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Application of CRISPR Interference for Metabolic Engineering of the Heterocyst-Forming Multicellular Cyanobacterium *Anabaena* sp. PCC 7120

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***Anabaena* sp. PCC 7120 (*A.* 7120) is a heterocyst-forming multicellular cyanobacterium that performs nitrogen fixation. This cyanobacterium has been extensively studied as a model for multicellularity in prokaryotic cells. We have been interested in photosynthetic production of nitrogenous compounds using *A.* 7120. However, the lack of efficient gene repression tools has limited its usefulness. We originally developed an artificial endogenous gene repression method in this cyanobacterium using small antisense RNA. However, the narrow dynamic range of repression of this method needs to be improved. Recently, clustered regularly interspaced short palindromic repeat (CRISPR) interference (CRISPRi) technology was developed and was successfully applied in some unicellular cyanobacteria. The technology requires expression of nuclease-deficient CRISPR-associated protein 9 (dCas9) and a single guide RNA (sgRNA) that is complementary to a target sequence, to repress expression of the target gene. In this study, we employed CRISPRi technology for photosynthetic production of ammonium through repression of *glnA*, the only gene encoding glutamine synthetase that is essential for nitrogen assimilation in *A.* 7120. By strictly regulating dCas9 expression using the TetR gene induction system, we succeeded in fine-tuning the GlnA protein in addition to the level of *glnA* transcripts. Expression of sgRNA by the heterocyst-specific *nifB* promoter led to efficient repression of GlnA in heterocysts, as well as in vegetative cells. Finally, we showed that ammonium is excreted into the medium only when inducers of expression of dCas9 were added. In conclusion, CRISPRi enables temporal control of desired products and will be a useful tool for basic science.**

Keywords: CRISPRi • Cyanobacteria • Gene regulation • Metabolic engineering.

Abbreviations: aTc, anhydrotetracycline; CRISPR, clustered regularly interspaced short palindromic repeat; CRISPRi, CRISPR interference; dCas9, nucleotide-deficient CRISPR-associated protein 9; RBS, ribosome-binding site; RT–qPCR, quantitative reverse transcription–PCR; sgRNA, single guide RNA; STAR, small transcription-activating RNA; Theo, theophylline.

Introduction

Anabaena sp. PCC 7120 (*A.* 7120) is a filamentous cyanobacterium that performs nitrogen fixation. This cyanobacterium performs oxygen-evolving photosynthesis in vegetative cells. When nitrogen sources are limited, vegetative cells at semi-regular intervals differentiate into heterocysts, which are terminally differentiated cells specialized for nitrogen fixation (Kumar et al. 2010). Hence, this cyanobacterium is a simple multicellular organism consisting of two types of cells. Because of this simplicity and ease of genetic manipulation, unique studies have been performed using *A.* 7120 as a model for multicellularity in prokaryotic cells. These include dynamic and complex spatio-temporal gene expression during differentiation of heterocysts (Ow et al. 2008, Ehira 2013), pattern formation (formation of heterocysts at semi-regular intervals) (Zhang et al. 2006) and intercellular communication, such as exchange of metabolites and signaling molecules through 'septal junctions' (Flores et al. 2015, Herrero et al. 2016). Studies on heterocyst development have also provided insight into global nitrogen and carbon metabolism in cyanobacteria (Herrero et al. 2001). In addition, we have focused on metabolic engineering of this cyanobacterium toward photosynthetic production of valuable nitrogenous compounds (Higo et al. 2016).

However, insufficiency of tools for gene regulation has limited the usefulness of *A.* 7120 to date. Creation of a knockout mutant takes at least 2 months by conventional methods in *A.* 7120 because of its long doubling time. Further, study of essential genes is difficult in this cyanobacterium due to lack of predictable and precisely controllable promoters and efficient gene repression systems. Furthermore, the insufficiency of gene regulation tools leads to genetic instability due to uncontrolled expression of genes when metabolic engineering is performed (Jones 2014). Recently, we have developed gene regulation tools in *A.* 7120 (Higo et al. 2016, Higo et al. 2017, 2018). We constructed TetR–anhydrotetracycline (aTc) gene induction systems that functions effectively in *A.* 7120. In the absence of the inducer aTc, expression of a target gene is strictly

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repressed by binding of TetR to its binding sites located in a synthetic *L03* promoter (Huang and Lindblad 2013). In contrast, the inducer dissociates TetR from the *L03* promoter, leading to induction of the target gene. Moreover, we succeeded in conditional knockdown of a gene using an artificially designed small RNA in combination with the TetR system, though with a narrow dynamic range (Higo et al. 2016). Thus, an efficient conditional knockdown tool in *A.* 7120 remains to be developed.

CRISPR (clustered regularly interspaced short palindromic repeat), a prokaryotic intrinsic defense system to degrade exogenous DNA sequences (Sorek et al. 2013), has been repurposed for control of gene expression [CRISPR interference (CRISPRi) (Qi et al. 2013)], as well as for genome editing (Hsu et al. 2014). CRISPRi technology has been developed for repression of target genes in a sequence-specific manner from the *Streptococcus pyogenes* CRISPR–Cas9 (CRISPR-associated protein 9) system. The system requires dCas9, a nuclease-deficient Cas9 protein, and a single guide RNA (sgRNA) that is complementary to the target DNA sequence. The dCas9 and sgRNA complex binds to a target DNA sequence when the target sequence is followed by a protospacer adjacent motif (PAM, 5′-NGG-3′ in the case of *S. pyogenes* Cas9), leading to inhibition of initiation or elongation of transcription (Qi et al. 2013). This technology was originally developed in *Escherichia coli* (Qi et al. 2013). Thereafter, it was found that gene expression is efficiently repressed using the CRISPRi system in diverse prokaryotic and eukaryotic cells (Gilbert et al. 2013, Choudhary et al. 2015, Peters et al. 2016).

Recently, CRISPRi technology was successfully used in the unicellular cyanobacteria *Synechocystis* sp. PCC 6803 (Yao et al. 2016), and *Synechococcus* sp. PCC 7002 (Gordon et al. 2016) and PCC 7942 (Huang et al. 2016). CRISPRi enabled enhancement of photosynthetic production of some valuable products, as well as tunable gene expression and conditional knockdown of essential genes in these cyanobacteria. Application of CRISPRi technology to *A.* 7120 might be more challenging because of the long doubling time (approximately 30 h under nitrogen fixation conditions) compared with that of unicellular cyanobacteria (<10 h). A decrease in the expression level of a protein could take a long time without active cell division after induction of knockdown of gene expression, especially if the protein has a long half-life time.

