

# A glutamine riboswitch is a key element for the regulation of glutamine synthetase in cyanobacteria

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Received May 15, 2018; Revised July 05, 2018; Editorial Decision July 23, 2018; Accepted July 25, 2018

## ABSTRACT

As the key enzyme of bacterial nitrogen assimilation, glutamine synthetase (GS) is tightly regulated. In cyanobacteria, GS activity is controlled by the interaction with inactivating protein factors IF7 and IF17 encoded by the genes *gifA* and *gifB*, respectively. We show that a glutamine-binding aptamer within the *gifB* 5' UTR of *Synechocystis* sp. PCC 6803 is critical for the expression of IF17. Binding of glutamine induced structural re-arrangements in this RNA element leading to enhanced protein synthesis *in vivo* and characterizing it as a riboswitch. Mutagenesis showed the riboswitch mechanism to contribute at least as much to the control of gene expression as the promoter-mediated transcriptional regulation. We suggest this and a structurally related but distinct element, to be designated type 1 and type 2 glutamine riboswitches. Extended bio-computational searches revealed that glutamine riboswitches are exclusively but frequently found in cyanobacterial genomes, where they are primarily associated with *gifB* homologs. Hence, this RNA-based sensing mechanism is common in cyanobacteria and establishes a regulatory feedback loop that couples the IF17-mediated GS inactivation to the intracellular glutamine levels. Together with the previously described sRNA NsiR4, these results show that non-coding RNA is an indispensable component in the control of nitrogen assimilation in cyanobacteria.

## INTRODUCTION

As part of amino acids, nucleobases and many other organic molecules, nitrogen (N) is a key element of life. Whereas some bacteria have evolved the ability to fix N<sub>2</sub> gas, most organisms rely on the uptake and assimilation of organic or inorganic nitrogen sources. For microorganisms, the availability and concentrations of different N sources is highly variable requiring consecutive and tight adjustments of the assimilation machinery. Bacteria have evolved complex regulatory networks to control N uptake and the activity of assimilatory enzymes (for reviews see 1–4). The key enzyme of bacterial N assimilation is the glutamine synthetase (GS), which catalyzes the condensation of glutamate and ammonium (NH<sub>4</sub><sup>+</sup>) to produce glutamine. To complete NH<sub>4</sub><sup>+</sup> assimilation, glutamine subsequently reacts with 2-oxoglutarate (2OG) producing two molecules of glutamate, a step which is catalyzed by the glutamine oxoglutarate aminotransferase (GOGAT). Expression and activity of GS is tightly regulated due to the general role of glutamine as major N source for fundamental biosynthetic pathways (5), but also due to its role as signaling molecule in some bacteria (6,7). Moreover, maintenance of glutamate homeostasis is required for optimal cell growth (8).

In enterobacteria and most other Gram-negative bacteria, GS activity is regulated by covalent modification. In response to increasing N availability, GS is inactivated via adenylation by a specific adenylyltransferase (ATase) (for a review of GS regulation see 9). Moreover, *glnA*, the gene encoding GS, is transcriptionally regulated by the NtrB-NtrC two-component system (3). Under N limitation the sensory kinase NtrB phosphorylates and activates NtrC,

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which acts as transcriptional activator for several N assimilatory genes including *glnA*. The activity of both, ATase and NtrB is controlled by the regulatory protein P<sub>II</sub> (for an overview of the P<sub>II</sub> signaling system see Refs. 10–12). However, the mechanisms of N control and GS regulation are very different in other groups of bacteria such as cyanobacteria.

### Cyanobacteria evolved unique mechanisms for GS regulation

Cyanobacteria are morphologically and genetically very diverse. They are the only prokaryotes performing oxygenic photosynthesis, play major roles in the global biogeochemical carbon (C) and N cycles (13–15) and are of substantial interest in green biotechnology (16–18). Compared to enterobacteria and most other Gram-negative bacteria, different mechanisms controlling N assimilation have evolved in cyanobacteria. The main transcriptional regulator involved in the coordinated up-regulation of N assimilatory genes under N-limitation is NtcA, a member of the CRP transcriptional regulator family (19). Moreover, GS activity is not modulated by covalent modification but by small inhibitory proteins, called GS inactivating factors (IFs), (20). Most cyanobacteria possess two homologous IFs, IF7 and IF17, which are encoded by the *gifA* and *gifB* genes, respectively. The expression of both genes is also regulated by NtcA, though inverse to *glnA* (21).

