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1 Bacteriophytochrome from *Magnetospirillum magneticum* affects phototactic
2 behaviour in response to light

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26 **Abstract**

27 Phytochromes are a class of photoreceptors found in plants and in some fungi,
28 cyanobacteria, photoautotrophic, and heterotrophic bacteria. Although phytochromes
29 have been structurally characterized in some bacteria, its biological and ecological
30 roles in magnetotactic bacteria remain unexplored. Here, we describe the biochemical
31 characterization of recombinant bacteriophytochrome (BphP) from magnetotactic
32 bacteria *Magnetospirillum magneticum* AMB-1 (*MmBphP*). The recombinant
33 *MmBphP* displays all the characteristic features, including the property of binding to
34 biliverdin (BV), of a genuine phytochrome. Site-directed mutagenesis identified that
35 cysteine-14 is important for chromophore covalent binding and photoreversibility.
36 Arginine-240 and histidine-246 play key roles in binding to BV. The N-terminal
37 photosensory core domain of *MmBphP* lacking the C-terminus found in other
38 phytochromes is sufficient to exhibit the characteristic red/far-red-light-induced fast
39 photoreversibility of phytochromes. Moreover, our results showed *MmBphP* is
40 involved in the phototactic response, suggesting its conservative role as
41 stress-protective. This finding provided us a better understanding of the physiological
42 function of this group of photoreceptors and photoresponse of magnetotactic bacteria.

43

44 **Key words:** Bacteriophytochrome, magnetotactic bacteria, biliverdin, phototactic
45 behaviour.

46

47 **Introduction**

48 Light is an important environmental signal and provides sensory information for
49 adaptation and energy for growth and metabolism. Protein-based photoreceptors have
50 evolved to sense light quality changes in the environment and trigger critical lifestyle
51 adaptations (Gomelsky and Hoff 2011; Gourinchas et al. 2019; Maresca et al. 2019).
52 Phytochromes, which sense ambient light quality and quantity by responding to red
53 and far-red light, are photoreceptor proteins used by bacteria, fungi, algae, and plants
54 (Duanmu et al. 2014; Nagano 2016; Rodriguez-Romero et al. 2010). Phylogenetic
55 analysis indicates that the phytochrome superfamily consists of five distinct clades,
56 namely, plant phytochromes, cyanobacterial phytochromes, bacteriophytochromes
57 (BphPs), fungal phytochromes, and a cluster of Phy-like sequences (Blumenstein et al.
58 2005; Hughes et al. 1997; Jaubert et al. 2008; Jiang et al. 1999; Karniol et al. 2005).

59

60 Most of bacteriophytochromes (BphPs) use a linear tetrapyrrole chromophore
61 (biliverdin, BV) and sense red and far-red light via a reversible shift from a
62 red-absorbing form (Pr) to a far-red-absorbing form (Pfr) (Rottwinkel et al. 2010;
63 Vuillet et al. 2007). BphPs are composed of an N-terminal photosensory core domain
64 (PCD) and a C-terminal output-transducing domain (OTD). The PCD comprises the
65 period/aryl hydrocarbon receptor nuclear translocator/single-minded (PAS) that
66 covalently binds to the open-chain BV chromophore via a thioether linkage with a
67 conserved cysteine (Cys) residue, a cGMP phosphodiesterase/adenylyl cyclase/FhlA
68 (GAF) domain stabilizing chromophore via polar and hydrophobic interactions, and a
69 phytochrome-specific (PHY) domain stabilizing the photoactivated Pfr state (Burgie
70 et al. 2016; Gourinchas et al. 2019; Lamparter et al. 2004). Upon light illumination,
71 the PHY-tongue element undergoes partial refolding into an α -helix after
72 chromophore photoisomerization (Burgie et al. 2016; Gourinchas et al. 2019). These
73 structural rearrangements around a chromophore binding site transduce the molecular
74 signal to a downstream effector module via the PHY domain and its C-terminal OTD
75 (Bjorling et al. 2016; Gourinchas et al. 2019).