In the present study, we applied CRISPRi technology as a conditional gene knockdown system in *A.* 7120. We targeted an essential gene, *glnA*, the only gene encoding glutamine synthetase, which is an initial enzyme in the nitrogen assimilation pathway. Repression of the *glnA* gene leads to excretion of ammonium (Chapman and Meeks 1983, Higo et al. 2016). By strictly regulating the amount of dCas9 using the TetR gene induction system, we succeeded in fine-tuning of the GlnA expression level in both vegetative cells and heterocysts. Ammonium production was completely switched on and off by the addition of inducers for gene expression. We also showed a successful conditional knockdown of *devH*, encoding a transcriptional regulator that is essential for heterocyst maturation (Hebbar and Curtis 2000, Ramírez et al. 2005). Thus, we established CRISPRi technology in *A.* 7120 to enable physiological study, as well as conditional photosynthetic production of desired products.

Results

sgRNA targeting of different sites

First, we examined suitable sites for sgRNA targeting. We constructed a CRISPRi system for repression of *glnA* using P_{nirA}-*tetR* and the TetR-repressive *L03* promoter (Higo et al. 2016) to induce both *dcas9* with an optimized ribosome-binding site (RBS), designed by RBS calculator (Espah Borujeni et al. 2014) (RBS1), and sgRNA (Supplementary Fig. S1A), in a shuttle vector that replicates in *A.* 7120. In this system, expression under the *L03* promoter is induced by depletion of nitrate from the medium [e.g. under nitrogen-fixing conditions (Frías and Flores 2010)], which decreases expression of *tetR* by the *nirA* promoter, and it is further induced by aTc (Higo et al. 2016). Three sgRNAs were designed. The first one targeted the non-template strand overlapping the NtcA-binding site, which is essential for induction of *glnA* by nitrogen deficiency (Valladares et al. 2004). The second one targeted the template strand overlapping the promoter region of the strongest transcriptional start site activated by NtcA (Valladares et al. 2004) and the third one targeted the non-template strand of the *glnA* coding region (a–c in Supplementary Fig. S1B). To avoid potential off-target binding, the sequences were chosen using a BLAST search against the *A.* 7120 genomic sequence. Four plasmids, each with or without sgRNA, were constructed and were introduced into *A.* 7120. We extracted total RNA from cells grown under nitrogen fixation conditions (ammonium-producing conditions from dinitrogen) with or without induction by aTc, and performed quantitative reverse transcription–PCR (RT–qPCR) analysis. In a strain without sgRNA, the expression level of *glnA* was similar to that in a strain with empty vector, irrespective of the addition of aTc (Supplementary Fig. S1C). In the three strains containing the different sgRNAs, the expression level of *glnA* decreased to 20–30% of the control strain, even without the inducer. Induction by aTc produced little further repression of *glnA* expression. This may be because slight induction of dCas9 and/or sgRNA by depletion of nitrate is sufficient for maximal repression of *glnA*. These results indicate that although each sgRNA is functional, very strict regulation of dCas9 and/or the sgRNA is essential for titratable regulation of genes (Gordon et al. 2016, Huang et al. 2016, Yao et al. 2016).

Efficient GlnA repression by strict regulation of dCas9

We aimed to regulate *dcas9 strictly* using the TetR system. Expression of *tetR* was controlled by P_{petE}. Because P_{petE} is active irrespective of the nitrogen source in the presence of copper ions (Buikema and Haselkorn 2001), *dcas9* under the *L03* promoter is strictly repressed by TetR in both nitrate-replete and depleted conditions without aTc (Higo et al. 2016). When both *dcas9* and sgRNA are induced by aTc, it is difficult to distinguish which expression is rate-limiting factor for repression by