### Signal perception of the N status in cyanobacteria

Although glutamine is a well-established signaling molecule for N availability in many Gram-negative bacteria, it seemed to lack any signaling function in cyanobacteria (6). Similar to other major bacterial groups, cyanobacteria sense the levels of 2OG, which connects C and N metabolism via the reactions performed by GS and GOGAT (7). In *Synechocystis* sp. PCC 6803 (*Synechocystis*), intracellular glutamine and 2OG concentrations vary with external N fluctuations, but only 2OG correlates well with NtcA-dependent gene expression (22). At the molecular level, 2OG modulates the affinity of NtcA to its recognition sequence (23,24) as well as the activity of P<sub>II</sub> which is also a key regulatory protein in cyanobacterial N control (12,25). NtcA is further activated by a 2OG dependent interaction with the transcriptional co-factor PipX (26–28). Alternatively, PipX can bind to P<sub>II</sub> under low 2OG conditions, an interaction that antagonizes PipX-dependent activation of NtcA (26).

NtcA binds as a homodimer to the conserved palindromic sequence GTA-N<sub>8</sub>-TAC with the A and T at positions 3 and 12 being somewhat variable (23,29,30). Depending on the location of the binding motif relative to the transcriptional start site, NtcA can act as activator (e.g. *glnA*) but also as repressor (e.g. *gifA* & *gifB*) (19). Under N excess (e.g. in presence of NH<sub>4</sub><sup>+</sup>) the 2OG level is low and NtcA is present in an inactive form, which has poor affinity to its target sites. Under N depletion, the 2OG level increases and stimulates complex formation between PipX and NtcA which increases its binding affinity to target promoters (26,31).

### Non-coding RNAs are involved in regulating N assimilation

In addition to transcriptional regulators, bacteria possess numerous and diverse small regulatory RNAs (sRNAs). These sRNAs can activate or repress gene expression at the post-transcriptional level by complementary base pairing with their mRNA targets (32,33). In cyanobacteria, the expression of several sRNAs is N-regulated (29,30,34,35). One of them, the NtcA-controlled nitrogen stress induced RNA 4 (NsiR4) impacts GS activity by interfering with the translation of the *gifA* mRNA (36). Another class of non-coding RNAs are riboswitches. These elements are part of untranslated regions of bacterial mRNAs and can control gene expression by ligand-induced structural modulation (37). Riboswitches are composed of an aptamer which specifically binds a ligand, and an expression platform which determines the read out of genetic information by interfering with the transcriptional or translational machinery in response to the structural modulation (38). The riboswitches identified so far respond to various ligands, including the amino acids glycine and lysine (39,40).

Previously, two RNA aptamers were reported that bind and change conformation specifically in response to glutamine (41,42). These were named the *glnA* and downstream-peptide (DP) aptamers (not to be confused with the *glnA* gene encoding glutamine synthetase), and the corresponding RNA sequence motifs were annotated as RF01739 and RF01704 in the Rfam database. Both were found to be restricted to cyanobacterial and marine metagenomic sequences and were to some extent positioned 5' of genes involved in N metabolism (41). Although structural modulation upon glutamine binding was observed (43), a riboswitch function and its possible physiological relevance have remained undetermined.

Here, we demonstrate that both the *glnA* and DP aptamers indeed function as glutamine riboswitches. In the cyanobacterial model organism *Synechocystis*, this riboswitch regulates the expression of the major GS inactivating factor, IF17, and is necessary for the appropriate regulation of GS activity. Homologous riboswitches in other cyanobacteria are mainly associated with *gifB* gene homologs. Hence, glutamine riboswitches play a major role in the control of GS activity throughout the cyanobacterial phylum. Our data establish glutamine as an additional signaling molecule in cyanobacterial N control that functions through a mechanism currently not known from any other bacterial phylum.

## MATERIALS AND METHODS

### Strains and cultivation

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803, originally obtained from N. Murata (Japan), was used as wild type. Cultivation was performed at 30°C under continuous white light illumination of 50–80 μmol quanta m<sup>-2</sup> s<sup>-1</sup> and gentle agitation in Cu<sup>2+</sup>-depleted BG11 medium (44) buffered with 20 mM TES to pH 8.0. All recombinant strains were selected on agar-solidified BG11 medium and maintained in presence of the corresponding antibiotics. However, for the *in vivo* experiments all mutants were grown without antibiotics, exactly as the WT.