76

77 The C-terminal OTD of most BphPs possesses a two-component histidine kinase
78 motif that transfers phosphate to a response regulator (RR) (Gourinchas et al. 2019;
79 Jaubert et al. 2007; Karniol and Vierstra 2004; Rottwinkel et al. 2010). Other output
80 modules, such as the PAS, GGDEF and EAL domains, which are involved in bacterial
81 second messenger metabolism, have also been described (Rockwell et al. 2006). BphP
82 in *Bradyrhizobium* ORS278 (*BrBphP3*) is formed from only N-terminal PCD without
83 the C-terminal OTD. *BrBphP3*'s feature lies in its binding to phycocycano-bilin rather
84 than BV, and it has been acquired by lateral gene transfer from a cyanobacterial
85 species (Jaubert et al. 2007). The functional roles of this BphPs that lack the
86 C-terminal OTD have yet to be found. The analysis of magnetotactic bacteria (MTB)
87 genomes reveals some BphP photoreceptors (Brutesco et al. 2017; Wang et al. 2019),
88 but only one BphP protein without a recognizable C-terminal OTD is found in the
89 AMB-1 model strain of *Magnetospirillum*. MTB can synthesize intracellular magnetic
90 crystals (magnetosomes) that allow cells to swim along geomagnetic field lines
91 (Faivre and Schüler 2008; Frankel and Blakemore 1989;). This magnetotaxis of MTB
92 can be dated back in the archaean era, suggesting that MTB coevolved with the
93 geomagnetic field over geological time (Lin et al. 2017). Interestingly, these bacteria
94 are rich in light perception systems. Frankel et al. (1997) found that the
95 *Magnetococcus* strain MC-1 has a negative photoresponse behaviour to
96 short-wavelength light. Multicellular magnetotactic prokaryotes present a
97 photophobic response and a photokinesis behaviour (Chen et al. 2015; de Melo and
98 Acosta-Avalos 2017; Qian et al. 2019; Shapiro et al. 2011; Zhou et al. 2012).
99 Illumination helps cell to eliminate intracellular reactive oxygen species (ROS) and
100 triggers swimming toward a light source in *Magnetospirillum magneticum* AMB-1
101 (Chen et al. 2011; Li et al. 2017). Although the biological roles of BphPs have been
102 addressed in some literatures (Bai et al. 2016; Bonomi et al. 2016; Moyano et al. 2020;
103 Mukherjee et al. 2019; Ricci et al. 2015; Wu et al. 2013), the mechanism and
104 biological function by which MTB respond to illumination remains unknown.
105 Accordingly, we determined whether the BphP of MTB is involved in the regulation
106 of photoresponse of this bacterium. By combining genetics, biochemical and

107 biophysical approaches, we found that BphP in AMB-1 (*MmBphP*) possesses
108 red/far-red-light-induced photochemical properties and participates in phototactic
109 behaviour.

110

111 **Materials and methods**

112 **Strain and culture conditions**

113 *M. magneticum* AMB-1 strains (ATCC 700264) were grown under microaerobic
114 condition on *Magnetospirillum* growth medium at 30°C (Chen et al. 2018; Matsunaga
115 et al. 1991; Yang et al. 2001). *Escherichia coli* strains BL21 (DE3) (Tiangen, China)
116 and WM3064 (Philippe and Wu 2010: 309-22) were grown in a LB medium at 37°C.
117 When appropriate, AMB-1 was grown with 15 µg/mL apramycin or kanamycin. *E.*
118 *coli* cultures were supplemented with apramycin (50 µg/mL) or kanamycin (50
119 µg/mL).

120

121 **Identification of *MmBphP* sequence**

122 BphP was identified in the AMB-1 genomic database BLAST (protein accession
123 number: WP_043744962.1). Phytochrome homologs were found with PDB 6FHT as a
124 template (Jaubert et al. 2007). The domain was analyzed using SMART
125 (<http://smart.embl-heidelberg.de>). For structural prediction, the protein sequence was
126 examined using the Phyre2 program (<http://www.sbg.bio.ic.ac.uk/phyre2/html/>)
127 (Kelley et al. 2015). Images were generated using AutoDock and PyMOL.

128

129 **Cloning, expression, and purification of *MmBphP***

130 The sequence of *MmBphP* was PCR-amplified by using primers designed to introduce
131 *Bam*HI and *Xho*I sites before ATG and designated stop codons, respectively. The
132 fragment was then cloned into the pET28a plasmid with N-terminal
133 His6-SUMO-tagged fusion proteins. C14A, D197A, C211A, R240A, H246A, Y249A,
134 S260A, C275A, H276A, C291A, C14&R240A, C14&H246A, and
135 C14&R240A&H246A mutants were cloned using a NEBuilder HiFi DNA assembly
136 reaction kit in accordance with the manufacturer's instructions. These proteins were