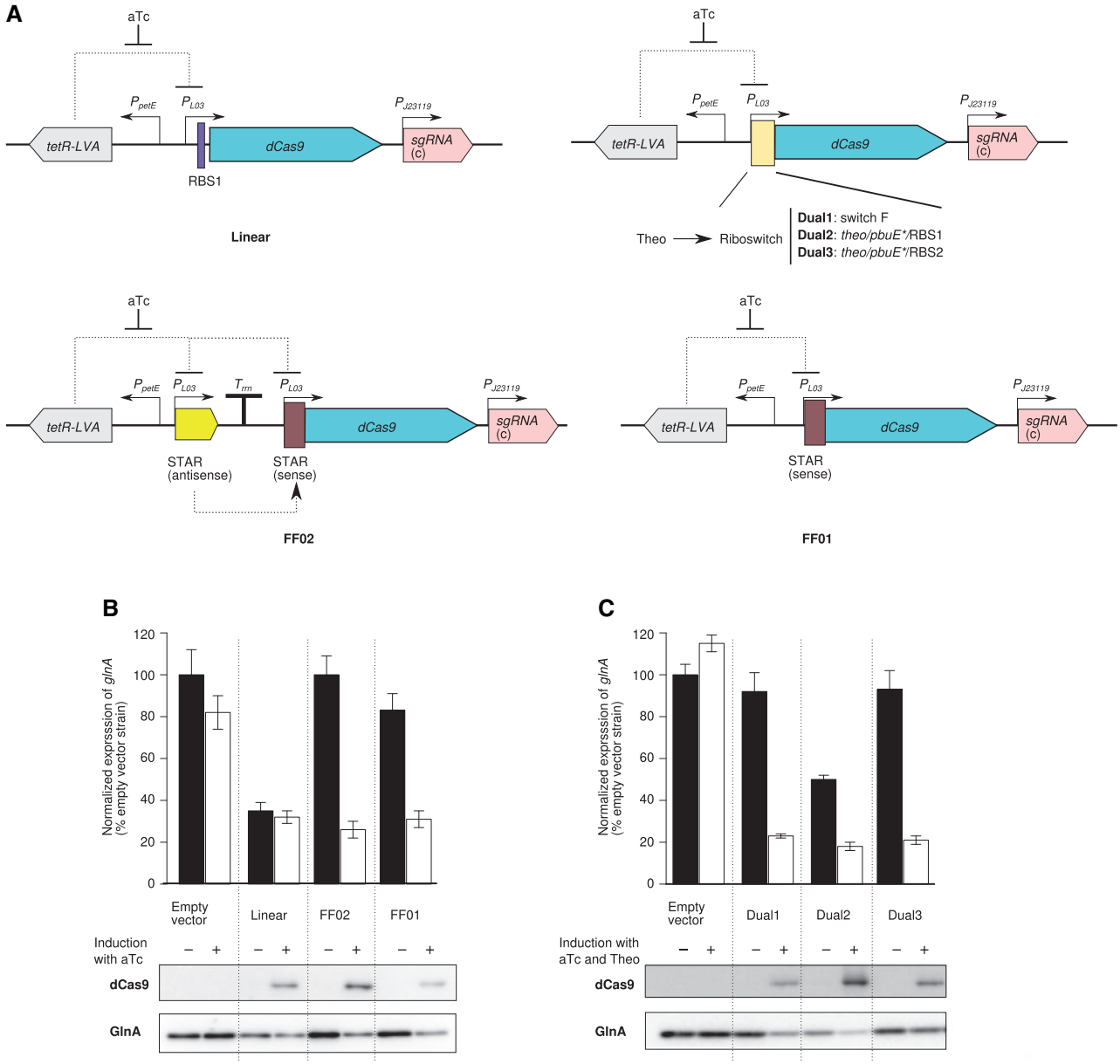

Fig. 1 Repression of *glnA* using systems where dCas9 was strictly regulated. (A) Schematic representation of six CRISPRi systems where dCas9 was strictly regulated. (B) and (C) RT–qPCR analysis of *glnA* after induction for 24 h (upper graph) and Western blotting using anti-Cas9 and anti-GlnA antibodies after induction for 72 h (lower panels). For induction of dCas9, 200 ng ml^{-1} aTc (B) or both 200 ng ml^{-1} aTc and 1,000 μM Theo (C) were added to cultures grown in nitrate-containing medium. *rnpB*, encoding the RNase E subunit, was used for normalization of RT–qPCR data. Data represent the mean ± SD ($n = 3$ from technical replicates).

CRISPRi. Hence, sgRNA targeting the coding region of *glnA* (c in Supplementary Fig. S1B), under the control of the constitutive and strong promoter *J23119* (a synthetic promoter from the Biobrick part BBa_J23119, iGEM Registry of Standard Biological Parts), was used hereafter unless otherwise noted.

Six plasmids for repression of *glnA*, Linear, Dual1, Dual2, Dual3, FF02 and FF01, were constructed (**Fig. 1A**). In the Linear system, expression of *dcas9* was induced by aTc. Because our attempts to improve the dynamic range of *glnA* repression by changing promoters for the expression of *dcas9* and/or sgRNA to weaker ones in the Linear system have failed, we tried to construct genetic circuits to regulate expression of *dcas9* very strictly. First, a riboswitch responsive to theophylline (Theo) was added to the Linear system. Dual1, Dual2 and Dual3 contain the translational ON riboswitch variant F (Ma et al. 2014, Higo et al. 2017), a transcriptional ON riboswitch (Higo et al. 2018) with a strong RBS (RBS1) and a weak RBS (RBS2) between the *L03* promoter (P_{L03}) and *dcas9*, respectively. In these three systems, expression of *dcas9* requires both aTc that dissociates TetR from P_{L03} (initiation of transcription) and Theo that changes the structure of the riboswitches at the 5′-untranslated region (recruitment of ribosome or elongation of transcription). Thus simple AND gate circuits were constructed. We also attempted to regulate dCas9 strictly by another approach, feed-forward gene regulation (Alon, 2007), where a regulator (in this case TetR) affects the expression of

another regulator [small transcription-activating RNA (STAR) antisense, see below], and both of the two regulators affect expression of a target gene (*dcas9*) co-operatively. We utilized the STAR dual system that produced a > 800-fold induction in *Escherichia coli* (Westbrook and Lucks 2017). This system is composed of two elements. One is a *cis*-element upstream of the target gene (sense) and the other is the STAR *trans*-element (antisense). The *cis*-element alone forms a secondary structure, resulting in both transcriptional termination and RBS occlusion and repression of downstream genes. In contrast, in the presence of STAR, which has a complementary sequence to the *cis*-element, the secondary structure of RNA is destroyed, resulting in activation of a target gene. In the FF02 system, both STAR antisense and *dcas9* with a STAR sense sequence were regulated by the *L03* promoter, which should minimize the leaking expression of *dcas9* without the inducer. An FF01 system in which STAR antisense was absent was also created for a negative control. RBS calculator (Espah Borujeni et al. 2014) predicted that the RBS derived from STAR is weaker compared with RBS1 and RBS2 for *dcas9* expression.

The six plasmids were introduced into *A.* 7120. The strain with the Linear system could not grow without nitrogen source. Because glutamine synthetase is essential, especially under nitrogen-limited conditions (Herrero et al. 2001), this lethality suggests leakiness of the Linear system. Thus, each strain was grown in the presence of nitrate. Expression of dCas9 in the Linear, FF02 and FF01 systems was induced by the addition of 200 ng ml^{-1} aTc. Both 200 ng ml^{-1} aTc and 1,000 μM Theo were added for induction of dCas9 in Dual1, Dual2 and Dual3 systems. After 24 h with or without induction, we extracted total RNA from the cells and performed RT–qPCR analysis (**Fig. 1B, C**). In the Linear system, *glnA* was repressed irrespective of induction of dCas9. In contrast, induction of dCas9 clearly repressed expression of *glnA* in the other five systems.

We also extracted total protein from the cells after induction for 72 h and performed Western blot analysis using anti-Cas9 or anti-GlnA antibodies (**Fig. 1B, C**). Similar to the RT–qPCR results, GlnA was repressed irrespective of induction of dCas9 in the Linear system. In this system, dCas9 was below the detection limit without aTc and was clearly induced by aTc, implying that failure to regulate the *glnA* level is not due to a lack of induction by the TetR system. Rather, these results suggested that a very small amount of dCas9 is sufficient for repression by CRISPRi technology, and that strict regulation of dCas9 is essential for efficient repression with a large dynamic range. In the other five systems, dCas9 was clearly induced and this induction led to distinct repression of GlnA. Unexpectedly, even in the FF01 system constructed for a negative control, dCas9 was induced and GlnA was clearly repressed. This could be due to leakiness of the STAR dual system in *A.* 7120 (Higo et al. 2018). The improved dynamic range of *glnA* repression in the FF02 or FF01 systems compared with the Linear system is possibly due to decreased expression of dCas9 by a weakened STAR RBS under non-inducing conditions.

The dynamic range of repression of *glnA* transcripts was wider in the Dual3 system (4.3-fold) compared with the Dual2 system (2.7-fold). This should be because weakened RBS in the former system led to reduced expression of dCas9 under non-induced conditions. However, GlnA protein was hardly repressed in the former system while it was clearly repressed in the latter system (**Fig. 1C**). Similarly, while the dynamic range of repression of *glnA* transcripts was wider in the Dual1 system (4.0-fold) compared with the Dual2 system, repression of the GlnA protein level was similar to the Dual2 system (50%) (**Fig. 1C**). Different GlnA protein levels despite the fact that *glnA* transcript levels were similar after induction (**Fig. 1C**) suggest post-transcriptional regulation of *glnA*. Otherwise, prolonged repression of *glnA* transcripts is required for repression of GlnA protein. Together with our failure to improve the dynamic range of *glnA* repression by changing promoters for the expression of *dcas9* and/or sgRNA to weaker ones in the Linear system, it was suggested that repression of GlnA protein is not straight forward.

