl Microbiol Biotechnol.* 61
434 (2003) 536–544. <https://doi.org/10.1007/s00253-002-1219-x>.
- 435 [26] M. Bennet, A. McCarthy, D. Fix, M.R. Edwards, F. Repp, P. Vach, J.W.C. Dunlop, M. Sitti, G.S.
436 Buller, S. Klumpp, D. Faivre, Influence of Magnetic Fields on Magneto-Aerotaxis, *PLoS ONE.* 9
437 (2014) e101150. <https://doi.org/10.1371/journal.pone.0101150>.
- 438 [27] N. Dardagan, A. Brdanin, D. Dzigal, A. Akagic, Multiple Object Trackers in OpenCV: A
439 Benchmark, in: 2021 IEEE 30th International Symposium on Industrial Electronics (ISIE), IEEE,
440 Kyoto, Japan, 2021: pp. 1–6. <https://doi.org/10.1109/ISIE45552.2021.9576367>.
- 441 [28] C.J. Pierce, E. Mumper, E.E. Brown, J.T. Brangham, B.H. Lower, S.K. Lower, F.Y. Yang, R.
442 Sooryakumar, Tuning bacterial hydrodynamics with magnetic fields, *Phys. Rev. E.* 95 (2017)
443 062612. <https://doi.org/10.1103/PhysRevE.95.062612>.
- 444 [29] M.P. Pichel, T.A.G. Hageman, I.S.M. Khalil, A. Manz, L. Abelmann, Magnetic response of
445 Magnetospirillum gryphiswaldense observed inside a microfluidic channel, *Journal of*
446 *Magnetism and Magnetic Materials.* 460 (2018) 340–353.
447 <https://doi.org/10.1016/j.jmmm.2018.04.004>.

448 [30] P. Leão, L. Le Nagard, H. Yuan, J. Cypriano, I. Da Silva-Neto, D.A. Bazylnski, D. Acosta-Avalos,
449 H.L. Barros, A.P. Hitchcock, U. Lins, F. Abreu, Magnetosome magnetite biomineralization in a
450 flagellated protist: evidence for an early evolutionary origin for magnetoreception in
451 eukaryotes, *Environ Microbiol.* 22 (2020) 1495–1506. [https://doi.org/10.1111/1462-](https://doi.org/10.1111/1462-2920.14711)
452 2920.14711.

453 [31] OriginPro, Originlab Corporation, Northhampton, MA, USA, (2016).

454 [32] D. Schüler, R. Uhl, E. Bäuerlein, A simple light scattering method to assay magnetism in
455 *Magnetospirillum gryphiswaldense*, *FEMS Microbiology Letters.* 132 (1995) 139–145.
456 <https://doi.org/10.1111/j.1574-6968.1995.tb07823.x>.

457 [33] C.N. Riese, R. Uebe, S. Rosenfeldt, A.S. Schenk, V. Jérôme, R. Freitag, D. Schüler, An automated
458 oxystat fermentation regime for microoxic cultivation of *Magnetospirillum gryphiswaldense*,
459 *Microb Cell Fact.* 19 (2020) 206. <https://doi.org/10.1186/s12934-020-01469-z>.

460 [34] I. Kolinko, C. Jogler, E. Katzmann, D. Schüler, Frequent Mutations within the Genomic
461 Magnetosome Island of *Magnetospirillum gryphiswaldense* Are Mediated by RecA, *J Bacteriol.*
462 193 (2011) 5328–5334. <https://doi.org/10.1128/JB.05491-11>.

463 [35] D.M.S. Esquivel, H.G. de P.L. de Barros, Motion of magnetotactic microorganisms, Brazil,
464 1985. http://inis.iaea.org/search/search.aspx?orig_q=RN:17012441.

465 [36] A. Lohße, I. Kolinko, O. Raschdorf, R. Uebe, S. Borg, A. Brachmann, J.M. Pletzko, R. Müller, Y.
466 Zhang, D. Schüler, Overproduction of Magnetosomes by Genomic Amplification of
467 Biosynthesis-Related Gene Clusters in a Magnetotactic Bacterium, *Appl. Environ. Microbiol.* 82
468 (2016) 3032–3041. <https://doi.org/10.1128/AEM.03860-15>.

469 [37] D.L.J. Thorek, R. Elias, A. Tsourkas, Comparative Analysis of Nanoparticle-Antibody
470 Conjugations: Carbodiimide Versus Click Chemistry, *Mol Imaging.* 8 (2009) 7290.2009.00021.
471 <https://doi.org/10.2310/7290.2009.00021>.

472 [38] J. Bolley, E. Guenin, N. Lievre, M. Lecouvey, M. Soussan, Y. Lalatonne, L. Motte, Carbodiimide
473 versus Click Chemistry for Nanoparticle Surface Functionalization: A Comparative Study for the
474 Elaboration of Multimodal Superparamagnetic Nanoparticles Targeting $\alpha_v \beta_3$ Integrins,
475 *Langmuir.* 29 (2013) 14639–14647. <https://doi.org/10.1021/la403245h>.

476 [39] M.A. Traore, C.M. Damico, B. Behkam, Biomanufacturing and self-propulsion dynamics of
477 nanoscale bacteria-enabled autonomous delivery systems, *Appl. Phys. Lett.* 105 (2014)
478 173702. <https://doi.org/10.1063/1.4900641>.

479 [40] N. Ebrahimi, C. Bi, D.J. Cappelleri, G. Ciuti, A.T. Conn, D. Faivre, N. Habibi, A. Hošovský, V.
480 Iacovacci, I.S.M. Khalil, V. Magdanz, S. Misra, C. Pawashe, R. Rashidifar, P.E.D. Soto-Rodriguez,
481 Z. Fekete, A. Jafari, Magnetic Actuation Methods in Bio/Soft Robotics, *Adv. Funct. Mater.* 31
482 (2021) 2005137. <https://doi.org/10.1002/adfm.202005137>.

483