137 expressed in *E. coli* BL21 (DE3) cells and purified. After nickel chelate affinity
138 chromatography, the proteins were further treated with SUMO protease and purified
139 through size exclusion chromatography on a Superdex 200 column in Tris buffer (10
140 mM Tris/Cl, 200 mM NaCl, pH8.0). For BphPs assembled in vitro, apoproteins were
141 incubated in darkness in a 10-fold molar excess of BV (Sigma, USA) at room
142 temperature for 1 h. Subsequently, proteins were separated from the free BV by using
143 a DP-10 desalting column (GE Healthcare, USA). The covalent attachment of BV to
144 *MmBphP* and mutant proteins was monitored through the zinc-induced fluorescence
145 of the chromoproteins subjected to SDS-PAGE. Blue native PAGE was performed on
146 a natural PAGE gel consisting of 3.5% stack and 10% gradient separation gel. The
147 cathode buffer and the anode were cooled at 4°C before it was used. Electrophoresis
148 was started at 80 V for 30 min, increased to 120 V for 1 h, and adjusted to 200 V for 1
149 h (Li et al. 2019). Covalent attachment of bilins to *MmBphP* and mutants were
150 monitored by zinc-induced fluorescence of the chromoproteins separated by
151 SDS-PAGE. When purified *MmBphP* was resolved on SDS-PAGE gels and incubated
152 with 1.5 M zinc acetate, it was visible under ultraviolet light as an orange fluorescent
153 band (Berkelman and Lagarias. 1986). The fluorescent biliprotein-containing bands
154 were visualized by transilluminating the gel on an AlphaImager HP UV light (365 nm)
155 box.

156

157 **Genetic manipulation of AMB-1**

158 The in-frame deletion of *bphP* was created with a CRISPR-Cas9 system by using the
159 pCRISPomyces-2 plasmid as previously described (Cobb et al. 2015). A sgRNA
160 cassette was designed and inserted into the pCRISPomyces-2 by *BbsI* site. PCR was
161 performed using PrimerSTAR HS DNA polymerase (Takara, Japan) to amplify 1.0 kb
162 upstream of the start codon in the coding region, and the 1.0 kb sequence downstream
163 of the stop codon and to delete *bphP*. These fragments were then incorporated into an
164 *XbaI* -digested pCRISP-sgRNA vector by using the NEBuilder HiFi DNA assembly
165 reaction system (NEB, USA).

166

167 The pCRISP-sgRNA*bphP*-mutant plasmid was constructed as follows.
168 *bphP*^{C14A&R240A&H246A}, 1.0 kb upstream sequence, and 1.0 kb downstream sequence
169 were PCR amplified using PrimerSTAR HS DNA polymerase (Takara, Japan). The
170 resulting 3.5 kb fragment was incorporated into the pET28a plasmid. The sgRNA
171 sequence in the *bphP*^{C14A&R240A&H246A} fragment was subjected to nonsense mutation to
172 prevent the sgRNA of the CRISPR plasmid from editing the inserted
173 *bphP*^{C14A&R240A&H246A}. The resulting plasmid was subjected to site-directed
174 mutagenesis in accordance with the NEBuilder HiFi DNA assembly reaction protocol
175 to introduce the sgRNA sequence of nonsense mutation (sgRNA sequence:
176 GGACTCGCAGCGTTGTACTA to GGACTCGgcGCGTTGTACTA) into the *bphP*
177 sequence. The site-directed 3.5 kb fragment was inserted into pCRISP-sgRNA*bphP*
178 plasmid with the NEBuilder HiFi DNA assembly reaction system.

179

180 The complementing vector ComBphP was constructed through PCR amplification
181 with PrimerSTAR HS DNA polymerase (Takara, Japan). The ComBphP coding
182 sequence was amplified and ligated into *EcoRI*- and *BamHI*-digested pAK0994, a
183 pBBR1MCS-based plasmid carrying a kanamycin resistance gene and expressing the
184 *amb0994-gfp* fusion from a *tac* promoter. This method was performed as described
185 previously (Chen et al. 2018).

186

187 All the plasmids were mobilized into AMB-1 through conjugation with the *E. coli*
188 strain WM3064, and CRISPR-based double-crossover events for deletions or allelic
189 exchange were employed using a selection and screening strategy as described
190 previously (Chen et al. 2018). All the deletions were verified through PCR and
191 sequencing. All the plasmids and primers used are listed in Tables S1 and S2.

