

cyAbrB transcriptional regulators as safety devices to inhibit heterocyst differentiation in *Anabaena*

Running title: cyAbrB transcriptional regulators prevent heterocyst formation

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19 Abstract

20 Cyanobacteria are monophyletic organisms that perform oxygenic photosynthesis. While they
 21 exhibit great diversity, they have a common set of genes. However, the essentiality of them for
 22 viability has hampered the elucidation of their functions. One example of the genes is *cyabrB1*
 23 encoding a transcriptional regulator. In the present study, we investigated the function of
 24 *cyabrB1* in heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120 through CRISPR
 25 interference, a method we recently utilized for the photosynthetic production of a useful
 26 chemical in the strain. Conditional knockdown of *cyabrB1* in the presence of nitrate resulted in
 27 formation of heterocysts. Two genes, *hetP* and *hepA*, which are required for heterocyst
 28 formation, were up-regulated by *cyabrB1* knockdown in the presence of combined nitrogen
 29 sources. The genes are known to be induced by HetR, a master regulator of heterocyst formation.
 30 *hetR* was not induced by *cyabrB1* knockdown. *hetP* and *hepA* were repressed by direct binding
 31 of cyAbrB1 to their promoter regions in a HetR-independent manner. In addition, the
 32 over-expression of *cyabrB1* abolished heterocyst formation upon nitrogen depletion. Also,
 33 knockout of *cyabrB2*, a paralogue gene of *cyabrB1*, in addition to *cyabrB1* knockdown,
 34 enhanced heterocyst formation in the presence of nitrate, suggesting functional redundancy of
 35 cyAbrB proteins. We propose that a balance between amounts of HetR and cyAbrB1 is a key
 36 factor influencing heterocyst differentiation during nitrogen step-down. cyAbrB proteins are
 37 essential safety devices inhibiting heterocyst differentiation.

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43 **Importance**

44 Spore formation in *Bacillus subtilis* and *Streptomyces* represents non-terminal differentiation
 45 and has been extensively studied as models of prokaryotic cell differentiation. In the two
 46 organisms, many cells differentiate simultaneously, and the differentiation is governed by a
 47 network in which one regulator stands at the top. Differentiation of heterocysts in *Anabaena* sp.
 48 PCC 7120 has also been extensively studied. The differentiation is unique because it is terminal
 49 and only 5-10% vegetative cells differentiate into heterocysts. In the present study, we identified
 50 cyAbrB1 as a repressor of two genes that are essential for heterocyst formation, *hetP* and *hepA*,
 51 independent of HetR, which is a master activator for heterocyst differentiation. The finding is
 52 reasonable for unique cell differentiation of *Anabaena* because cyAbrB1 could suppress
 53 heterocyst differentiation tightly in vegetative cells, while only cells in which HetR is
 54 over-expressed could differentiate into heterocysts.

55

56 **Introduction**

57 Cyanobacteria are ancient and monophyletic prokaryotes, which are characterized by a capacity
58 to perform oxygenic photosynthesis. They are found in diverse habitats, including fresh and
59 marine water, hot springs, frozen lakes, soil, and deserts (1). Specific responses to
60 environmental changes enable them to adapt to their habitats (2). In addition, they exhibit a
61 great diversity of morphology and cell arrangements. Moreover, some cyanobacteria can
62 differentiate into specific cell types in response to environmental stimuli, which is one type of
63 stress response. The most studied differentiated cell type in cyanobacteria is the heterocyst. At
64 semi-regular intervals, some filamentous cyanobacteria can differentiate into larger and round
65 cells called heterocysts, which are cells specialized for nitrogen fixation, which enables
66 heterocystous cyanobacteria to inhabit nitrogen-poor environments.

67 *Anabaena* sp. PCC 7120 (*Anabaena*) has been extensively studied as a model for
68 heterocyst differentiation (3,4). Upon the depletion of combined nitrogen, 5-10% of vegetative
69 cells that perform oxygenic photosynthesis differentiate into heterocysts. A transcriptional
70 regulator, NtcA, widely conserved in cyanobacteria, perceives nitrogen deficiency as an
71 increase of a metabolite 2-oxoglutarate (5). Subsequently, NtcA indirectly induces HetR, a
72 master regulator of heterocyst differentiation (6). Accumulation of HetR spatially initiates
73 specific developmental program and enables patterned heterocyst formation (7–9). During
74 differentiation, deposition of exopolysaccharide and glycolipid layers results in morphological
75 changes in the cells. In addition, cellular metabolism is dynamically altered by the inactivation
76 of oxygenic photosystem II and enhancement of respiration (3,4). Such changes enable
77 heterocysts to protect oxygen-labile nitrogenase from oxygen.

78 Despite their great diversity, cyanobacteria have a core set of genes that are conserved
79 across the phylum (10, 11). Many of the conserved genes have been found to be associated with

80 core biological process such as DNA replication, transcription, translation, photosynthesis, the
81 Calvin cycle, and various metabolic pathways (11). Therefore, many of their functions can be
82 predicted. However, the functions of some of the conserved genes are yet to be elucidated.
83 Although the study of such genes could offer novel insights into cyanobacterial biology,
84 essentiality of the core genes (11, 12) has hampered such investigations. An example of the
85 genes is *cyabr1* (13).

86 *cyabrB* encoding a transcriptional regulator is conserved among cyanobacteria (13). The
87 DNA-binding domain of cyAbrB located at the C-terminus belongs to AbrB-like family
88 (Pfam14250), which is unique to cyanobacteria and is not conserved in other organisms
89 including melainabacteria, a non-photosynthetic sister phylum to the cyanobacteria. Each
90 cyanobacterium has two copies of *cyabrB* genes, *cyabrB1* and *cyabrB2* (also known as *calA* and
91 *calB* in *Anabaena*, respectively). Because some attempts to disrupt *cyabrB1* in *Anabaena* and
92 unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis*) have failed so far
93 (13–16), the gene should be essential for cyanobacteria. Conversely, gene disruption of *cyabrB2*
94 is possible in *Synechocystis*. Studies on *cyabrB2* mutants have revealed that *cyAbrB2* is
95 involved in acclimation to changes in carbon and nitrogen availability (13, 17–19). While the
96 studies demonstrated that *cyabrB2* had specific functions, some evidence suggested a functional
97 overlap between cyAbrB1 and cyAbrB2 (20).

98 Recent studies towards photosynthetic production of useful chemicals have rapidly
99 developed tools for artificial gene regulation systems for cyanobacteria (21–23). Among them, a
100 gene knockdown technology, CRISPR interference (CRISPRi), has attracted much attention
101 because the system exhibited repression over a wide dynamic range in an inducer
102 concentration-dependent manner (24–28). Target genes can be repressed by the formation of a
103 complex consisting of a nuclease-deficient Cas9 (dCas9), a single guide RNA (sgRNA), which

104 corresponds to the target DNA sequence, and target DNA (29). While CRISPRi has been
105 successfully applied to enhance the production of desired products in cyanobacteria (24–26, 30),
106 its basic scientific applications are still awaited.

107 A question of whether a transcriptional regulator cyAbrB1 conserved in cyanobacteria
108 regulates core genes or specific genes in *Anabaena* motivated us to study the function of
109 *cyabrB1*. In the present study, we created *Anabaena* strains in which *cyabrB1* is conditionally
110 knocked down through CRISPRi technology. cyAbrB1 amounts were significantly repressed in
111 any conditions tested and in any genetic background tested when the CRISPRi system was
112 induced. Repression of *cyabrB1* resulted in formation of heterocysts even in the presence of
113 nitrate. Not *hetR*, but two direct target genes of HetR, *hetP* and *hepA*, which are required for
114 heterocyst development (4), were induced by *cyabrB1* knockdown in the presence of combined
115 nitrogen in a HetR-independent manner. Over-expression of *cyabrB1* abolished heterocyst
116 formation under nitrogen-depleted conditions. Therefore, we concluded that cyAbrB1 is
117 essential for the suppression of heterocyst differentiation and propose a model that cyAbrB1
118 offers HetR an appropriate threshold for the induction of heterocyst development.

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121 **Results**

122 **Heterocyst formation by *cyabrB1* knockdown in the presence of nitrate**

123 A conditional *cyabrB1* knockdown strain C104 was constructed by integrating a plasmid
124 containing *P_{petE}-tetR*, *P_{L03}-dcas9*, and *P_{J23119}-sgRNA* targeting *cyabrB1* (Figure S1) to the neutral
125 site *cyaA* (31). In the strain, an inducer anhydrotetracycline (aTc) derepresses *L03* promoter by
126 binding to TetR, and *dcas9* is induced. sgRNA is constitutively expressed. Therefore, the
127 addition of aTc switches on repression by CRISPRi. A negative control strain C100 without

128 sgRNA was also constructed.

129 Strain C104 was grown in nitrate-containing medium and bubbled with air containing
130 1% (v/v) CO₂. When the expression of *cyabrB1* was repressed for 48 h, 0.6% of heterocysts
131 were formed (Fig. S2A). In contrast, when *cyabrB1* was not repressed, little heterocysts
132 (<0.1%) were formed. To facilitate clearer observation of the phenotype, C104 was cultured in
133 nitrate-containing medium and bubbled with air containing 5% (v/v) CO₂, in which carbon is
134 excess to nitrogen in the cells. In this condition, C104 and C100 formed 0.5 and 0.4% of the
135 heterocysts, respectively, in the absence of the inducer. In the presence of the inducer, C104
136 formed 2.9% of the heterocysts but C100 did not form any heterocysts (Fig. 1A) (<0.1%).
137 Knockdown of cyAbrB1 was confirmed by western-blotting analysis (Fig. 1B and C). While an
138 addition of aTc to control strain C100 did not repress cyAbrB1 expression, in the case of C104,
139 addition of aTc considerably repressed cyAbrB1 expression (less than 10%), particularly at 48
140 and 72 h. Repression of *cyabrB1* did not lead to heterocyst formation when C104 was cultured
141 in ammonium-containing medium (Fig. S2B).