Temporal and dose-dependent control of glnA expression under nitrogen fixation conditions

Of the above five GlnA repression systems, GlnA was most repressed by Dual2. As shown below, the dynamic range of repression of the GlnA protein level was the widest in the Dual2 system among our systems. Therefore, we decided to focus on this system towards the maximum production of ammonium. We analyzed repression of GlnA using Dual2 in detail, under nitrogen fixation conditions. After the addition of 200 ng ml^{-1} aTc and 1,000 μM Theo, total RNA was extracted at the indicated times and RT–qPCR analysis of *glnA* was performed (**Fig. 2**, upper graph). Time course analysis of the amounts of dCas9 and GlnA was also performed by Western blotting (**Fig. 2**, lower panels). Within 8 h of induction, *dcas9* was dramatically induced and this continued up to 72 h. The level of *glnA* decreased after 8 h and this was also maintained up to 72 h. In contrast, the amount of GlnA protein did not change at 8 h after induction compared with that at 0 h. A decrease of GlnA was typically observed after 48 h, possibly due to the high stability of this protein. However, effective repression was achieved (65% of that before induction and 76% of that in the empty vector strain). This is a great improvement compared with our previous results using small antisense RNA (29% before induction and 45% of the empty vector strain) (Higo et al. 2016).

We investigated whether the amount of dCas9 inversely correlated with expression of *glnA*. We added different concentrations of the two inducers for dose-dependent induction of dCas9. As expected, after 24 h of induction, dCas9 expression was induced in a Theo concentration-dependent manner (**Fig. 3A**). RT–qPCR analysis showed that the expression level of *glnA* inversely correlated with dCas9 (**Fig. 3A**). After 72 h of induction, the amount of GlnA also inversely correlated with dCas9 (**Fig. 3B**). Thus, we achieved dose-dependent repression of GlnA.

Strict repression systems under nitrogen fixation conditions

In the three systems FF02, FF01 and Dual1, GlnA was not repressed without induction of dCas9 and was clearly repressed

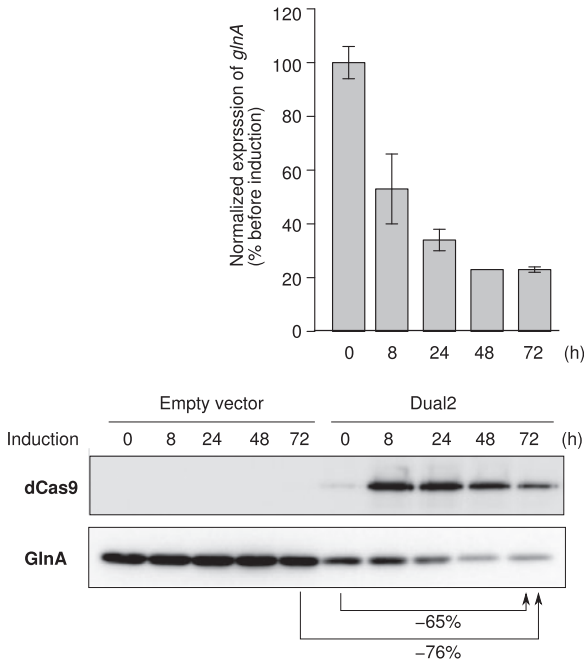

Fig. 2 Time course analysis of repression of *glnA* in the Dual2 system. RT–qPCR analysis of *glnA* (upper graph) and Western blotting using anti-Cas9 and anti-GlnA antibodies (lower panels) at the indicated times. For induction of dCas9, both 200 ng ml^{-1} aTc and 1,000 μM Theo were added to cultures grown in nitrogen fixation conditions. *rnpB*, encoding the RNase E subunit, was used for normalization of RT–qPCR data. Data represent the mean ± SD ($n = 3$ from independent cultures).

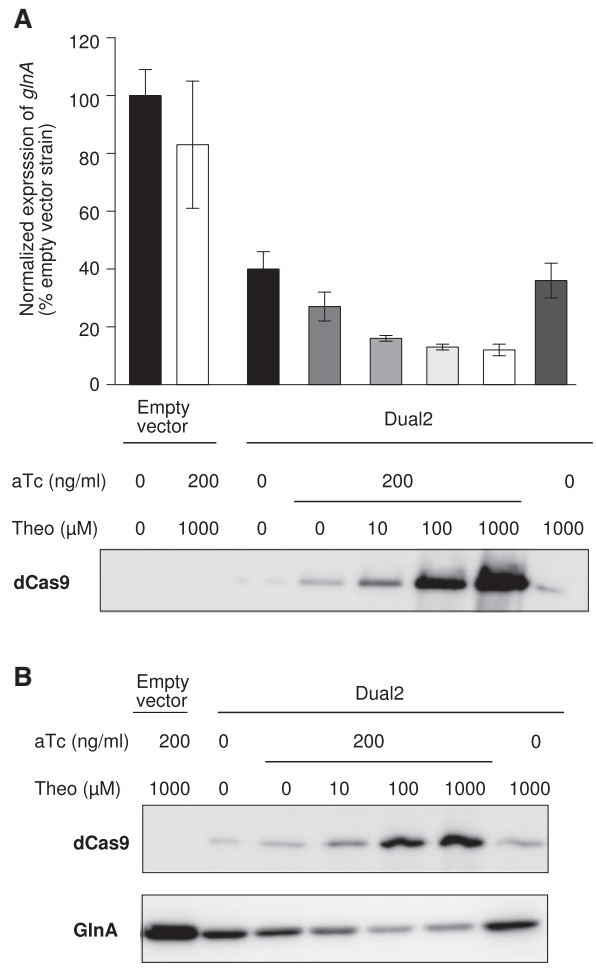

Fig. 3 Dose-dependent repression of *glnA* in the Dual2 system. (A) Total RNA and total protein were extracted from cells grown in nitrogen fixation conditions after induction of dCas9 for 24 h by the indicated concentrations of aTc and Theo. RT–qPCR analysis of *glnA* (upper graph) and Western blotting using anti-Cas9 antibody (lower panel) were then performed. *rnpB*, encoding the RNase E subunit, was used for normalization of RT–qPCR data. Data represent the mean ± SD ($n = 3$ from independent cultures). (B) Total protein was extracted from cells grown in nitrogen fixation conditions after induction of dCas9 for 72 h by the indicated concentrations of aTc and Theo. Western blotting using anti-Cas9 and anti-GlnA antibodies was then performed.