192

193 **Spectroscopic characterization**

194 The absorbance spectra of the purified *MmBphP* wild-type (WT) and mutant proteins
195 were obtained at room temperature from 850 nm to 250 nm by using a UV-visible
196 spectrophotometer (Unico UV-2800, USA). The assay was recorded either in the dark

197 or after 15 min of illumination with 625 nm, 680 nm, 710 nm, or 750 nm LED light
198 with a half-width of 30 nm at 10 $\mu\text{mol photons/m}^2/\text{s}$ (Hasunopto, China). Fig. S1
199 shows the spectra of these light sources. Dark recovery kinetic was followed at 708
200 nm and 753 nm after 15 min under 710 nm light. For dark conversion, the half-life
201 time was estimated as the time in which 50% of the Pfr-to-Pr conversion took place
202 considering the saturation value as 100%. The two phase association exponential fit
203 was used to derive the half-life parameter. Room-temperature fluorescence emission
204 and excitation spectra were recorded using a fluorescence spectrophotometer (Hitachi
205 F-4500, Japan). Fluorescence spectra were obtained at 680 nm excitation or 750 nm
206 emission.

207

208 **Phototactic behaviour analyses of AMB-1**

209 Phototactic behaviour was quantitatively analyzed as previously described (Li et al.
210 2017). Using a modified mini MTB collection vessel, the cultures with or without
211 H_2O_2 induction were pooled into the reservoir that is connected to the collecting tube
212 at one side. And then the device was placed in a cassette, where the collection tube
213 was exposed parallelly to the light beam. Before conducting the phototactic
214 experiment, a microaerobic band formed after growing in the dark for 30 min. The
215 upper interface of the microaerobic zone is parallel to the collection tube. After 30
216 min of 710 nm or 750 nm light irradiation, the OD in the collecting tube and the
217 reservoir was measured ($\text{OD}_{\text{collecting tube}}$ and $\text{OD}_{\text{reservoir}}$, respectively). The relative
218 numbers of phototactic cells were analyzed by the ratio of $(\text{OD}_{\text{collecting tube}} -$
219 $\text{OD}_{\text{reservoir}})/\text{OD}_{\text{reservoir}}$. Control experiments were performed in the dark. In this study,
220 we analyzed the effect of H_2O_2 on the photoresponse between WT and mutants. To
221 prevent the difference in photoresponse caused by H_2O_2 on the state of bacterial
222 movement for WT and mutants, we observed that at 40 μM concentrations H_2O_2
223 affected nonsignificantly the velocities of these strains (Fig. S2, Supporting
224 Information). Thus, we studied the difference in phototactic behaviour between the
225 WT and mutants under the conditions of without or with 40 μM H_2O_2 . The statistical
226 analyse of phototactic behaviour was conducted through Mann–Whitney *U*-test using

227 SPSS 22.0 (SPSS, IBM).

228

229 **Results**

230 **Identification of BphP**

231 According to the composition of the C-terminal OTD, the domain organization of
232 BphPs can be classified into nine types (Fig. 1A). Three of them (e, f and h) are found
233 in freshwater AMB-1, MSR-1, MS-1, XM-1, MV-1 or marine QH-2 MTB.
234 Interestingly, BphP proteins in some of these MTB are found to be type (h) BphP
235 which lack the C-terminal OTD (Fig. 1B). The representative *MmBphP* in the model
236 strain AMB-1 of *Magnetospirillum* sp. consists of a 496 aa PAS–GAF–PHY domain
237 (Fig. 1B). Fig. 1C shows the arrangement of the genes located downstream or
238 upstream of *MmBphP* in AMB-1, revealing a heme oxygenase gene close to *MmBphP*.
239 The heme oxygenase opens the heme ring to form the linear tetrapyrrole BV which is
240 the first intermediate in phytochrome chromophore synthesis (Muramoto et al. 1999),
241 suggesting *MmBphP* may be combined with BV in its natural state to perform its
242 functions. Accordingly, by taking AMB-1 as the research object, we studied the
243 possible involvement of BphP with no recognizable OTDs in the photoresponse
244 regulation in MTB. To further enrich the function assessment of BphPs lacking OTDs,
245 we purified the *MmBphP* protein and constructed the *MmBphP* deletion mutant in
246 AMB-1 strain.

247

248 **Characteristics and photochemical properties of recombinant *MmBphP* protein**

249 Recombinant *MmBphP* was detected through SDS–PAGE electrophoresis, and a
250 single band migrating at ~54 kDa was observed (Fig. S3 B, Supporting Information).
251 According to the peak position of the elution profiles of a Superdex 200
252 size-exclusion column, *MmBphP* displayed a molecular weight of approximately 108
253 kDa, suggesting the dimer property of these proteins in the solution (Fig. S3 A,
254 Supporting Information). Blue native electrophoresis results revealed that the protein
255 could form stable homodimers (Fig. S3 B, Supporting Information).