142 To rule out the possibility that the observed phenotype is due to off-target effects of
143 CRISPRi, we constructed C105 and C106 that retain sgRNA targeting different sites of *cyabrB1*.
144 Formation of heterocyst was promoted in both C105 and C106 by *cyabrB1* repression similarly
145 in C104, when cells were cultured in nitrate-containing medium and bubbled with air containing
146 5% (v/v) CO₂ (Fig. S3). The results suggest that cyAbrB1 is required for the suppression of
147 heterocyst formation in the presence of nitrate.

148

149 **Repression of *hetP* and *hepA* by cyAbrB1 in the presence of nitrogen sources**

150 To elucidate the cause of heterocyst formation in the presence of nitrate, RNA was extracted
151 from cells of C100 and C104 cultured for 48 h in nitrate-containing medium with 5% CO₂ in the

152 absence or presence of the inducer, and an RT-qPCR analysis was performed. The expression of
153 four genes that are induced at early stages of heterocyst development, including *hetR*, *hetP*, *hetZ*,
154 and *hepA*, was investigated (Fig. 2A). *hetR* encodes a master regulator of heterocyst
155 differentiation and its over-expression causes heterocyst formation in the presence of nitrogen
156 sources (9). Both *hetP* and *hetZ* are directly up-regulated by HetR, and ectopic expression of
157 either gene leads to heterocyst formation in the presence of nitrogen sources (4, 32–36). *hepA*
158 encodes a component of an ABC transporter required for the construction of the heterocyst
159 exopolysaccharide layer (37), which is the first step during morphological differentiation (4).
160 HetR also directly induces expression of *hepA* (4, 38). While the expression of *hetR* and *hetZ*
161 did not change significantly, the expression of *hetP* and *hepA* was significantly induced when
162 *cyabrB1* was repressed in C104. Similar results were observed in C105 and C106 (Fig. 2A). We
163 confirmed that *cyabrB1* transcript and cyAbrB1 protein in C104, C105, and C106 were
164 repressed in the presence of the inducer (Fig. S4A and B, respectively).

165 Subsequently, we extracted RNA from C104 cultured in the presence of nitrate with 1%
166 CO₂ bubbled or in the presence of ammonium with 5% CO₂ bubbled (Fig. 2B). Although no
167 heterocysts were formed in the latter condition, *hetP* and *hepA* were greatly induced when
168 *cyabrB1* was repressed in both conditions, similarly in Fig 2A. We confirmed that *cyabrB1*
169 transcript and cyAbrB1 protein were repressed in both conditions in the presence of the inducer
170 (Fig. S4C and D, respectively). The results indicate that the up-regulation of *hetP* and *hepA* by
171 *cyabrB1* knockdown induced heterocyst formation rather than *hetP* and *hepA* were induced
172 following the initiation of heterocyst development, and that heterocyst formation was
173 suppressed by an unknown mechanism in the presence of ammonium.

174 Expression of *nifH* encoding a subunit of nitrogenase was quantified to determine
175 whether the heterocysts formed by *cyabrB1* repression were functional. Repression of *cyabrB1*

176 in C104 in the presence of nitrate bubbled with 5% CO₂ induced *nifH*, but not under bubbling
177 with 1% CO₂ (Fig. S5), suggesting that maturation of heterocysts depends on C-N balance
178 inside the cells and does not directly depend on cyAbrB1. Therefore, we concluded that
179 cyAbrB1 is essential for repression of *hetP* and *hepA* in the presence of nitrogen sources.

180

181 **Induction of *hetP* and *hepA* by cyAbrB1 knockdown independently of HetR**

182 To clarify whether the up-regulation of *hetP* and *hepA* is independent of or dependent on HetR,
183 we constructed a C104h strain in which *cyabrB1* could be knocked down using the CRISPRi
184 system in a *hetR* mutant strain (39) (Fig. S1). RNA was extracted from C104h cultured in the
185 presence of nitrate bubbled with 5% CO₂ in the absence or presence of aTc. RT-qPCR analysis
186 revealed that *hetP* and *hepA* were up-regulated by *cyabrB1* repression in the *hetR*-deficient
187 background (Fig. 3A), indicating that cyAbrB1 regulates the expression of the two genes
188 independently of HetR. We confirmed that *cyabrB1* transcript and cyAbrB1 protein in C104h
189 were repressed similarly in C104 in the presence of the inducer (Fig. 3A and B).

190 Heterocysts were not formed following the repression of *cyabrB1* in C104h (Fig. 3C).
191 Although the expression of *hetP* in C104h was induced by *cyabrB1* in the presence aTc
192 compared to in the absence of the inducer, the expression levels of *hetP* in the presence of the
193 inducer in C104h were low compared to those in C104 (Figs. 2A and 3A), possibly due to the
194 effect of *hetR* disruption. The result could explain why heterocysts were not formed even when
195 *hetP* and *hepA* were induced in C104h (Fig. 3C).

196

197 **Specificity and redundancy of cyAbrB proteins**

198 Subsequently, we constructed a *cyabrB2* knockout mutant DR2080 and a *cyabrB1*
199 knockdown/*cyabrB2* knockout mutant C104B2 (Fig. S1) to examine specificity and redundancy

200 of cyAbrB1 and cyAbrB2. In C104B2, *cyabrB1* knockdown caused 4.5% heterocyst formation
 201 in nitrate-containing medium bubbled with 1% CO₂ (Fig. 4A). In contrast, *cyabrB1* knockdown
 202 mutant C104 and *cyabrB2* knockout mutant DR2080 produced only 0.6 and 0% heterocyst
 203 under similar conditions, respectively (Fig. S2A and Fig. 4A). An RT-qPCR analysis revealed
 204 that *hetP* and *hepA* were similarly induced by *cyabrB1* knockdown in C104 and C104B2
 205 (Compare Figs. 2B and 4B). The expression of *hetR* and *hetZ* were not induced by *cyabrB1*
 206 knockdown in C104B2, similarly to in C104 (Fig. 4B). Deletion of *cyabrB2* hardly influenced
 207 expression of *hetR*, *hetP*, *hetZ*, and *hepA*. Repression of *cyabrB1* transcript and cyAbrB1
 208 protein in C104B2 was confirmed (Fig. S6A and B). C104B2 did not form heterocysts in
 209 ammonium-containing medium (Fig. S6C). Comparison of the results with *cyabrB1* knockdown,
 210 *cyabrB2* knockout, and *cyabrB1* knockdown/*cyabrB2* knockout mutants revealed that cyAbrB1
 211 but not cyAbrB2, specifically regulates the expression of *hetP* and *hepA*. However, with regard
 212 to heterocyst formation in the presence of nitrate, cyAbrB1 and cyAbrB2 could be redundant
 213 since the double mutant produced more heterocysts compared to single mutants.

214

215 **Direct binding of cyAbrB1 to promoters of *hetP* and *hepA***

216 To test whether the expression of *hetP* and *hepA* was directly regulated by cyAbrB1, we
 217 expressed a recombinant His-cyAbrB1 in *Escherichia coli* and purified it. The purified
 218 His-cyAbrB1 protein had an apparent molecular weight of 18,000, which was largely consistent
 219 with the theoretical value (Fig. 5A). We performed a gel mobility shift assay using His-cyAbrB1
 220 and Cy3-labeled DNA probe PhetP that includes *hetP* promoter region (Fig. 5B). His-cyAbrB1
 221 retarded the mobility of the probe. Thereafter, we examined the specificity of the interaction
 222 using a competition assay. Addition of a 5 or 10-fold molar excess of non-labeled DNA probe
 223 PhetP and PhepA (*hepA* promoter region) eliminated the retardation, but that of *cyabrB1*RT

(internal region of *cyabrB1*) did not (Fig. 5B). The results indicate that cyAbrB1 binds promoter regions of *hetP* and *hepA* and that the two genes are directly repressed by cyAbrB1.

Inhibition of heterocyst formation by over-expression of *cyabrB1*

We investigated whether or how cyAbrB1 participates in heterocyst development in the absence of nitrogen sources. *cyabrB1* was knocked down upon removal of combined nitrogen sources in C104. After 24 or 48 h of nitrogen step-down, heterocysts were formed in the strain in the absence or presence of aTc (Fig. S7A and B). However, vegetative cell intervals were shorter in the presence of the inducer than in the absence of the inducer (Fig. S7C). Figure S7D shows that cyAbrB1 was repressed in the presence of the inducer in C104 at 48 h in the absence of nitrogen sources.

Then, we constructed a *cyabrB1* over-expression strain T121 (Fig. S1). In the strain, aTc induced the expression of *cyabrB1* driven by P_{L03} . Strain C100, in which aTc induced the expression of *dcas9* but not *cyabrB1*, was used as the control strain. C100 or T121 was transferred from a nitrate-containing medium to a nitrogen-free medium, and was grown in the absence or presence of aTc. While C100 grew regardless of the absence or presence of aTc, T121 did not grow at all in the presence of the inducer (Fig. 6A). In contrast, the over-expression of *cyabrB1* only minimally inhibited the growth of T121 in the presence of nitrate, as previously demonstrated (40). Microscopic observations revealed that the addition of aTc to T121 abolished heterocyst formation 24 h after nitrogen depletion (Fig. 6B). Expression levels of *hetR*, *hetP*, and *hepA* were measured after depletion of nitrogen sources for 8 h, at which the genes were up-regulated in the wild type strain (41). While the expression of *hetP* was not repressed, the expression of *hepA* was repressed in T121 in the presence of aTc compared to in the absence of aTc, or in C100 in the absence or presence of aTc (Fig. 6B).