with induction of dCas9 under nitrate-replete conditions (**Fig. 1B, C**). Repression of *glnA* under nitrogen fixation conditions in these three systems was also investigated. RT–qPCR analysis showed that 80% repression of *glnA* was achieved after 24 h of dCas9 induction by 200 ng ml^{-1} aTc in the FF02 and FF01 systems, or by 200 ng ml^{-1} aTc and 1,000 μM Theo in the Dual1 system, while slight repression (approximately 30% of the empty vector strain) was observed without induction (Supplementary Fig. S2A, B, upper graph). In other words, a 3- to 4-fold dynamic range of repression of *glnA* transcripts was achieved in the three systems similarly to the Dual2 system (3.3-fold). Western blot analysis using an anti-GlnA antibody demonstrated little repression of GlnA without induction of dCas9 and clear repression with induction for 72 h (49, 54 and 40% of that without induction, respectively) (Supplementary Fig. S2A, B, lower panel). These results demonstrate that systems with little repression under non-induced conditions were obtained at the expense of a dynamic range of repression, in contrast to the Dual2 system, where the widest dynamic range of GlnA repression was achieved (65% of repression without induction) although half of GlnA was repressed even under non-induced conditions.

Repression of GlnA in heterocysts

Repression of proteins with high stability in heterocysts should be more challenging than in vegetative cells because heterocysts never divide. We tested whether GlnA is repressed in heterocysts as well as in whole filaments, under nitrogen fixation conditions, in the Dual2 strain after 72 h of induction (**Fig. 4A, B**). Enrichment of heterocysts was confirmed by weaker expression of RbcL and strong expression of NifH in heterocysts. Western blotting showed that GlnA was effectively repressed up to 67% of the level without induction in whole filaments, similar to the above results (**Figs 2, 3B**). However, repression of GlnA in heterocysts was only 48%. Since induction of dCas9 in whole filaments and heterocysts was similar (**Fig. 4A**), the possibly that inefficient repression of GlnA in heterocysts was not due to inefficient induction of dCas9 in heterocysts is excluded.

We aimed to improve repression of GlnA in heterocysts by simultaneous expression of two sgRNAs targeting *glnA*.

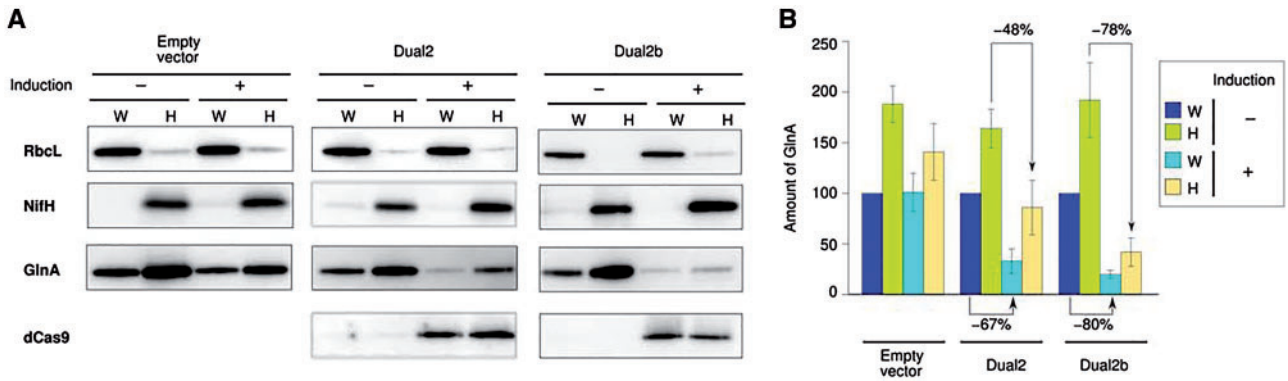

Fig. 4 Repression of GlnA in heterocysts. (A) After induction of dCas9 for 72 h by 200 ng ml^{-1} aTc and 1,000 μM Theo, heterocysts were enriched. Total protein was then extracted from whole filaments (W lanes) and enriched heterocysts (H lanes). Western blotting using anti-RbcL, anti-NifH, anti-GlnA and anti-Cas9 antibodies was performed. RbcL and NifH are marker proteins for vegetative cells and heterocysts, respectively. (B) Relative amounts of GlnA in whole filaments and heterocysts were quantified from Western blots. Amounts of GlnA relative to that in whole filaments without induction (set as 100) are shown for each strain. Data represent the mean ± SD ($n = 4$, two biological replicates each, including a technical replicate).

In addition to expression of an sgRNA targeting the coding sequence under the *J23119* promoter, an sgRNA targeting the NtcA-binding site upstream of *glnA* (a in Supplementary Fig. S1B) was expressed under the heterocyst-specific *nifB* promoter (Wang and Xu 2005). In this new system (designated Dual2b), GlnA was effectively repressed in whole filaments (80% without induction; **Fig. 4A, B**). GlnA was also effectively repressed in heterocysts (78% without induction) (**Fig. 4A, B**). Hence, successful repression of GlnA in heterocysts as well as whole filaments was achieved.

Application of CRISPRi to ammonium production

We applied CRISPRi to ammonium production. Because repression of GlnA required at least 48–72 h (**Fig. 2**), we incubated strains of Dual2 and Dual2b with inducers (200 ng ml^{-1} aTc and 1,000 μM Theo) for 72 h beforehand, under nitrogen fixation conditions. After washing cells with fresh medium, the two strains were inoculated into nitrogen-free medium with different concentrations of the inducers. Growth of cells and excretion of ammonium were then monitored for 6 d (**Fig. 5A, B**).

In the Dual2 system strain, little ammonium was excreted without the inducers. In contrast, with addition of 200 ng ml^{-1} aTc and 1,000 μM Theo, approximately 200 μM ammonium was excreted after 4 d (**Fig. 5B**). Growth was slightly delayed in this condition compared with that without the inducers. In the Dual2b system strain, no ammonium was excreted without the inducers. With addition of 200 ng ml^{-1} aTc and 1,000 μM Theo, nearly 200 μM ammonium was excreted after 5 d (**Fig. 5B**). Growth of this strain was significantly delayed in this condition compared with that without the inducers and that of the Dual2 strain with the same concentrations of the inducers (**Fig. 5A**). Furthermore, with smaller amounts of inducers (200 ng ml^{-1} aTc and 100 μM Theo), the Dual2b strain excreted higher amounts of ammonium than the Dual2 strain. These results indicate that repression of GlnA in heterocysts, as well as in vegetative cells (**Fig. 4**), is important for successful production of ammonium using this cyanobacterium.