256

257 In Fig. 2, the purified *MmBphP* binding to the BV chromophore appears blue green
258 (insert in Fig. 2A). The absorption spectrum of the dark-adapted *MmBphP* sample is
259 typical of the Pr ground state with a main band centered at 708 nm (Fig. S4,
260 Supporting Information). Illumination of the Pr state with 625 nm, 680 nm or 710 nm
261 light induces only a small absorption increase in the near infra-red region around 753
262 nm (Fig. 2A; Fig. S4, Supporting Information), which is similar to the ‘Meta’ state of
263 Agp1 BphP (Borucki et al. 2005), an intermediate during the dark transition from Pfr
264 to Pr. The photoconversion properties of WT *MmBphP* obtained by these three light
265 treatments are similar. The calculated difference spectrum of *MmBphP* further shows
266 the characteristic phytochrome signature with maxima of 706 and 753 nm for the two
267 different forms. Irradiation with far-red light (750 nm) results in a spectrum similar to
268 that of the dark-adapted protein (Fig. S4, Supporting Information). The reason of the
269 spectrum change is the rotation of 4th ring D of BV caused by photoisomerization
270 after absorption of red light (Gourinchas et al. 2019). In this study, the 710 nm light
271 was used as red light illumination, and a 750 nm light was used as far-red illumination
272 source.

273

274 To assess whether *MmBphP* has similar structural characteristics as other BphPs, we
275 constructed a predictive structural model of *MmBphP* using a homologue protein
276 (PBD code 6FHT) as template through Phyre2 search. As shown in Fig. 2B. Like
277 other BphPs (Yang, et al. 2007; Gourinchas et al. 2019), *MmBphP* is composed of
278 PAS, GAF, and PHY domains. The enlarged dotted line on the right of the predictive
279 structure, by using AutoDock and PyMOL, shows that *MmBphP* is associated with a
280 bounded BV.

281

282 The occurrence of a fast and near complete reversion to the dark-adapted state in Fig.
283 2C shows that the absorption kinetics changes at 708 nm or 753 nm after 710 nm
284 illumination treatment. The half-time of the dark recovery is 7.8 s at 25°C (Table 1)
285 and several orders of magnitude faster than those reported in phytochromes or BphPs
286 in which dark recoveries occur in periods ranging from minutes to days (Karniol and

287 Vierstra 2003). However, a faster dark recovery than *MmBphP* has been reported for
288 *BrBphP3* in *Rhizobium* ORS278 that shows a half-time of 460 ms in dark recovery
289 after illumination (Jaubert et al. 2007).

290

291 To further investigate the photochemical characteristics of *MmBphP*, we analyzed the
292 fluorescent properties of this protein. Fluorescence spectra were obtained at 680 nm
293 excitation or 750 nm emission. Fig. 2D shows the excitation (black line) and emission
294 (gray line) spectra for a suspension of *MmBphP* recorded at room temperature. The
295 fluorescence excitation spectra of *MmBphP* closely match the absorption spectra at
296 708 nm. The fluorescence emission spectra are centered at 723 nm, which indicates
297 that the fluorescent originates from the BphP-bound BV chromophore (Toh et al.
298 2011). Similar to the emission spectra of *MmBphP*, *DrBphP* emits fluorescence in the
299 near-infrared region at 720 nm, which is substantially longer than the wavelength
300 emitted by GFP-derived fluorescent proteins (Shu et al. 2009).

301

302 **Identification of *MmBphP* variants unable to bind to BV**

303 In general, a conserved Cys is in the PAS domain in most bacteria, or in GAF domain
304 in cyanobacterial and plant phytochromes forms the covalent linkage to the bilin
305 chromophore (Kreslavski et al. 2018). To determine the Cys residues involved in
306 covalent attachment to the bilin chromophore in AMB-1, we carried out site-directed
307 mutant experiments (C14A, C211A, C275A, and C291A). In addition AMB-1 BphP
308 residues D197, R240, H245, Y249, S260, and H276, which are conserved to those
309 interacting with the propionate side chains of BV in *Rhodospseudomonas palustris*
310 (Yang, et al. 2007), were also substituted for alanine to analyze their potential binding
311 to BV (Fig. S5 A, Supporting Information).