248 Expression of *hetR* was also repressed in T121 following the addition of aTc, although it was
249 minimal compared to the expression of *hepA* for an unknown reason. We confirmed that the
250 addition of aTc led to the accumulation of *cyabrB1* transcripts and cyAbrB1 protein (Fig. 6B).
251 The results suggested that over-production of cyAbrB1 inhibited the transcription of *hepA*. The
252 reason why the expression of *hetP* was not inhibited is discussed below.

253 We quantified the amounts of cyAbrB1 after nitrogen depletion in PCC 7120. Cells of
254 PCC 7120 were washed by nitrogen-free medium and transferred to nitrate-containing or
255 nitrogen-free medium, and were cultured for 8 h. Total proteins were extracted from cells before
256 and after their cultivation. Western blotting using anti-cyAbrB1 antibody revealed that the
257 depletion of nitrogen sources did not alter the amounts of cyAbrB1 (Fig. S8A and B). We also
258 quantified the amounts of cyAbrB1 in mature heterocysts after nitrogen depletion for 24 h
259 relative to that in whole filaments. The amounts of cyAbrB1 were marginally lower in
260 heterocysts (Fig. S8C and D). The results suggested that high induction of HetR in
261 proheterocysts (42) rather than decreasing cyAbrB1 amounts was a limiting step in the
262 up-regulation of *hetP* and *hepA* during heterocyst development in the wild type strain following
263 combined nitrogen step-down.

264

265 **Effect of *cyabrB1* repression or over-expression on the expression of *alr0947***

266 It has previously been shown that *cyabrB1* and *alr0947* constitute an operon (40). Therefore, we
267 evaluated the polar effect of *cyabrB1* repression. While the expression of *cyabrB1* was highly
268 repressed following the addition of aTc in C104, C105, and C106 in some conditions (Fig. S4A
269 and C), the expression of *alr0947* was only slightly repressed (Fig. S9A and B). It was
270 demonstrated that the over-expression of cyAbrB1 repressed the expression of *alr0947* since
271 cyAbrB1 inhibits the transcription of the *cyabrB1-alr0947* operon (40). When *cyabrB1* was

272 over-expressed, *alr0497* was repressed marginally (Fig. S9). While both repression and
273 induction of *cyabrB1* resulted in a slight decrease in *alr0947* expression, repression induced
274 heterocyst formation and the induction inhibited heterocyst formation, indicating that the
275 observed phenotypes in the present study were caused by changes in expression levels of
276 *cyabrB1* rather than those of *alr0947*.

277

278 **Discussion**

279 In the present study, we revealed that cyAbrB1 is essential for the repression of *hetP* and *hepA*
280 in the presence of nitrogen sources and that heterocysts are formed even in the presence of
281 nitrate when *cyabrB1* is knocked out. Since the first description of *cyabrB1* in cyanobacteria
282 (13), its essentiality has hampered investigation of the function of the gene. We overcome the
283 challenge by applying CRISPRi, which is recently developed technology that has been
284 employed to facilitate photosynthetic production of desired chemicals in some model
285 cyanobacteria (24–26). Here, we demonstrated that the technology is also very useful for basic
286 research. In all the conditions tested, and in any genetic backgrounds, CRISPRi facilitated the
287 robust knock down of *cyabrB1*. The inhibition of heterocyst formation by the over-expression of
288 *cyabrB1* in the absence of nitrogen sources has been overlooked so far. Agervald et al. (40) used
289 the *nirA* promoter, which over-expresses only in the presence of nitrate while He and Xu (16)
290 used the *petE* promoter, which might not be strong enough to hinder heterocyst formation.
291 Consequently, the development of a variety of tools to regulate gene expression (23) is
292 fundamental and could broaden the range of basic research and biotechnological application.

293

294 **Balance between cyAbrB1 and HetR determines heterocyst differentiation**

295 Some evidence indicated that cyAbrB1 hinders heterocyst formation in the presence of nitrogen

sources by repressing the expression of *hetP* and *hepA* through specific binding to promoter regions of the genes, which are direct targets of HetR. The possibility that the up-regulation of the genes by *cyabrB1* knockdown is due to an increase in HetR proteins or its activity (e.g. decreased level of PatS, a HetR inhibitor (43)) should be rejected based on the results that *cyAbrB1* regulation of the genes is independent of HetR (Fig. 3A) and that expression of *hetZ*, a direct target of HetR (34), was not induced following *cyabrB1* knockdown (Fig 2). Upon nitrogen step-down, amounts of *cyAbrB1* did not significantly decrease (Fig. S8). We propose that strong induction of HetR in proheterocysts (42) out-competes the repression of *hetP* and *hepA* by *cyAbrB1* during heterocyst differentiation in a wild type strain. The view is partly supported by Figure 6, in which the over-expression of *cyAbrB1* hampered the induction of *hepA* but not the induction of *hetP*. The observation could be explained by the fact that the binding affinity of HetR for the *hetP* promoter is much higher than that for the *hepA* promoter (38). Decrease of amounts of *cyAbrB1* or *cyAbrB1* activity attributed to glutathionylation (44) in proheterocysts is a potential reason. Taken together, we propose that a balance between the amounts of *cyAbrB1* and HetR is a key determinant for the initiation of heterocyst differentiation.

It is typical that both a global activator and a repressor regulate prokaryotic cell differentiation. In *Streptomyces*, a global transcriptional activator AdpA and repressor BldD control morphological differentiation (45, 46), while in *Bacillus subtilis*, an activator Spo0A and a repressor AbrB regulate spore formation (47). Notably, both the DNA-binding domain of *cyAbrB* (AbrB-like family, Pfam14250) and that of AbrB from *B. subtilis* (MazE_antitoxin family, Pfam04014) belong to the same clan (AbrB, CL0132), although the former is located at the C-terminus and the latter is located at the N-terminus. However, the relationship between the activator and the repressor is different between *Anabaena* and *B. subtilis*, as well as

320 *Streptomyces*. The expression of AdpA is regulated by BldD in *Streptomyces* (46, 48) and the
 321 expression of AbrB is regulated by Spo0A in *B. subtilis* (47). In contrast, our results
 322 demonstrated that HetR does not regulate cyAbrB1, and vice versa. Many cells differentiate in *B.*
 323 *subtilis* and *Streptomyces* simultaneously. Hence, the fact that one regulator governs the whole
 324 network is a practical strategy for cell differentiation in the above organisms. In contrast, only
 325 one-tenth of cells differentiate into heterocysts and the remaining vegetative cells maintain
 326 viability and photosynthetic activity in *Anabaena*. In addition, heterocyst differentiation is
 327 terminal (non-reversible) while cell differentiation in *B. subtilis* and *Streptomyces* is
 328 non-terminal (reversible). Therefore, robust inhibition of heterocyst differentiation by cyAbrB1,
 329 whose functioning is independent of HetR, could be an essential safety device in *Anabaena*, in
 330 concert with HetR inhibitors PatS (43) and HetN (49, 50) and regulation by HetR
 331 phosphorylation (51).

332

333 **Repression of heterocyst formation in ammonium medium**

334 Heterocysts were formed in the presence of nitrate but not in the presence of ammonium in
 335 *cyabrB1* knockdown mutant C104 even though the expression of *hetP* was induced in the
 336 presence of ammonium similar to in the presence of nitrate when *cyabrB1* was knocked down.
 337 The results suggest the existence of an unidentified mechanism that regulates heterocyst
 338 differentiation. A previous study demonstrated that the over-expression of *hetP* induced
 339 heterocyst formation even in the presence of ammonium (33). The inconsistency between our
 340 results and those of the previous study could be explained by the unidentified mechanism,
 341 which would be cyAbrB1 dependent.

342

343 **Perspectives**

344 In the present study, we could not identify target genes of cyAbrB1 other than *hetP* and *hepA*,
 345 which are not conserved in many non-heterocystous cyanobacteria. Up-regulation of the genes
 346 in a cyAbrB1 knockdown mutant could not explain why *cyabrB1* is essential in *Anabaena*. A
 347 transcriptome analysis would identify other target genes comprehensively, and would answer
 348 our question on whether cyAbrB1 conserved in cyanobacteria regulates core genes (10, 11). If
 349 cyAbrB1 regulated core genes, whether cyAbrB1 is involved in the reconstruction of
 350 metabolism during heterocyst development such as inactivation of photosystem II (52) or
 351 enhancement of photosystem I (53) would be an interesting question.

352 Our results demonstrated that cyAbrB1, but not cyAbrB2, specifically regulates the
 353 expression of *hetP* and *hepA*. However, heterocyst formation in the presence of nitrate was
 354 enhanced in double mutant C104B2 than in the single mutant C104 (Fig. 4). The results indicate
 355 that cyAbrB proteins can function both specifically and redundantly/cooperatively in *Anabaena*,
 356 as has been suggested in *Synechocystis* (20), although the underlying mechanism remains to be
 357 elucidated. A transcriptome analysis of C104B2, C104, and that of a *cyabrB1* knockdown
 358 mutant in *Synechocystis* could shed more light on the evolution and adaptation of cyanobacteria.
 359

360 **Materials and Methods**

361 **Bacterial strains and growth condition**

362 *Anabaena* strains were cultured at 30°C under 30-35 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in BG11 medium
 363 (54) (17.6 mM sodium nitrate as nitrogen source). BG11₀ (lacking nitrogen sources) or BG11_a
 364 (5 mM ammonium chloride as nitrogen sources) were used after washing the cells twice with
 365 BG11₀, where indicated. Each medium was supplemented with 20 mM HEPES-NaOH (pH 7.5).
 366 Two $\mu\text{g/ml}$ each of spectinomycin and streptomycin and 25 $\mu\text{g/ml}$ neomycin-sulfate were added
 367 when required. Liquid cultures were bubbled with air containing 1.0% (v/v) CO₂ unless

368 otherwise stated.