### Generation of recombinant strains

The 5' UTR of *gifB* and 54 nt of its coding region were amplified from *Synechocystis* gDNA using the oligonucleotides gifB6803.NsiI\_fw and gifB6803.NheI\_rev. The information about the transcriptional start site (TSS) has been taken from (21). The PCR product was cloned into the plasmid pXG10-SF (45) via the NsiI and NheI sites resulting in a translational fusion of a truncated *gifB* ORF with the *sfgfp* gene. The primers used for cloning and the resulting plasmids are given in Supplemental Tables S3 and S4. The 5' UTR of a DUF4278-encoding gene from *Prochlorococcus* sp. MED4 was also cloned into pXG10-SF using the primers gifBMED4.NsiI\_fw/gifBMED4.NheI\_rev and following the same cloning strategy (TSS extracted from (46)). Please note that this gene is covered by the Yfr6 RNA which was initially regarded as non-coding (47) but harbors an ORF. To establish point mutations within the *glnA* aptamer (RF01739) the pXG10-SF-gifB6803(WT) plasmid was reamplified using the primer combination gifB.M1\_fw/rev. The PCR products were introduced into *Escherichia coli* via transformation and the resulting plasmid pXG10-SF-gifB6803(M1) was isolated and again used as template for a subsequent PCR to establish complementary point mutation M3.

To introduce and express the *gifB::sfgfp* constructs in *Synechocystis* they were amplified from the five pXG10-SF derivatives using the primers gifB-J23101\_fw and pJV863SeqRVshift. Thereby the constructs were fused to the BioBrick BBa\_J23101 promoter (iGEM Registry, (48)) which was found to be constitutively active at a high level in *Synechocystis* (49) and expected to function in a wide range of cyanobacteria (50). The same strategy was used for the construct harboring the DP aptamer (RF01704) of *Prochlorococcus* sp. MED4 by using the primers gifMED4-J23101\_fw/pJV863SeqRVshift. The PCR products were cloned into the replicative broad-host vector pVZ322 via the *XhoI/XbaI* sites thereby omitting its  $Km^R$  gene. The final pVZ322-based plasmids were introduced into *Synechocystis* WT via conjugal transfer from *E. coli*. The recombinant strains were selected on BG11 agar containing 2  $\mu\text{g ml}^{-1}$  gentamycin.

For the mutagenesis of the glutamine riboswitch in the chromosome of *Synechocystis* we generated constructs to exchange the WT locus of *gifB* via homologous recombination. First, recombination sites (RS) were amplified from gDNA using the primer combinations gifB\_fwd/gifB\_KmR\_rev and slr1597\_fwd/slr1597\_rev. These carried overlapping ends with a kanamycin resistance cartridge ( $Km^R$ ) amplified from a customized plasmid using the primers KmR\_fwd/KmR\_rev. A plasmid backbone with overlapping ends to both RS was amplified from a pJET1.2 plasmid using primers pJET\_fwd/pJET\_rev. The RS, the  $Km^R$  and the plasmid backbone were assembled using the AQUA cloning technique (51). The final plasmid pJET-gifB-RS-Ctrl was purified from a transformed *E. coli* DH5 $\alpha$  strain. To establish point mutation M1 the plasmid was reamplified using primers gifB.M1\_fw and gifB.M1\_rev(6803). After transformation of *E. coli* and purification of the plasmid pJET-gifB-RS-M1, the compensatory mutation M3 was established in the same

way using gifB\_M3\_fw/ gifB\_M3\_rev and resulted in the plasmid pJET-gifB-RS-M3. Because first attempts failed to establish *Synechocystis* mutants using the described plasmids, we extended the recombination flank partially covering *slr1597* by the entire CDS. This increased the length of homologous sequence from 202 to 1184 bp. For this we amplified an extended fragment from gDNA using primer combination slr1597-NdeI\_fw/slr1597-XbaI\_rev. The resulting PCR product was purified and ligated into plasmids pJET-gifB-RS-Ctrl/-M1/-M3 via *NdeI/XbaI* restriction sites. This replaced the initial recombination site by the extended one. The resulting plasmids were used to transform the WT and established recombinant *Synechocystis* strains harboring either the WT *gifB*-5' UTR (*gifB::Ctrl*) or point-mutated versions (*gifB::M1*, [riboswitch structure abolished] and *gifB::M3* [riboswitch structure restored]). A schematic illustration of the constructs integrated into the genome is shown in Figure 4A. The recombinant strains were verified by sequencing PCR products amplified from gDNA.