312

313 The binding of BV to WT or mutated AMB-1 *MmBphP* was analyzed by a
314 zinc-induced fluorescence assay. As demonstrated by zinc-induced fluorescence,
315 apo-*MmBphP* and all the variants containing C14A show negative results in
316 zinc-induced fluorescence test, confirming the importance of Cys14 for covalently

317 binding to BV (Fig. 3A). To further compare kinetic properties among variants and
318 WT phytochromes, we analyzed the absorption spectroscopy and calculated the
319 half-life time obtained from the experiment that the absorption kinetics changes at 708
320 nm after 710 nm illumination treatment (Fig. 3B; Table 1). Fig. 3B shows that the
321 absorbance of C14A, D197A or H246A at 708 nm is greatly reduced upon 710 nm
322 light illumination compared with WT. Moreover, the illuminated states possess
323 different life times. The half-life times are 387.9 s, 44.2 s and 5790.1 s for C14A,
324 D197A and H246A, respectively (Table 1). The final photoproduct of these three
325 variants is much longer stable than that of WT. C211A, R240A, C275A, and C291A
326 exhibit minimal photoconversion and a fast reversion to the dark-adapted state, which
327 is similar to WT. The H276A mutant adopts the Pr dark state but shows no detectable
328 formation of the Pfr state upon illumination at 710 nm. This may result either from
329 completely blocked photoconversion to the Pfr state or from formation of a Pfr state
330 that is too short-lived to be detected in our experiments (Yang et al. 2009).
331 Interestingly, Y249A and S260A resulted in a characteristic of the Pfr state upon 710
332 nm light illumination and exhibited slower dark recovery compared with WT.

333

334 Red light/darkness induced spectral analysis showed that compared with WT protein,
335 C14A could form a photoconvertible holoform with blue-shifted extrema and had
336 detectable absorption at 702 nm in the dark, as well as R240A and H246A (Fig. 3;
337 Table 1). The crystal structures of the chromophore-binding pocket of *RpBphP2* and
338 *RpBphP3* showed that the Arg240 and His246 residues interact with the propionate
339 side chains of BV (Bellini et al. 2012; Yang et al. 2007). Experimental data confirmed
340 that the H247Q mutation played a role in the stabilization and coordination of the
341 chromophore (Tasler et al. 2005). Fig. 3 shows that the single substitution of these
342 residues affects zinc-induced fluorescence assay or photochemical spectra at
343 different levels. To assess the accumulation effect of these mutations, we further
344 constructed the double mutants C14&R240 and C14&H246, and the triple mutant
345 C14A&R240A&H246. We observed additional effect of the substitutions, i.e. they
346 almost completely abolished the absorption spectra at 600–800 nm (Fig. 3B).

347

348 The Pr and Pfr states of BphPs typically display maximum absorption peaks at 700
349 nm and 750 nm, respectively, with some overlap in the region between the two peaks.
350 To avoid cross excitation the red-light sources used to illuminate Pr state are shifted
351 towards lower wavelengths from the maximum absorption peak to 625 nm or 680 nm
352 in order to avoid excitation of the Pfr state. However, for some BphPs that the Pr state
353 displays an absorbance maximum at 700–710 nm, some researchers used 700 nm or
354 705 nm LED light for red light illumination of PR, and 750 nm for Pfr (Giraud et al.
355 2005; Fixen et al. 2014). We compared spectra of WT *MmBphP* or mutants
356 illuminated at 710 nm with those at 625 nm, 680 nm or 750 nm. The spectra results by
357 using 625 nm, 680 nm, and 710 nm light sources were similar (Fig. S4, Supporting
358 Information; Fig. S6, Supporting Information). Therefore, we reported the results
359 obtained using LEDs at 710 nm as red light illumination, and 750 nm as far-red
360 illumination source in most case except otherwise specified in this study.

361

362 **Analysis of phototactic behaviour in AMB-1**

363 Previously we reported that illumination triggers cells to swim toward a light source
364 and helps cells to eliminate intracellular ROS in AMB-1 (Chen et al. 2011; Li et al.
365 2017). In addition, research reveals that *RpBphP4* is redox sensitive (Vuillet et al.
366 2007). To investigate the effect of *MmBphP* on phototactic response, we constructed
367 mutant strains and analyzed the phototactic behaviour with or without H₂O₂ in
368 AMB-1 cells by using a modified MTB collection vessel (Chen et al. 2020; Li et al.
369 2017). The phototactic behaviour was analyzed by illuminating with 710 nm and 750
370 nm light sources. For AMB-1 WT and mutant strains, the relative number of cells
371 swimming toward 710 nm or 750 nm light was significantly higher ($p < 0.01$) than that
372 under the dark condition (Fig. 4). Moreover, for the 710 nm illumination and H₂O₂
373 treatment groups, the photoresponse of Δ BphP or BphP^{C14A&R240A&H246A} mutant cells
374 was significantly weaker than that of WT cells (Fig. 4, $p < 0.01$). In contrast, for 750
375 nm illumination and H₂O₂ treatment groups, there was no significant difference in the
376 phototactic behaviour between WT and mutant cells ($p > 0.05$). For strains without

377 H₂O₂ treatment, the number of WT cells swimming toward light was not significantly
378 different from that of the mutant strain ($p>0.05$). In addition, there was no significant
379 difference in the phototactic response between WT and ComBphP cells ($p>0.05$).