369

370 **Plasmid construction**

371 Plasmids for the knockdown of *cyabrB1* by CRISPRi or the over-expression of *cyabrB1* were
 372 constructed using the hot fusion method (55). DNA fragments were inserted between the
 373 BamHI and KpnI sites of a genome-integrating vector pSU102-cyaA (26). Schematic
 374 representations of them are shown in Fig. S1 and detailed sequences are described in
 375 Supplemental information. Inactivation of *cyabrB2* was accomplished by replacing a 200-bp
 376 portion of the *cyabrB2* coding region with a spectinomycin resistant cassette as follows.
 377 Upstream and downstream regions of the *cyabrB2* gene were amplified by PCR using the
 378 primer pair 2080-5F and 2080-5R and the 2080-3F and 2080-3R pair, respectively. The
 379 spectinomycin cassette was inserted between upstream and downstream regions and the
 380 resultant construct was cloned between SacI and XhoI sites of pRL271 (56) to construct
 381 pR2080S. To construct the expression plasmids for the hexahistidine-tagged cyAbrB1 protein,
 382 DNA fragment containing the *cyabrB1* coding regions was amplified by PCR using the primer
 383 pair 0946-F and 0946-R. The amplified DNA fragment was cloned between NdeI and BamHI
 384 sites of the pET-28a expression vector (EMD Millipore) to construct pEcyAbrB1.

385

386 **RNA extraction and RT-qPCR analysis**

387 Total RNA was extracted from cells using a phenol-based solution PGTX (57) and
 388 Zirconia/Silica beads (□ 0.1 mm). After treatment with DNase I (Takara Bio, Shiga, Japan),
 389 RNA was cleaned-up with NucleoSpin RNA kit (Takara Bio, Shiga, Japan). Synthesis of cDNA
 390 and qPCR were performed as described previously (26). Primers used in qPCR are listed in
 391 Table 1.

392

393 **Western-blotting analysis**

394 Cell pellets were resuspended with 400 μ l of extraction buffer [25 mM HEPES-NaOH (pH 7.5),
395 1 mM EDTA, 5 mM 2-mercaptoethanol and 1 $\times$ protease inhibitor cocktail (Roche)]. Cells were
396 disrupted using Bullet Blender (Next Advance) in the presence of 0.5 g of stainless steel beads
397 (ϕ 0.2 mm) with 3 cycles of agitation for 3 min and cooling for 3 min. Beads and cell debris
398 were removed by centrifugation at 21,000 $\times g$ for 3 min. Western-blotting analysis was
399 conducted as follows: Equal amounts of total protein were separated on a denaturing
400 SDS-polyacrylamide gel and blotted onto a polyvinylidene fluoride (PVDF) membrane.
401 cyAbrB1 was detected using a rabbit polyclonal antibody raised against His-cyAbrB1 from
402 *Synechocystis* (20).

403

404 **Enrichment of heterocysts**

405 The enrichment of heterocysts from *Anabaena* filaments was performed as described previously
406 (26).

407

408 **Expression and purification of His-cyAbrB1**

409 *E. coli* BL21(DE3) harboring pEcyabrB1 was grown at 37°C in 250 ml of Luria-Bertani
410 medium. The recombinant gene was expressed in exponentially growing cells (an OD₆₀₀ of 0.6)
411 by adding 1 mM isopropyl- β -D-thiogalactopyranoside. After 5 h of incubation, the cells were
412 harvested by centrifugation. His-cyAbrB1 was purified with the Ni-NTA Fast Start kit (Qiagen).
413 The elution fractions containing the purified protein were loaded onto a PD MidiTrap G-25
414 column (GE healthcare) equilibrated with 20 mM phosphate buffer (pH 7.4) containing 0.5 M
415 NaCl and 10% glycerol, and the protein was eluted with the same buffer.

416

417 **Gel mobility shift assay**

418 The *hetP* promoter region was amplified by PCR using primer pair PhetP-F and PhetP-R and
 419 was cloned into the EcoRV site of the PBluescript II KS+ to construct pBPhetP. A Cy3-labeled
 420 probe PhetP was prepared by PCR using a Cy3-labeled M13-F primer and PhetP-R with
 421 pBPhetP as a template. His-cyAbrB1 was incubated with a Cy3-labeled probe (3 nM) in 20 µl
 422 of incubation buffer [20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 40 ng/µl
 423 BSA, and 5% glycerol] for 30 min at room temperature. The mixtures were subjected to
 424 electrophoresis on a native 5% polyacrylamide gel and Cy3-labeled probes were detected on an
 425 FLA-9000 imaging system (FUJI Film). Non-labeled DNA probes PhetP, PhepA, and
 426 cyabrB1RT were prepared by PCR using primer pair PhetP-F and PhetP-R, primer pair PhepA-F
 427 and PhepA-R, and primer pair RTcyabrB1-F and RTcyabrB1-R, respectively.

428

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432

433 **References**

- 434 1. Whitton, D. A. 2012 *Ecology of cyanobacteria II: their diversity in space and time* Springer.
- 435
- 436 2. Murata, N., and Suzuki, I. 2006 Exploitation of genomic sequences in a systematic analysis to
- 437 access how cyanobacteria sense environmental stress. *J Exp Bot* 57:235–247.
- 438
- 439 3. Kumar, K., Mella-Herrera, R. A., and Golden, J.W. 2010 Cyanobacterial heterocysts. *Cold*
- 440 *Spring Harb. Perspect. Biol.* 2:a000315.
- 441
- 442 4. Flores, E., Picossi, S., Valladares, A., and Herrero, A. 2018 Transcriptional regulation of
- 443 development in heterocyst-forming cyanobacteria. *Biochim Biophys Acta*
- 444 doi:10.1016/j.bbagr.2018.04.006..
- 445
- 446 5. Valladares, A., Flores, E., and Herrero, A. 2008 Transcription activation by NtcA and
- 447 2-oxoglutarate of three genes involved in heterocyst differentiation in the cyanobacterium
- 448 *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 190:6126–6133.
- 449
- 450 6. Ehira, S., and Ohmori, M. 2006 NrrA directly regulates expression of *hetR* during heterocyst
- 451 differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.*
- 452 188:8520–8525.
- 453
- 454 7. Black, T. A., Cai, Y., and Wolk, C. P. 1993 Spatial expression and autoregulation of *hetR*, a
- 455 gene involved in the control of heterocyst development in *Anabaena*. *Mol. Microbiol.* 9:77–84.
- 456

- 457 8. Zhou, R., Cao, Z., and Zhao, J. 1998 Characterization of HetR protein turnover in *Anabaena*
458 sp. PCC 7120. Arch Microbiol 169:417–423.
459
- 460 9. Buikema, W. J., and Haselkorn, R. 2001 Expression of the *Anabaena hetR* gene from a
461 copper-regulated promoter leads to heterocyst differentiation under repressing conditions. Proc.
462 Natl. Acad. Sci. USA 98:2729–2734.
463
- 464 10. Simm, S., Keller, M., Selymes, M., and Schleiff, E. 2015 The composition of the global and
465 feature specific cyanobacterial core-genomes. Front Microbiol
466 6:doi:10.3389/fmicb.2015.00219.
467
- 468 11. Tiruveedula, G. S. S., and Wangikar, P. P. 2017 Gene essentiality, conservation index and
469 co-evolution of genes in cyanobacteria. PLoS ONE 12:e0178565.
470
- 471 12. Rubin, B. E., Wetmore, K. M., Price, M. N., Diamond, S., Shultzaberger, R. K., Lowe, L. C.,
472 Curtin, G., Arkin, A. P., Deutschbauer, A., and Golden, S. S. 2015 The essential gene set of a
473 photosynthetic organism. Proc. Natl. Acad. Sci. USA 112:E6634–6643.
474
- 475 13. Ishii, A., and Hihara, Y. 2008 An AbrB-like transcriptional regulator, Sll0822, is essential
476 for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. Plant Physiol
477 148:660–670.
478
- 479 14. Oliveira, P., and Lindblad, P. 2008 An AbrB-like protein regulates the expression of the
480 bidirectional hydrogenase in *Synechocystis* sp. strain PCC 6803. J. Bacteriol. 190:1011–1019.

481

482 15. Agervald, A., Zhang, X., Stensjö, K., Devine, E., and Lindblad, P. 2010 CalA, a

483 cyanobacterial AbrB protein, interacts with the upstream region of *hypC* and acts as a repressor

484 of its transcription in the cyanobacterium *Nostoc* sp. strain PCC 7120. *Appl Environ Microbiol.*

485 76:880–890.

486

487 16. He, D., and Xu, X. 2010 CalA, a cyAbrB protein, binds to the upstream region of *ftsZ* and is

488 down-regulated in heterocysts in *Anabaena* sp. PCC 7120. *Arch Microbiol* 192:461–469.

489

490 17. Lieman-Hurwitz, J., Haimovich, M., Shalev-Malul, G., Ishii, A., Hihara, Y., Gaathon, A.,

491 Lebendiker, M., and Kaplan, A. 2009 A cyanobacterial AbrB-like protein affects the apparent

492 photosynthetic affinity for CO₂ by modulating low-CO₂-induced gene expression. *Environ*

493 *Microbiol* 11:927–936.

494

495 18. Kaniya, Y., Kizawa, A., Miyagi, A., Kawai-Yamada, M., Uchimiya, H., Kaneko, Y.,

496 Nishiyama, Y., and Hihara, Y. 2013 Deletion of the transcriptional regulator *cyAbrB2*

497 deregulates primary carbon metabolism in *Synechocystis* sp. PCC 6803. *Plant Physiol*

498 162:1153–1163.