Previously, we achieved ammonium excretion (200 μM at maximum) only when mutants possessing a mutation in *glnA* were used (Higo et al. 2016). Production of ammonium could be enhanced by further repression of *glnA* by expression of a small antisense RNA. However, constant repression of the activity of GlnA, which is essential for nitrogen assimilation in cyanobacteria, led to the appearance of spontaneous mutants defective in ammonium excretion (data not shown). Hence, we believe that the ability to regulate expression of the essential gene *glnA*, and temporal production of ammonium by CRISPRi, could lead to improved production of ammonium in the future.

Application of CRISPRi to repression of a gene essential for heterocyst development

As an application of CRISPRi technology, we here show an instance of conditional knockdown of a gene with a weaker RBS system (FF01). Conditional knockdown of *devH*, encoding a transcriptional regulator that is essential for heterocyst maturation (Hebbar and Curtis 2000, Ramírez et al. 2005), was attempted. A halfway *devH* mutant, where the last nine codons of *devH* were replaced by 43 codons derived from vector sequences, was constructed (Hebbar and Curtis 2000). This mutant could not grow in nitrogen-free medium. However, the possibility that the additional C-terminal sequence of DevH of the mutant strain adversely affected diazotrophic growth could not be ruled out. Our attempts to complete deletion of this gene have failed. Strains with or without the sgRNA targeting the *devH* coding region were constructed by single homologous recombination at the neutral site *cyaA* (Katayama and Ohmori 1997) (Supplementary Fig. S3A). Each strain was grown in the absence or presence of 200 ng ml^{-1} aTc without nitrogen sources. While the inducer did not affect growth at all in a control strain without sgRNA, the inducer abolished growth almost totally in a strain expressing sgRNA targeting *devH* (Supplementary Fig. S3B). In addition, we confirmed that expression of *devH* in the strain expressing sgRNA targeting *devH* was strongly repressed in the presence of the inducer (Supplementary Fig. S3C). These results indicated that

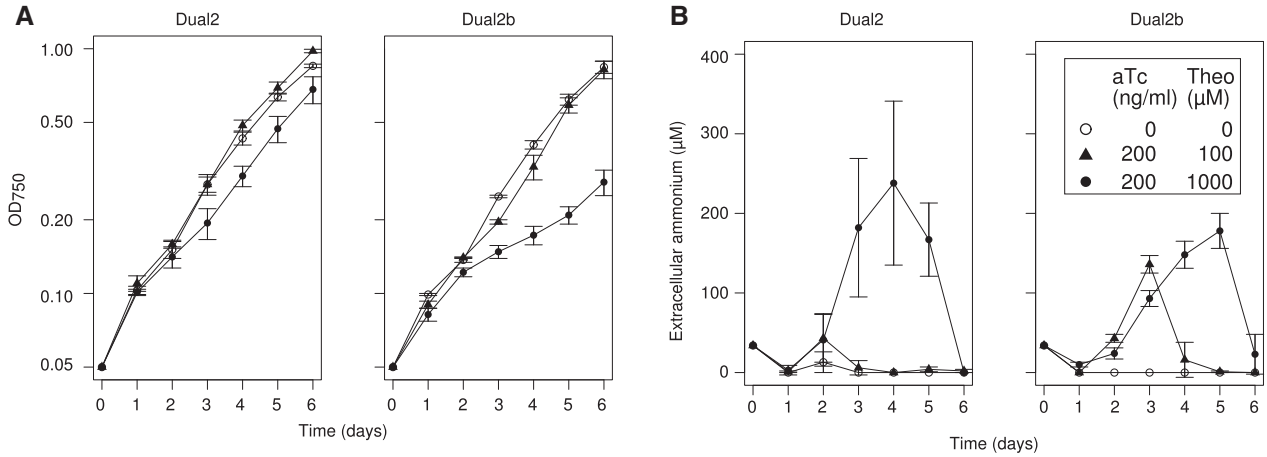

Fig. 5 Growth (A) and excreted ammonium (B) by Dual2 and Dual2b strains under nitrogen fixation conditions. The two strains were incubated with inducers (200 ng ml^{-1} aTc and 1,000 μM Theo) for 72 h beforehand, under nitrogen fixation conditions. After washing the cells with fresh medium, different concentrations of aTc and Theo were added at day 0. Data represent the mean ± SD ($n = 3$ from independent culture).

sufficient repression of *devH* was achieved in the weaker RBS system.

Discussion

In the present study, we succeeded in achieving conditional knockdown of an essential gene, *glnA*, in *A.* 7120, leading to conditional photosynthetic production of ammonium (**Fig. 5**). By combining two sgRNAs, GlnA was repressed in heterocysts that are unable to divide (**Fig. 4**).

Strict induction of dCas9 is a key factor for repression with a wide dynamic range in CRISPRi technology (Gordon et al. 2016, Huang et al. 2016, Yao et al. 2016). In our study, we achieved this by constructing an AND gate, or by RBS engineering (**Fig. 1**). Although we attempted to create an efficient *glnA* repression system by replacing promoters for *dcas9* and/or sgRNA with weaker ones in the Linear system, the constructed systems showed a narrow dynamic range of repression. In the FF01 and FF02 systems where *dcas9* is expressed with a weaker RBS, gene expression of *glnA* under non-induced conditions was barely repressed in these systems (**Fig. 1**; Supplementary Fig. S2). In contrast to the FF01 and FF02 systems where only aTc was required for induction of repression, in the Dual2 system both aTc and Theo were required for induction of repression, and expression of *glnA* was repressed under non-induced conditions. However, the strongest repression of GlnA was achieved in the Dual2 system, which showed the widest dynamic range, under nitrogen fixation conditions (**Fig. 3**; Supplementary Fig. S2). All of these systems could be used for various purposes, depending on requirements. An example is shown in Supplementary Fig. S3, where efficient repression of *devH* was achieved by a weaker RBS system.

Although FF01 and Dual2 systems showed a similar dynamic range of repression of *glnA* transcripts (**Fig. 3**; Supplementary Fig. S2), GlnA protein was more efficiently repressed in the Dual2 strain than in the FF01 system. GlnA is essential for nitrogen assimilation, especially under nitrogen fixation conditions in heterocysts (Herrero et al. 2001). Moreover, *glnA* expression is regulated by a complex feed forward mechanism in cyanobacteria (Klähn et al. 2015). An essential role for *glnA* and the complex regulation mechanism of *glnA* might prevent efficient repression of GlnA in a simple system. A robust gene repression system, such as AND gate, should be the key solution for efficient repression of *glnA*, unlike the repression of *devH*.