380

381 **Discussion**

382 Photosensing proteins play key roles in the regulation of physiological activities in
383 response to light, a critical element in the acclimatization to the environment. In this
384 study, we described the properties of photosensing protein *MmBphP*, a new type of
385 BphPs, presenting in the MTB model strain AMB-1. *MmBphP* has
386 red/far-red-light-induced photoreversibility features among all members of the
387 phytochrome superfamily. The characteristic absorbance spectra and photoconversion
388 of *MmBphP* reflect its association with BV chromophore, which are covalently
389 attached at the conserved Cys14 in the PAS domain as most BphPs and Fungal
390 phytochromes do, while plant and cyanobacterial phytochromes are covalently
391 attached at a conserved Cys in the GAF domain. BV adopts the special conformation
392 in the Pr state through the vinyl group of ring A linking to the cysteine covalently and
393 goes through an isomerization at its C15-C16 double bond upon light activation,
394 which results in rotation of ring D (Gourinchas et al. 2019). The *MmBphP* protein
395 lacking the C-terminal part showed a fast recovery to the stable state after
396 illumination. Our result is consistent with previous findings that the dark recovery is
397 fast in BphPs lacking C-terminal region. *BrBphP3* lacking natural C-terminal region
398 in *Rhizobium* ORS278 showed a fast dark recovery with half-life time of 460 ms
399 (Jaubert et al. 2007). In *Agrobacterium tumefaciens* and *Xanthomonas campestris*, the
400 Agp2 variant lacking the output module and *XccBphP* lacking the PAS9 domain
401 showed much faster kinetics, pointing to the modulation of the dark reversion of the
402 chromophore by the output module (Velazquez et al. 2015; Otero et al. 2016).

403

404 In addition, our results showed that light induced photoconversion had a small
405 absorption increase in the near infra-red region around 753 nm, and long-term
406 irradiation would not affect these absorption spectra (data not shown). An obvious Pfr

407 state could not be fully detected in various conditions assayed in this study. We
408 speculate *MmBphP* may experience a ‘fast’ Pfr reaction, which evolved on a
409 picosecond or microsecond time scale (Heyne et al. 2002; Jaubert et al. 2007; Singer
410 et al. 2016). This may result from formation of an obvious Pfr state that is too
411 short-lived to be detected in our experiments. Another possibility is that a
412 supplementary protein or component in AMB-1 cells is involved in controlling Pfr to
413 Pr conversion and they are absent in hetero-expressed and purified recombinant
414 *MmBphP* protein complex. Nevertheless, we do have observed a rapid absorption
415 kinetics change at 708 nm or 753 nm after 710 nm illumination treatment in Fig. 2C,
416 which indicates the occurrence of photoconversion in *MmBphP* after irradiation.

417

418 In the spectral measurement (Fig. 3B; Table 1), substitution of Cys14, Asp197 or
419 His246 exhibited a significantly weakened and broadened absorption band in the red,
420 and detected a slower dark recovery upon 710 nm light illumination compared with
421 WT, suggesting their possibility of being the BV binding pocket which is lined by
422 many highly conserved residues in *MmBphP*. Alanine substitutions at Cys211,
423 Cys275, and Cys291 show Pr/Pfr photoconversion efficiency similar to the WT
424 *MmBphP*, demonstrating the variants could still covalently bind to BV and exhibit a
425 little effect on BV configuration or photoconversion. Moreover, the maximum
426 absorption peaks of C14A, R240A and H246A mutants showed a blue shift of 6 nm to
427 702 nm in the dark state, indicating that they may affect the binding to BV. Strikingly,
428 illumination of red light on single mutant Y249A and S260A resulted in the
429 appearance of an obvious absorption band (753 nm), characteristic of the Pfr state.
430 These two mutants underwent reversible Pr/Pfr photoconversion, but its rate of
431 reversion to the Pr state in the dark is significantly slower than that of WT, with a
432 half-life time of about 247.8 s and 411.5 s, respectively. Thus, introduction of a single
433 alanine residue into the location of Tyr249 or Ser260 in *MmBphP* was sufficient to
434 replace the photoconversion of WT *MmBphP*, suggesting Tyr249 and Ser260 may
435 locate at the binding pocket of *MmBphP*, which has a direct impact on the Pr/Pfr
436 photoconversion. Photoconversion of the H276A, is barely detectable in our