499

500 19. Orf, I., Schwarz, D., Kaplan, A., Kopka, J., Hess, W. R., Hagemann, M., and Klähn, S. 2016

501 CyAbrB2 contributes to the transcriptional regulation of low CO₂ acclimation in *Synechocystis*

502 sp. PCC 6803. *Plant Cell Physiol.* 57:2232–2243.

503

504 20. Yamauchi, Y., Kaniya, Y., Kaneko, Y., and Hihara, Y. 2011 Physiological roles of the

505 cyAbrB transcriptional regulator pair Sll0822 and Sll0359 in *Synechocystis* sp. strain PCC 6803.
506 J. Bacteriol. 193:3702–3709.
507
508 21. Knoot, C. J., Ungerer, J., Wangikar, P. P., and Pakrasi, H. B. 2018 Cyanobacteria:
509 Promising biocatalysts for sustainable chemical production. J. Biol. Chem. 293:5044–5052.
510
511 22. Humphreys, C. M., and Minton, N. P. 2018 Advances in metabolic engineering in the
512 microbial production of fuels and chemicals from C1 gas. Curr Opin Biotechnol 50:174–181.
513
514 23. Carroll, A. L., Case, A. E., Zhang, A., and Atsumi, S. 2018 Metabolic engineering tools in
515 model cyanobacteria. Metab Eng 50:47-56.
516
517 24. Yao, L., Cengic, I., Anfelt, J., and Hudson, E. P. 2016 Multiple gene repression in
518 cyanobacteria using CRISPRi. ACS Synth Biol 5:207–212.
519
520 25. Gordon, G. C., Korosh, T. C., Cameron, J. C., Markley, A. L., Begemann, M. B., and
521 Pfleger, B. F. 2016 CRISPR interference as a titratable, trans-acting regulatory tool for
522 metabolic engineering in the cyanobacterium *Synechococcus* sp. strain PCC 7002. Metab. Eng.
523 38:170–179.
524
525 26. Higo, A., Isu, A., Fukaya, Y., Ehira, S., and Hisabori, T. 2018 Application of CRISPR
526 interference for metabolic engineering of the heterocyst-forming multicellular cyanobacterium
527 *Anabaena* sp. PCC 7120. Plant Cell Physiol. 59:119–127.
528

529 27. Naduthodi, M. I. S., Barbosa, M. J., and van der Oost, J. 2018 Progress of CRISPR-Cas
530 based genome editing in photosynthetic microbes.. *Biotechnol J* 13:e1700591.
531

532 28. Behler, J., Vijay, D., Hess, W. R., and Akhtar, M. K. 2018 CRISPR-based technologies for
533 metabolic engineering in cyanobacteria. *Trends Biotechnol* 36:996-1010.
534

535 29. Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., and
536 Lim, W. A. 2013 Repurposing CRISPR as an RNA-guided platform for sequence-specific
537 control of gene expression. *Cell* 152:1173–1183.
538

539 30. Kaczmarzyk, D., Cengic, I., Yao, L., and Hudson, E. P. 2018 Diversion of the long-chain
540 acyl-ACP pool in *Synechocystis* to fatty alcohols through CRISPRi repression of the essential
541 phosphate acyltransferase PlsX. *Metab Eng* 45:59–66.
542

543 31. Katayama, M., and Ohmori, M. 1997 Isolation and characterization of multiple adenylate
544 cyclase genes from the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.*
545 179:3588–3593.
546

547 32. Higa, K. C., and Callahan, S. M. 2010 Ectopic expression of *hetP* can partially bypass the
548 need for *hetR* in heterocyst differentiation by *Anabaena* sp. strain PCC 7120. *Mol. Microbiol.*
549 77:562–574.
550

551 33. Videaud, P., Rivers, O. S., Hurd, K., Ushijima, B., Oshiro, R. T., Ende, R. J., O’Hanlon, S.
552 M., and Cozy, L. M. 2016 The heterocyst regulatory protein HetP and its homologs modulate

553 heterocyst commitment in *Anabaena* sp. strain PCC 7120. Proc. Natl. Acad. Sci. USA
554 113:E6984–E6992.
555
556 34. Du, Y., Cai, Y., Hou, S., and Xu, X. 2012 Identification of the HetR recognition sequence
557 upstream of *hetZ* in *Anabaena* sp. strain PCC 7120. J. Bacteriol. 194:2297
558
559 35. Videau, P., Rivers, O. S., Tom, S. K., Oshiro, R. T., Ushijima, B., Swenson, V. A., Philmus,
560 B., Gaylor, M. O., and Cozy, L. M. 2018 The *hetZ* gene indirectly regulates heterocyst
561 development at the level of pattern formation in *Anabaena* sp. strain PCC 7120. Mol. Microbiol.
562 109:91–104.
563
564 36. Zhang, H., Wang, S., Wang, Y., and Xu, X. 2018 Functional overlap of *hetP* and *hetZ* in
565 regulation of heterocyst differentiation in *Anabaena* sp. strain PCC 7120. J. Bacteriol.
566 200:e00707–e00717.
567
568 37. Holland, D., and Wolk, C. P. 1990 Identification and characterization of *hetA*, a gene that
569 acts early in the process of morphological differentiation of heterocysts. J. Bacteriol.
570 172:3131–3137.
571
572 38. Videau, P., Ni, S., Rivers, O. S., Ushijima, B., Feldmann, E. A., Cozy, L. M., Kennedy, M.
573 A., and Callahan, S. M. 2014 Expanding the direct HetR regulon in *Anabaena* sp. strain PCC
574 7120. J. Bacteriol. 196:1113–1121.
575
576 39. Asai, H., Iwamori, S., Kawai, K., Ehira, S., Ishihara, J., Aihara, K., Shoji, S., and Iwasaki, H.

577 2009 Cyanobacterial cell lineage analysis of the spatiotemporal *hetR* expression profile during
578 heterocyst pattern formation in *Anabaena* sp. PCC 7120.
579 PLoS One 4:e7371.
580
581 40. Agervald, A., Baebprasert, W., Zhang, X., Incharoensakdi, A., Lindblad, P., and Stensjö, K.
582 2010 The cyAbrB transcription factor CalA regulates the iron superoxide dismutase in *Nostoc*
583 sp. strain PCC 7120. Environ Microbiol 12:2826–2837.
584
585 41. Ehira, S., and Ohmori, M. 2006 NrrA, a nitrogen-responsive response regulator facilitates
586 heterocyst development in the cyanobacterium *Anabaena* sp. strain PCC 7120. Mol. Microbiol.
587 59:1692–1703.
588
589 42. Black, T., Cai, Y., and Wolk, C. P. 1993 Spatial expression and autoregulation of *hetR*, a
590 gene involved in the control of heterocyst development in *Anabaena*. Mol. Microbiol. 9:77–84.
591
592 43. Yoon, H. S., and Golden, J. W. 1998 Heterocyst pattern formation controlled by a diffusible
593 peptide. Science 282:935–938.
594
595 44. Sakr, S., Dutheil, J., Saenkham, P., Bottin, H., and Leplat, C. et al. 2013 The activity of the
596 *Synechocystis* PCC6803 AbrB2 regulator of hydrogen production can be post-translationally
597 controlled through glutathionylation. Int. J. Hydrog. Energ 38:13547–13555.
598
599 45. Higo, A., Horinouchi, S., and Ohnishi, Y. 2011 Strict regulation of morphological
600 differentiation and secondary metabolism by a positive feedback loop between two global

601 regulators AdpA and BldA in *Streptomyces griseus*. Mol. Microbiol. 81:1607–1622.

602

603 46. den Hengst, C. D., Tran, N. T., Bibb, M. J., Chandra, G., Leskiw, B. K., and Buttner, M. J.

604 2010 Genes essential for morphological development and antibiotic production in *Streptomyces*

605 *coelicolor* are targets of BldD during vegetative growth. Mol. Microbiol. 78:361–379.

606

607 47. Phillips, Z. E., and Strauch, M. A. 2002 *Bacillus subtilis* sporulation and stationary phase

608 gene expression. Cell Mol Life Sci. 2 59:392–402.

609

610 48. Bush, M. J., Tschowri, N., Schlimpert, S., Flärdh, K., and Buttner, M. J. 2015 c-di-GMP

611 signalling and the regulation of developmental transitions in streptomycetes. Nat Rev Microbiol

612 13:749-760.

613

614 49. Borthakur, P. B., Orozco, C. C., Young-Robbins, S. S., Haselkorn, R., and Callahan, S. M.

615 2005 Inactivation of *patS* and *hetN* causes lethal levels of heterocyst differentiation in the

616 filamentous cyanobacterium *Anabaena* sp. PCC 7120. Mol. Microbiol. 57:111–123.

617

618 50. Higa, K. C., Rajagopalan, R., Risser, D. D., Rivers, O. S., Tom, S. K., Videau, P., and

619 Callahan, S. M. 2012 The RGSGR amino acid motif of the intercellular signaling protein, HetN,

620 is required for patterning of heterocysts in *Anabaena* sp. strain PCC 7120. Mol. Microbiol.

621 83:682–693.

622

623 51. Valladares, A., Flores, E., and Herrero, A. 2016 The heterocyst differentiation

624 transcriptional regulator HetR of the filamentous cyanobacterium *Anabaena* forms tetramers

625 and can be regulated by phosphorylation. *Mol. Microbiol.* 99:808–819

626

627 52. Thomas, J. 1970 Absence of the pigments of photosystem II of photosynthesis in

628 heterocysts of a blue-green alga. *Nature* 228:181–183.

629

630 53. Cardona, T., Battchikova, N., Zhang, P., Stensjö, K., Aro, E. M., Lindblad, P., and

631 Magnuson, A. 2009 Electron transfer protein complexes in the thylakoid membranes of

632 heterocysts from the cyanobacterium *Nostoc punctiforme*. *Biochim Biophys Acta*

633 1787:252–263.