We previously established heterocyst- or vegetative cell-specific gene induction systems (Higo et al. 2018). By combining this spatio-temporal gene induction system and CRISPRi technology, a cell type-specific conditional knockdown system could be created. Such a system would be highly useful for studying genes that are expressed in both types of cell, but with different functions in each cell type. For example, NtcA, a global transcriptional regulator for nitrogen assimilation in cyanobacteria, has different target genes in the two types of cells (Muro-Pastor et al. 1999). Further, photosystem I is an essential component of oxygenic photosynthesis for generation of ATP and reducing power in vegetative cells, while it is believed to be involved in generation of ATP via cyclic electron transport in heterocysts for nitrogen fixation (Magnuson and Cardona 2016). Spatio-temporal gene induction and repression systems would also be useful for metabolic engineering of *A.* 7120. Modification of compartmentalization of some of the metabolic pathways in heterocysts and vegetative cells would enhance photosynthetic production of ammonium. For example, repression of *glnA* in heterocysts, where ammonium fixed by nitrogenase is assimilated by GlnA, than in vegetative cells, should lead to enhanced excretion of ammonium.

Recently, CRISPR genome editing technology involving markerless knock-ins, knockouts and point mutations was developed in cyanobacteria, including *A.* 7120 (Ungerer and Pakrasia 2016). We believe that the technologies derived from CRISPR enable complex genetic engineering and facilitate research on multicellularity and photosynthetic production in *A.* 7120.

Materials and Methods

Bacterial strains and growth condition

Anabaena strains were routinely grown at 30°C at 30–35 μmol photons m^{-2} s^{-1} in BG11 medium (Rippka et al. 1979), supplemented with 20 mM HEPES-NaOH (pH 7.5). For nitrogen deprivation experiments, cells were grown in the same medium but lacking $NaNO_3$ ($BG11_0$). For experiments of repression of *glnA* or *devH*, 5 μg ml^{-1} neomycin sulfate or 2 μg ml^{-1} each of spectinomycin and streptomycin was added to the medium, respectively. Liquid culture was bubbled with air containing 1.0% (v/v) CO_2.

Plasmid construction

All plasmids were constructed by the Hot Fusion method (Fu et al. 2014). For repression of *glnA*, DNA fragments were amplified by PCR using KOD Plus Neo (Toyobo), and were inserted between *Eco*RI and *Bam*HI sites of pRL25c (Wolk et al. 1988), a shuttle vector that replicates in *A.* 7120. For repression of *devH*, DNA fragments were inserted between *Bam*HI and *Kpn*I sites of a genome integration vector pSU102-cyaA, in which a DNA fragment of the *cyaA* region amplified by primers 5′-gagctcgaattgatcctACCCCTATTTTGGCAATGGC-3′ and 5′-acctgcaggtcgac tAATTAAGAGATGCGATCGCC-3′, is inserted in *Xba*I sites of pSU102 (Ehira et al. 2018). Detailed sequences are described in the Supplementary data.

RNA extraction and RT–qPCR analysis

Extraction of total RNA, synthesis of cDNA and RT-qPCR analysis were performed as described previously (Higo et al. 2018). The primers for PCR were as follows: for *rnpB*, forward primer 5′-TAGGGAGAGAGTAGGCGTTG-3′ and reverse primer 5′-TTCTGTGGCACTATCCTCAC-3′; for *glnA*, forward primer 5′-A AACCTCTCTTCGCAGGTGA-3′ and reverse primer 5′-GTTGGTGATTGCCAA CAGTG-3′; for *devH*, forward primer 5′- CCTTTCACGCTCCAGTCCTA-3′ and reverse primer 5′- ACGCTGGTGTTGGTATCTGA-3′.

Western blot analysis

Total protein was extracted using a Mini-Beadbeater (Biospec Products) as described previously (Higo et al. 2016). Equal amounts of total protein were separated on a denaturing SDS–polyacrylamide gel, and blotted onto a polyvinylidene fluoride (PVDF) membrane. GlnA, dCas9, RbcL and NifH were detected using anti-GlnA (Agrisera, AS01 018), anti-Cas9 (Clontech, 632607), anti-RbcL (Agrisera, AS03 037) and anti-NifH (Agrisera, AS01 021 A) antibodies, respectively, with appropriate secondary antibodies and a chemiluminescence kit (ImmunoStar LD, Wako).

Enrichment of heterocysts

The enrichment of heterocysts from *Anabaena* cells was performed as described previously (Golden et al. 1991). Enrichment of heterocysts was confirmed by Western blotting using anti-RbcL and anti-NifH antibodies.

Measurements of the concentration of ammonium excreted from Anabaena cells

The concentration of ammonium in the culture medium was measured as described previously (Higo et al. 2016).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References

Alon, U. (2007) Network motifs: theory and experimental approaches. *Nat. Rev. Genet.* 8: 450–461.

Buikema, W.J. and Haselkorn, R. (2001) Expression of the *Anabaena hetR* gene from a copper-regulated promoter leads to heterocyst differentiation under repressing conditions. *Proc. Natl. Acad. Sci. USA* 98: 2729–2734.

Chapman, J.S. and Meeks, J.C. (1983) Glutamine and glutamate transport by *Anabaena variabilis*. *J. Bacteriol.* 156: 122–129.

Choudhary, E., Thakur, P., Pareek, M. and Agarwal, N. (2015) Gene silencing by CRISPR interference in mycobacteria. *Nat. Commun.* 6: 6267.

Ehira, S. (2013) Transcriptional regulation of heterocyst differentiation in *Anabaena* sp. strain PCC 7120. *Russ. J. Plant Physiol.* 60: 443–452.

Ehira, S., Takeuchi, T. and Higo, A. (2018) Spatial separation of photosynthesis and ethanol production by cell type-specific metabolic engineering of filamentous cyanobacteria. *Appl. Microbiol. Biotechnol.* (in press).

Espah Borujeni, A., Channarasappa, A.S. and Salis, H.M. (2014) Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites. *Nucleic Acids Res.* 42: 2646–2659.

Flores, E., Herrero, A., Forchhammer, K. and Maldener, I. (2015) Septal junctions in filamentous heterocyst-forming cyanobacteria. *Trends Microbiol.* 24: 79–82.

Frías, J.E. and Flores, E. (2010) Negative regulation of expression of the nitrate assimilation *nirA* operon in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 192: 2769–2778.

Fu, C., Donovan, W.P., Shikapwashya-Hasser, O., Ye, X. and Cole, R.H. (2014) Hot fusion: an efficient method to clone multiple DNA fragments as well as inverted repeats without ligase. *PLoS One* 9: e115318.

Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A., Torres, S.E., et al. (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154: 442–451.

Golden, J.W., Whorff, L.L. and Wiest, D.R. (1991) Independent regulation of *nifHDK* operon transcription and DNA rearrangement during heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 173: 7098–7105.

Gordon, G.C., Korosh, T.C., Cameron, J.C., Markley, A.L., Begemann, M.B. and Pfleger, B.F. (2016) CRISPR interference as a titratable, trans-acting regulatory tool for metabolic engineering in the cyanobacterium *Synechococcus* sp. strain PCC 7002. *Metab. Eng.* 38: 170–179.

Hebbar, P.B. and Curtis, S.E. (2000) Characterization of *devH*, a gene encoding a putative DNA binding protein required for heterocyst function in *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 182: 3572–3581.

Herrero, A., Muro-Pastor, A.M. and Flores, E. (2001) Nitrogen control in cyanobacteria. *J. Bacteriol.* 183: 411–425.

Herrero, A., Stavans, J. and Flores, E. (2016) The multicellular nature of filamentous heterocyst-forming cyanobacteria. *FEMS Microbiol. Rev.* 40: 831–854.

Higo, A., Isu, A., Fukaya, Y. and Hisabori, T. (2016) Efficient gene induction and endogenous gene repression systems for the filamentous cyanobacterium *Anabaena* sp. PCC 7120. *Plant Cell Physiol.* 57: 387–396.

Higo, A., Isu, A., Fukaya, Y. and Hisabori, T. (2017) Designing synthetic flexible gene regulation networks using RNA devices in cyanobacteria. *ACS Synth Biol* 6: 55–61.

Higo, A., Isu, A., Fukaya, Y. and Hisabori T. (2018) Spatio-temporal gene induction systems in the heterocyst-forming multicellular cyanobacterium *Anabaena* sp. PCC 7120. *Plant Cell Physiol.* 59: 82–89.

Hsu, P.D., Lander, E.S. and Zhang, F. (2014) Development and applications of CRISPR–Cas9 for genome engineering. *Cell* 157: 1262–1278.

Huang, H.H. and Lindblad, P. (2013) Wide-dynamic-range promoters engineered for cyanobacteria. *J. Biol. Eng.* 7: 10.

Huang, C.H., Shen, C.R., Li, H., Sung, L.Y., Wu, M.Y. and Hu, Y.C. (2016) CRISPR interference (CRISPRi) for gene regulation and succinate production in cyanobacterium *S. elongatus* PCC 7942. *Microb. Cell. Fact.* 15: 196.

Jones, P.R. (2014) Genetic instability in cyanobacteria—an elephant in the room? *Front. Bioeng. Biotechnol.* 2: 12.

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

Klähn, S., Schaal, C., Georg, J., Baumgartner, D., Knippen, G., Hagemann, M., et al. (2015) The sRNA NsiR4 is involved in nitrogen assimilation control in cyanobacteria by targeting glutamine synthetase inactivating factor IF7. *Proc. Natl. Acad. Sci. USA* 112: E6243–E6252.

Kumar, K., Mella-Herrera, R.A. and Golden, J.W. (2010) Cyanobacterial heterocysts. *Cold Spring Harb. Perspect. Biol.* 2: a000315.

Ma, A.T., Schmidt, C.M. and Golden, J.W. (2014) Regulation of gene expression in diverse cyanobacterial species by using theophylline-responsive riboswitches. *Appl. Environ. Microbiol.* 80: 6704–6713.

Magnuson, A. and Cardona, T. (2016) Thylakoid membrane function in heterocysts. *Biochim. Biophys. Acta* 1857: 309–319.

Muro-Pastor, A.M., Valladares, A., Flores, E. and Herrero, A. (1999) The *hetC* gene is a direct target of the NtcA transcriptional regulator in cyanobacterial heterocyst development. *J. Bacteriol.* 181: 6664–6669.

Ow, S.Y., Cardona, T., Taton, A., Magnuson, A., Lindblad, P., Stensjö, K., et al. (2008) Quantitative shotgun proteomics of enriched heterocysts from *Nostoc* sp. PCC 7120 using 8-plex isobaric peptide tags. *J. Proteome. Res.* 7: 1615–1628.

Peters, J.M., Colavin, A., Shi, H., Czarny, T.L., Larson, M.H., Wong, S., et al. (2016) A comprehensive, CRISPR-based functional analysis of essential genes in bacteria. *Cell* 165: 1493–1506.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., et al. (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152: 1173–1183.

Ramírez, M.E., Hebbar, P.B., Zhou, R., Wolk, C.P. and Curtis, S.E. (2005) *Anabaena* sp. strain PCC 7120 gene *devH* is required for synthesis of the heterocyst glycolipid layer. *J Bacteriol.* 187: 2326–2331.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y. (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol*. 111: 1–61.

Sorek, R., Lawrence, C.M. and Wiedenheft, B. (2013) CRISPR-mediated adaptive immune systems in bacteria and archaea. *Annu. Rev. Biochem*. 82: 237–266.

Ungerer, J. and Pakrasia, H.B. (2016) Cpf1 is a versatile tool for CRISPR genome editing across diverse species of cyanobacteria. *Sci. Rep*. 6: 39681.

Valladares, A., Muro-Pastor, A.M., Herrero, A. and Flores, E. (2004) The NtcA-dependent P1 promoter is utilized for *glnA* expression in N_2-fixing heterocysts of *Anabaena* sp. strain PCC 7120. *J. Bacteriol*. 186: 7337–7343.

Wang, Y. and Xu, X. (2005) Regulation by *hetC* of genes required for heterocyst differentiation and cell division in *Anabaena* sp. strain PCC 7120. *J. Bacteriol*. 187: 8489–8493.

Westbrook, A.M. and Lucks, J.B. (2017) Achieving large dynamic range control of gene expression with a compact RNA transcription–translation regulator. *Nucleic Acids Res.* 45: 5614–5628.

Wolk, C.P., Cai, Y., Cardemil, L., Flores, E., Hohn, B., Murry, M., et al. (1988) Isolation and complementation of mutants of *Anabaena* sp. strain PCC 7120 unable to grow aerobically on dinitrogen. *J. Bacteriol*. 170: 1239–1244.

Yao, L., Cengic, I., Anfelt, J. and Hudson, E.P. (2016) Multiple gene repression in cyanobacteria using CRISPRi. *ACS Synth. Biol.* 5: 207–212.

Zhang, C.C., Laurent, S., Sakr, S., Peng, L. and Bédu, S. (2006) Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. *Mol. Microbiol*. 59: 367–375.