437 experiments, but it has normal Pr absorption spectra in the dark. The phenotype
438 indicates that it is likely to be involved in engaging the GAF domain for
439 photoconversion, as the residue in the GAF domain usually interact with the
440 chromophore primarily (Yang et al. 2007). The spectral properties of the variants
441 tested can be noncovalent associations with BV. Because photoconversion does not
442 appear to take covalent attachment as the prerequisite, covalent attachment may
443 provide a more stable holoprotein that make phytochrome suited to reversible
444 photoswitching better by utilize bilin chromophores (Rockwell et al. 2006). In
445 addition to the output module affecting dark recovery (Velazquez et al. 2015; Otero et
446 al. 2016), our results suggest that the amino acids at the BV binding pocket also affect
447 Pr/Pfr photoconversion efficiency.

448

449 Interestingly, BphP^{C14A&R240A}, BphP^{C14A&H246A} or BphP^{C14A&R240A&H246A} exhibited
450 little red, far-red, or near-red absorption and failed to covalently bind to BV,
451 indicating the importance of Cys14, Arg240 and His246 forming the BV binding
452 pocket to some extent. The substitution of arginine with alanine, which abolishes two
453 salt bridges that form with the propionate side chain of the B pyrrole ring, is sufficient
454 to prevent the covalent and noncovalent association of BV with *Rp*BphPs (Fixen et al.
455 2014). Thus, the conserved arginine in *Mm*BphP may be required for the autocatalytic
456 incorporation of BV. The histidine was characterized to be of important for the
457 chromophore attachment in *Calothrix* sp. PCC7601 CphB (Quest and Gartner 2004).
458 H247Q mutation in *Pseudomonas aeruginosa* resulted in a spectral shift of the Pr and
459 Pfr forms. This result suggested that these mutations play a role in the stabilization
460 and coordination of the chromophore (Tasler et al. 2005). In comparison with the
461 other BphPs, these different characteristics observed in these variants of *Mm*BphP
462 may be related to an increase in the number of hydrogen bonds in the BV-binding
463 pocket, which increases the rigidity of the BV environment (Fixen et al. 2014).

464

465 Currently, studies have shown that ultraviolet radiation and free-iron-generated ROS
466 have been major environmental challenges in life on early Earth. Therefore, the initial

467 biomineralization of iron nanoparticles in early life cells has developed into a
468 mechanism to reduce the toxicity of ROS (Lin et al. 2019). Li et al. (2017) found that
469 light helps to eliminate intracellular ROS in AMB-1 cells. Fei et al. (2019) reported
470 that phytochrome A and phytochrome B can help reduce ROS in the cell and are
471 potent regulators of plant defense. Our result showed that under the oxidative stress of
472 H₂O₂ treatment, the number of WT cells moving toward 710 nm light is significantly
473 higher than that of *MmBphP* mutant strains, while the phototactic behaviour has no
474 significant difference between WT and mutants by illuminating with 750 nm light.
475 The result indicates *MmBphP* can respond to red light to activate antioxidant enzymes
476 such as catalase and peroxidase when transformation of Pr into Pfr occurred
477 (Kreslavski et al. 2018), and further help fight toxic effects of oxidative stress or ROS,
478 allowing WT cells to swim toward the light source. The lacking C-terminal region
479 results in a ‘classical’ output module missing, one may, however, consider *MmBphP* a
480 genuine photochrome because of the properties of photo response shown in this study.
481 The mechanism of how the C14, R240, H246 affect the photoconversion behaviours
482 through interaction with BV configurations (Fig. S5 B, Supporting Information) and
483 how *MmBphP* are involved in the light transduction pathway need further study.

484

485 **Supplementary data**

486 Supplementary data are available at *FEMSLE* online

487

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490

491 **Author contributions**

492 The manuscript was written through contributions of all authors. All authors have
493 given approval to the final version of the manuscript. HC, L-FW and TS conceived
494 and designed the experiments. HC, DL and YC prepared samples and carried out the
495 experiment. HC, L-FW and TS reviewed, analyzed and interpreted data. HC and TS

496 wrote the paper and all authors discussed the results and commented on the
497 manuscript.

498

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505

506 **Competing interests**

507 The authors declare no conflict of interest.

508

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