634

635 54. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. 1979 Generic

636 assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.*

637 111:1–61.

638

639 55. Fu, C., Donovan, W. P., Shikapwashya-Hasser, O., Ye, X., and Cole, R. H. 2014 Hot fusion:

640 An efficient method to clone multiple DNA fragments as well as inverted repeats without ligase.

641 PLoS ONE 9:e115318.

642

643 56. Cai, Y., and Wolk, C. P. 1990 Use of a conditionally lethal gene in *Anabaena* sp. strain PCC

644 7120 to select for double recombinants and to entrap insertion sequences. *J. Bacteriol.*

645

646

Table 1 Primers used in this study

Primer	Sequence (5' - 3')
# <i>cyabrB2</i> knockout	
2080-5F	AAGAGCTCGAGCTGGGATATCTTGAC
2080-5R	AGTCGCGAGCCACACTGTTTAGCTT
2080-3F	AGTCGCGAACCAGGAGATGAGTTTG
2080-3R	AACTCGAGTAGAGGATGCTATGCTTG
#His-cyAbrB1 expression	
0946-F	AACATATGACTGAAACTGCAACC
0946-R	AGGGATCCTTATTCTTCATCTTCCAA
#qPCR	
mpB_RT_F	CGTGAGGATAGTGCCACAGA
mpB_RT_R	ATAGTTCCTTCGGCCTTGCT
RTcyabrB1-F	ACCACGACGAGAAAGAGCTA
RTcyabrB1-R	TTGGTGCTTCTGGGCTAAGA
RThetR-F	CATCGATCTGATCAAGCGTCT
RThetR-R	CAGGGCTTGTCTGACTTCCT
RThetP-F	TGGTTGAAGCAATTCTGGCT
RThetP-R	TGTGCGGTAGGGAATGTAGT
hetZ_F_RT	AACCACCAGAAACCAACGTC
hetZ_R_RT	TCTGGTTCTGGTTGGGTAGC
RThetA-F	CAGGAATTAGCTGGGTTGACA
RThetA-R	ATTGAAGGTAGCACGCATCC
RTnifH-F	GCTTTCTACGGTAAAGGCGG
RTnifH-R	TGGAGTCAGCTTTAGGGTCG
alr0947_RT_F	ACGGGAAACTTTACCCCAAC
alr0947_RT_R	AGTAGCGATCGCCCAGACTA
#EMSA	
PhetP-F	TTCCGGATTTATGGTTGCAAC
PhetP-R	TTTGAGCATTTTCTACTAGG

PhepA-F	CAAAATTTAAAATTAAATACAG
PhepA-R	CTCACAATTTTATAGTTGTGCAGGG
Cy3-M13-F	Cy3-TTGTAACGACGGCCAGTG

647

648 **Figure legends**

649 Figure 1. Promotion of heterocyst formation by knockdown of *cyabrB1* in the presence of
 650 nitrate. Control strain lacking sgRNA (C100) and *cyabrB1* knockdown strain (C104) were
 651 cultured in nitrate-containing medium in the absence or presence of the inducer aTc (50 ng/ml).
 652 Cultures were bubbled with air containing 5% (v/v) CO₂. (A) Formation of heterocysts by
 653 *cyabrB1* knockdown in the presence of nitrate. Images were photographed after 48 h of
 654 cultivation. Arrowheads indicate heterocysts. (B) Confirmation of cyAbrB1 knockdown. After
 655 48 h of cultivation, total protein was extracted and western blotting using anti-cyAbrB1
 656 antibody was performed. Different amounts of total proteins from C100 cultured in the absence
 657 of aTc were loaded to show the linearity of the results. (C) Time course analysis of cyAbrB1
 658 knockdown. Each strain was cultivated for the indicated time in the presence of 50 ng/ml aTc.
 659 Subsequently, total protein was extracted and western blotting using anti-cyAbrB1 antibody was
 660 performed.

661
 662 Figure 2. Up-regulation of some genes related to heterocyst differentiation following
 663 knockdown of *cyabrB1*. After 48 h of cultivation in the absence or presence of 50 ng/ml aTc,
 664 RNA was extracted and RT-qPCR was performed. *rnpB* was used for normalization. Data
 665 represent mean $\pm$ SD (n = 3 from independent culture). Amounts of each gene relative to that in
 666 C100 grown in nitrate-containing medium bubbled with air containing 5% (v/v) CO₂ in the
 667 absence of the inducer are shown. (A) Each strain was grown in nitrate-containing medium and
 668 bubbled with air containing 5% (v/v) CO₂. (B) Strain C104 was grown in nitrate-containing
 669 medium bubbled with air containing 1% (v/v) CO₂, or in ammonium-containing medium
 670 bubbled with air containing 5% (v/v) CO₂.

671

672 Figure 3. Knockdown of *cyabrB1* in the *hetR* mutant background. Cells were cultured in
673 nitrate-containing medium bubbled with air containing 5% (v/v) CO₂ in the absence or presence
674 of aTc for 48 h. (A) Effect of *hetR* inactivation on expression of *cyabrB1* and genes related to
675 heterocyst formation. RNA was extracted and RT-qPCR was performed. *rnpB* was used for
676 normalization. Data represent mean $\pm$ SD (n = 3 from independent culture). Amounts of each
677 gene relative to that in C100 cultured in nitrate-containing medium bubbled with air containing
678 5% (v/v) CO₂ in the absence of the inducer are shown. (B) Confirmation of cyAbrB1
679 knockdown. Total protein was extracted and western blotting using anti-cyAbrB1 antibody was
680 performed. (C) No heterocyst formation by *cyabrB1* knockdown in *hetR* deletion background.
681 Cells of C104h were micro-photographed.

682
683 Figure 4. Specific and redundant functions of *cyabrB* genes. Cells were cultured in
684 nitrate-containing medium bubbled with air containing 1% (v/v) CO₂ in the absence or presence
685 of aTc for 48 h. (A) Formation of heterocysts by *cyabrB1* knockdown and *cyabrB2* knockout.
686 Cells of C104B2 or DR2080 were micro-photographed. (B) RNA was extracted and RT-qPCR
687 was performed. *rnpB* was used for normalization. Data represent the mean $\pm$ SD (n = 3 from
688 independent culture). Amounts of each gene relative to that in C100 grown in nitrate-containing
689 medium bubbled with air containing 5% (v/v) CO₂ in the absence of the inducer are shown.

690
691 Figure 5. Direct binding of cyAbrB1 to promoters of *hetP* and *hepA*. (A) Purification of
692 His-cyAbrB1. Purified His-cyAbrB1 was subjected to 15% SDS-PAGE. Lane M, protein
693 molecular weight marker; lane His-cyAbrB1, purified His-cyAbrB1. (B) Electrophoresis
694 mobility shift assay with His-cyAbrB1. cyAbrB1 was mixed with 3 nM DNA probe (promoter
695 region of *hetP*). Non-labeled DNAs (PhetP, PhepA, or internal region of *cyabrB1* (*cyabrB1RT*))

696 were added.

697

698 Figure 6. Effect of *cyabrB1* over-expression on heterocyst development. Cells of the negative
699 control strain C100 and *cyabrB1* over-expression strain T121 were cultured in the absence or
700 presence of 200 ng/ml aTc. (A) Impaired growth under nitrogen-depleted conditions by *cyabrB1*
701 over-expression. Cells were cultured in nitrogen-free or nitrate-containing medium and OD₇₅₀
702 was monitored. Data represent mean $\pm$ SD (n= 3 from independent culture). (B) Effect of
703 *cyabrB1* over-expression on heterocyst formation. Cells of C100 and T121 cultured for 24 h in
704 the absence or presence of the inducer were micro-photographed. (C) Effect of *cyabrB1*
705 over-expression on expression of genes related to heterocyst formation. After 8 h of nitrogen
706 depletion, RNA was extracted and RT-qPCR was performed. *rnpB* was used for normalization.
707 Data represent mean $\pm$ SD (n = 3 from independent culture). Amounts of each gene relative to
708 that in C100 grown in nitrate-free medium in the absence of the inducer are shown.
709 Over-expression of cyAbrB1 was confirmed by western blotting.

710

711

712 Figure S1. Schematic representation of the genetic backgrounds of strains used in the present
713 study. Components related to CRISPRi system or induction of *cyabrB1* were integrated at
714 neutral site *cyaA* locus by single homologous recombination. Note that sizes of genes in the map
715 are not proportional to sequence lengths.

716

717 Figure S2. Knockdown of *cyabrB1* leads to formation of heterocysts in the presence of nitrate,
718 but not in the presence of ammonium. Cells were cultured for 48 h in the absence or presence of
719 aTc and micro-photographed. Arrowheads indicate heterocysts. (A) *cyabrB1* knockdown strain

720 (C104) was cultured in nitrate-containing medium and bubbled with air containing 1% (v/v)
 721 CO₂. (B) *cyabrB1* knockdown strain (C104) was cultured in ammonium-containing medium
 722 bubbled with air containing 5% (v/v) CO₂.
 723
 724 Figure S3. Knockdown of *cyabrB1* using sgRNA targeting different sites of *cyabrB1* led to
 725 formation of heterocysts in the presence of nitrate. Cells were cultured for 48 h in the absence or
 726 presence of aTc and micro-photographed. Arrowheads indicate heterocysts. Each *cyabrB1*
 727 knockdown strain was cultured in nitrate-containing medium bubbled with air containing 5%
 728 (v/v) CO₂.
 729
 730 Figure S4. Confirmation of *cyabrB1* knockdown. (A and C) After 48 h of cultivation in the
 731 absence or presence of 50 ng/ml aTc, RNA was extracted and RT-qPCR was performed. *rnpB*
 732 was used for normalization. Data represent mean $\pm$ SD (n = 3 from independent culture).
 733 Amounts of each gene relative to that in C100 grown in nitrate-containing medium bubbled
 734 with air containing 5% (v/v) CO₂ in the absence of the inducer are shown. (B and D) After 48 h
 735 of cultivation in the absence or presence of 50 ng/ml aTc, total protein was extracted and
 736 western blotting using anti-cyAbrB1 antibody was performed. (A and B) Each strain was grown
 737 in nitrate-containing medium and bubbled with air containing 5% (v/v) CO₂. (C and D) Strain
 738 C104 was cultured in nitrate-containing medium bubbled with air containing 5 or 1% (v/v) CO₂
 739 or in ammonium-containing medium bubbled with air containing 5% (v/v) CO₂.
 740
 741 Figure S5. Expression of *nifH* encoding nitrogenase subunit in *cyabrB1* knockdown strain C104
 742 under different conditions. After 48 h of cultivation under the indicated conditions in the
 743 absence or presence of 50 ng/ml aTc, RNA was extracted and RT-qPCR was performed. *rnpB*

744 was used for normalization. Data represent mean $\pm$ SD (n = 3 from independent culture).

745 Amounts of *nifH* relative to that in C100 grown in nitrate-containing medium bubbled with air

746 containing 5% (v/v) CO₂ in the absence of the inducer are shown.

747

748 Figure S6. Knockdown of *cyabrB1* in *cyabrB2* deletion background. Cells were cultured in

749 nitrogen-containing media bubbled with air containing 1% (v/v) CO₂ in the absence or presence

750 of aTc for 48 h. (A) Confirmation of *cyabrB1* knockdown. Cells were cultured in the presence

751 of nitrate. RNA was extracted and RT-qPCR was performed. *rnpB* was used for normalization.

752 Data represent mean $\pm$ SD (n = 3 from independent culture). Amounts of *cyabrB1* relative to

753 that in C100 grown in nitrate-containing medium bubbled with air containing 5% (v/v) CO₂ in

754 the absence of the inducer are shown. (B) Confirmation of cyAbrB1 knockdown. Each strain

755 was grown in the presence of nitrate or ammonium-containing medium. Total protein was

756 extracted and western blotting using anti-cyAbrB1 antibody was performed. (C) No heterocyst

757 formation of 104B2 following *cyabrB1* knockdown in ammonium-medium. Cells of C104B2

758 were cultured in the presence of ammonium and were micro-photographed.

759

760 Figure S7. Heterocyst formation in *cyabrB1* knockdown strain C104 in the absence of combined

761 nitrogen sources. Nitrate was depleted from the culture medium in strain C104. Subsequently,

762 C104 was cultured in nitrogen-free medium in the absence or presence of 50 ng/ml aTc. Images

763 were photographed after 24 (A) or 48 h (B) of cultivation. Arrowheads indicate heterocysts. (C)

764 Lengths of vegetative cell intervals between heterocysts in C104 cultured in nitrogen-free

765 medium for 48 h in the absence (gray bars) or presence of aTc (black bars). (D) Confirmation of

766 cyAbrB1 knockdown under nitrogen-fixation conditions. After 48 h of cultivation in

767 nitrogen-free medium in the absence or presence of 50 ng/ml aTc, total protein was extracted

768 and western blotting using anti-cyAbrB1 antibody was performed.

769

770 Figure S8. Expression of cyAbrB1 in wild type background. (A and B) Expression of cyAbrB1

771 after nitrogen depletion. Cells of PCC 7120 cultured in nitrate medium were washed two times

772 with nitrogen-free medium. Subsequently, cells were resuspended in nitrate-medium (+N) or

773 nitrogen-free medium (-N) and were cultured for 8 h. (A) Total protein was extracted and

774 western blotting using anti-cyAbrB1 was performed. (B) Relative amounts of cyAbrB1

775 compared to that before nitrogen depletion were quantified from western blots. Data represent

776 mean $\pm$ SD (n = 3 from independent culture). (C and D) Expression of cyAbrB1 in heterocysts.

777 (C) After 24 h of nitrogen depletion, heterocysts were enriched. Total protein was extracted

778 from whole filaments (lane W) and enriched heterocysts (lane H). Western blotting using

779 anti-cyAbrB1, anti-RbcL, and anti-NifH antibodies was performed. RbcL and NifH are marker

780 proteins for vegetative cells and heterocysts, respectively. (D) Relative amounts of cyAbrB1 in

781 heterocysts compared to that in whole filaments were quantified from western blots. Data

782 represent mean $\pm$ SD (n = 3 from independent culture).

783

784 Figure S9. Effect of *cyabrB1* knockdown or over-expression on expression of *alr0947*. RNA

785 was extracted and RT-qPCR was performed. *rnpB* was used for normalization. Data represent

786 mean $\pm$ SD (n = 3 from independent culture). (A and B) Cells were grown in the absence or

787 presence of aTc for 48 h. (A) Each strain was grown in nitrate-containing medium bubbled with

788 air containing 5% (v/v) CO₂. (B) C104 was grown under indicated conditions. Amounts of

789 *alr0947* relative to that in C100 cultured in nitrate-containing medium bubbled with air

790 containing 5% (v/v) CO₂ in the absence of the inducer are shown. (C) Each strain was grown in

791 nitrogen-free medium for 8 h. Data represent mean $\pm$ SD (n = 3 from independent culture).

792 Amounts of *alr0947* relative to that in C100 cultured in nitrate-free medium in the absence of
793 the inducer are shown.

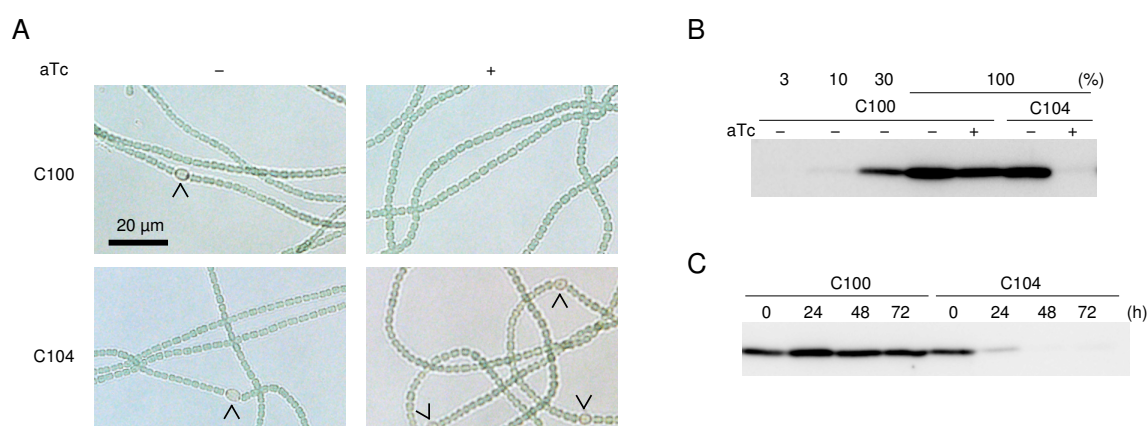


Fig. 1. Promotion of heterocyst formation by knockdown of *cyabrB1* in the presence of nitrate. Control strain lacking sgRNA (C100) and *cyabrB1* knockdown strain (C104) were cultured in nitrate-containing medium in the absence or presence of the inducer aTc (50 ng/ml). Cultures were bubbled with air containing 5% (v/v) CO₂. (A) Formation of heterocysts by *cyabrB1* knockdown in the presence of nitrate. Images were photographed after 48 h of cultivation. Arrowheads indicate heterocysts. (B) Confirmation of cyAbrB1 knockdown. After 48 h of cultivation, total protein was extracted and western blotting using anti-cyAbrB1 antibody was performed. Different amounts of total proteins from C100 cultured in the absence of aTc were loaded to show the linearity of the results. (C) Time course analysis of cyAbrB1 knockdown. Each strain was cultivated for the indicated time in the presence of 50 ng/ml aTc. Subsequently, total protein was extracted and western blotting using anti-cyAbrB1 antibody was performed.

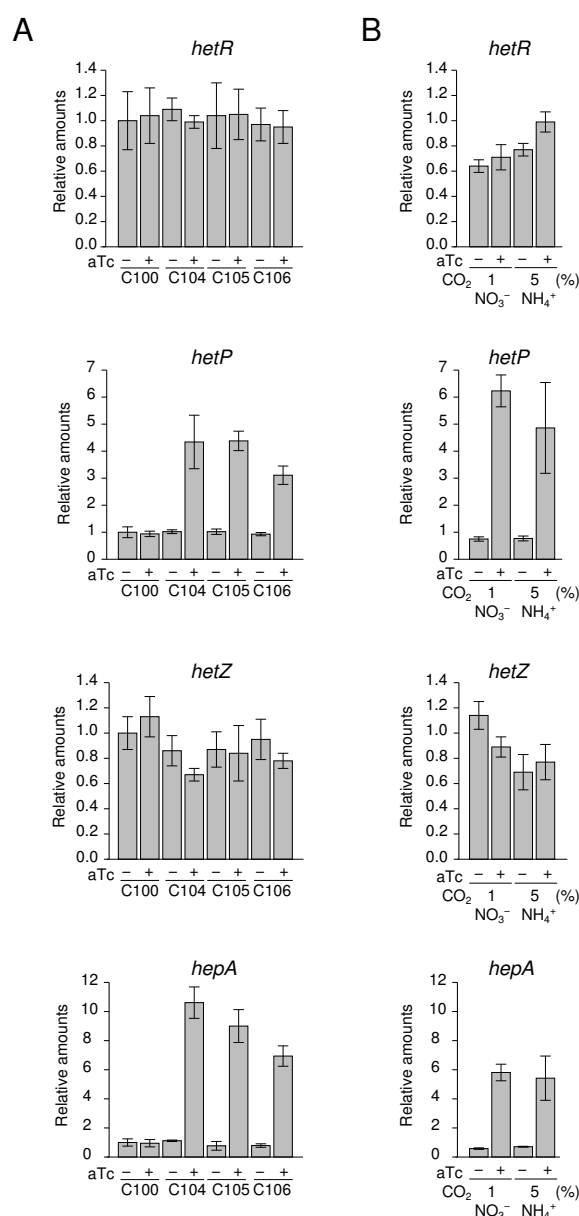


Fig. 2. Up-regulation of some genes related to heterocyst differentiation following knockdown of *cyabrB1*. After 48 h of cultivation in the absence or presence of 50 ng/ml aTc, RNA was extracted and RT-qPCR was performed. *rnpB* was used for normalization. Data represent mean $\pm$ SD (n = 3 from independent culture). Amounts of each gene relative to that in C100 grown in nitrate-containing medium bubbled with air containing 5% (v/v) CO₂ in the absence of the inducer are shown. (A) Each strain was grown in nitrate-containing medium and bubbled with air containing 5% (v/v) CO₂. (B) Strain C104 was grown in nitrate-containing medium bubbled with air containing 1% (v/v) CO₂, or in ammonium-containing medium bubbled with air containing 5% (v/v) CO₂.

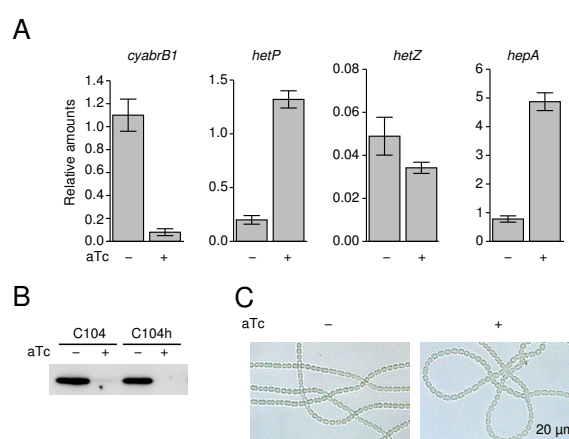


Fig. 3. Knockdown of *cyabrB1* in the *hetR* mutant background. Cells were cultured in nitrate-containing medium bubbled with air containing 5% (v/v) CO₂ in the absence or presence of aTc for 48 h. (A) Effect of *hetR* inactivation on expression of *cyabrB1* and genes related to heterocyst formation. RNA was extracted and RT-qPCR was performed. *rnpB* was used for normalization. Data represent mean $\pm$ SD (n = 3 from independent culture). Amounts of each gene relative to that in C100 cultured in nitrate-containing medium bubbled with air containing 5% (v/v) CO₂ in the absence of the inducer are shown. (B) Confirmation of cyAbrB1 knockdown. Total protein was extracted and western blotting using anti-cyAbrB1 antibody was performed. (C) No heterocyst formation by *cyabrB1* knockdown in *hetR* deletion background. Cells of C104h were micro-photographed.

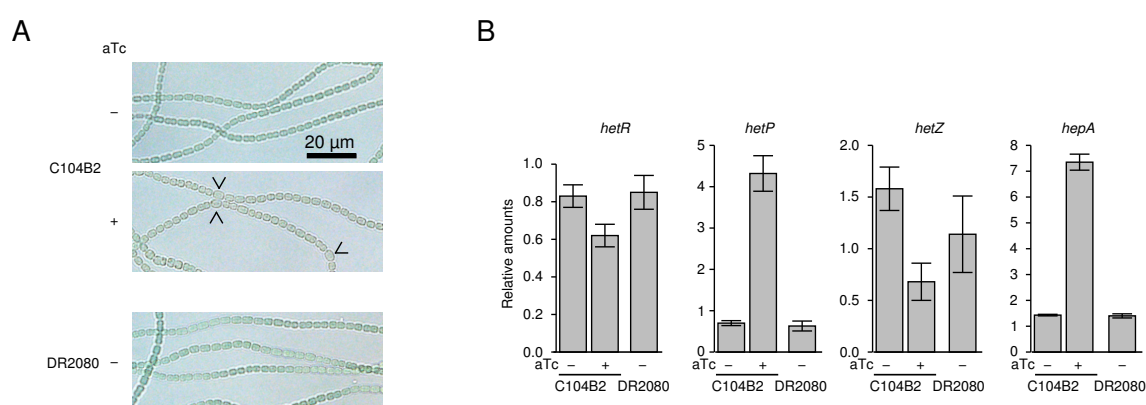


Fig. 4. Specific and redundant functions of *cyabrB* genes. Cells were cultured in nitrate-containing medium bubbled with air containing 1% (v/v) CO₂ in the absence or presence of aTc for 48 h. (A) Formation of heterocysts by *cyabrB1* knockdown and *cyabrB2* knock-out. Cells of C104B2 or DR2080 were micro-photographed. (B) RNA was extracted and RT-qPCR was performed. *rnpB* was used for normalization. Data represent the mean $\pm$ SD (n = 3 from independent culture). Amounts of each gene relative to that in C100 grown in nitrate-containing medium bubbled with air containing 5% (v/v) CO₂ in the absence of the inducer are shown.

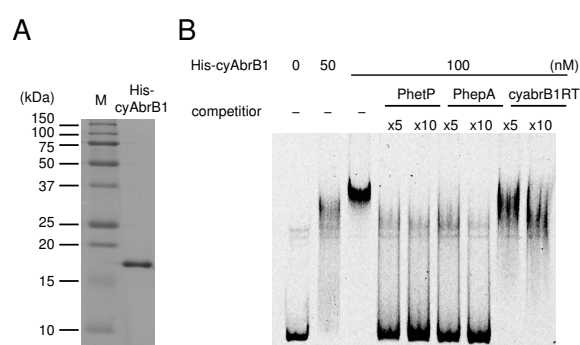


Fig. 5. Direct binding of cyAbrB1 to promoters of *hetP* and *hepA*. (A) Purification of His-cyAbrB1. Purified His-cyAbrB1 was subjected to 15% SDS-PAGE. Lane M, protein molecular weight marker; lane His-cyAbrB1, purified His-cyAbrB1. (B) Electrophoresis mobility shift assay with His-cyAbrB1. cyAbrB1 was mixed with 3 nM DNA probe (promoter region of *hetP*). Non-labeled DNAs (PhetP, PhepA, or internal region of *cyabrB1* (cyabrB1RT)) were added.

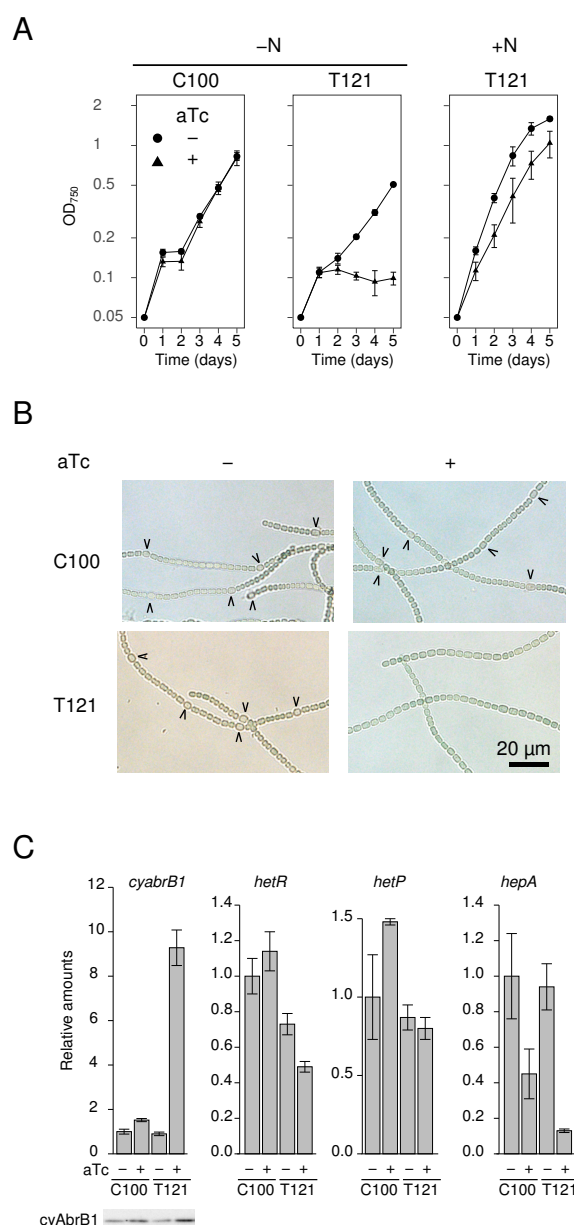


Fig. 6. Effect of *cyabrB1* over-expression on heterocyst development. Cells of the negative control strain C100 and *cyabrB1* over-expression strain T121 were cultured in the absence or presence of 200 ng/ml aTc. (A) Impaired growth under nitrogen-depleted conditions by *cyabrB1* over-expression. Cells were cultured in nitrogen-free or nitrate-containing medium and OD₇₅₀ was monitored. Data represent mean $\pm$ SD (n= 3 from independent culture). (B) Effect of *cyabrB1* over-expression on heterocyst formation. Cells of C100 and T121 cultured for 24 h in the absence or presence of the inducer were micro-photographed. (C) Effect of *cyabrB1* over-expression on expression of genes related to heterocyst formation. After 8 h of nitrogen depletion, RNA was extracted and RT-qPCR was performed. *rnpB* was used for normalization. Data represent mean $\pm$ SD (n = 3 from independent culture). Amounts of each gene relative to that in C100 grown in nitrate-free medium in the absence of the inducer are shown. Over-expression of cyAbrB1 was confirmed by western blotting.