### GFP fluorescence measurements

GFP fluorescence was measured in *Synechocystis* *in vivo* using a VICTOR<sup>3</sup> multiplate reader (PerkinElmer). The cells were grown as described above to an OD<sub>750</sub> of ~0.5. For the NH<sub>4</sub><sup>+</sup> upshift 10 mM NH<sub>4</sub>Cl (f. c.) or the same volume of water were added to the cultures. For the measurement, 200  $\mu\text{l}$  culture aliquots were filled into a 96-well microtiter plate (VWR<sup>®</sup> Tissue Culture Plates, 96 wells-F). The fluorescence was measured with the following setup: excitation filter F485, emission filter F535, measurement time 1.0 s, CW-lamp energy 21673. The cell absorbance at 750 nm (OD<sub>750</sub>) was measured in parallel (CW-lamp filter 750/8 nm, measurement time 1.0 s). A WT strain served as a negative control. From each fluorescence value the background fluorescence of the WT was subtracted and then divided by the measured OD<sub>750</sub>.

### Expression analyses and glutamine determination

The *Synechocystis* strains *gifB::Ctrl*, *gifB::M1* and *gifB::M3* were grown in Erlenmeyer flasks under the conditions described above to an OD<sub>750</sub> of 0.8. Samples for protein and amino acid extraction were taken prior to and in short intervals after adding NH<sub>4</sub>Cl in a final concentration of 10 mM. Proteins were extracted from cells harvested by centrifugation, separated by SDS-PAGE and transferred to nitrocellulose membranes as described previously (52). Membranes were blocked with TBS-T containing 5% milk powder and probed with sera containing anti-IF17 (1:2000) or anti-TrxA (1:5000). The antisera were obtained from M.I. Muro-Pastor and F.J. Florencio (University of Seville, Spain). As secondary antibody, anti-rabbit IgG-HRP conjugates (Thermo Fisher; catalog #65–6120) were used according to the manufacturer's protocol. Glutamine was extracted with 80% EtOH from frozen cell pellets and quantified as described previously (36,53).

### In-line probing

A 104-nucleotide RNA corresponding to the *Synechocystis gifB* 5' UTR as well as versions containing the point mutations M1 and M3 were generated by *in vitro* transcription from PCR products using T7 RNA polymerase. The respective transcription templates were PCR amplified from the pXG10-SF plasmids described above, which already harbored the *gifB*-UTR or its point mutations M1 and M3, using primer *gifB*-UTR\_rev in combination with either *gifB*-UTR-T7\_fw (WT, M3) or *gifB*-M1-T7\_fw (M1). *In vitro* transcription was performed from ~6 pmol of dsDNA in a 50  $\mu$ l reaction containing 50 mM Tris-HCl (pH 7.5 at 23°C), 15 mM MgCl<sub>2</sub>, 2 mM spermidine, 5 mM DTT, 2.5 mM each of the four ribonucleoside 5' triphosphates (NTPs) and 10 units  $\mu$ l<sup>-1</sup> bacteriophage T7 RNA polymerase. The reaction was performed for 2 h at 37°C. The RNA was purified via denaturing polyacrylamide gel electrophoresis and 5'-radiolabeled with <sup>32</sup>P as described previously (42). The radiolabeled RNA was subjected to in-line probing analyses as described previously (54). Accordingly, the RNA was incubated for 2 d at 23°C in a solution containing 50 mM Tris-HCl (pH 8.3 at 23°C), 20 mM MgCl<sub>2</sub>, 100 mM KCl and increasing concentrations of a ligand ranging from 3.2  $\mu$ M to 10 mM in half-log concentration increments. As possible ligands for the 5' UTR of *gifB*, we tested L-glutamine, L-glutamate and NH<sub>4</sub>Cl. After incubation the products of the in-line probing reactions were separated by denaturing gel electrophoresis (10% polyacrylamide, 8 M urea). The gels were dried and subsequently imaged using a Storm 820 PhosphorImager (GE Healthcare). The relative intensities of the various degradation products were quantified using ImageQuANT software. Values for apparent  $K_D$  were determined using a sigmoidal-dose response equation and GraphPad Prism 6.

### Bioinformatics analyses

The phylogenetic tree was generated using the Neighbor-Joining method (55) and the 16S rDNA sequences of 60 representative cyanobacteria. The 16S rDNA sequences were retrieved from the whole genome RefSeq records. Alignments and evolutionary analyses were conducted in MEGA7 (56) using standard parameters. All positions containing gaps and missing data were eliminated.

In the same set of organisms, the occurrence and frequency of the RNA motifs RF01739 (*glnA* aptamer) and RF01704 (DP aptamer) was analyzed by performing an Infernal (v.1.1.2) (57) cmsearch for both motifs against the entire set of replicons for all species. The *E*-value threshold was set to 0.0001. The covariance models for the two RNA families were retrieved from the Rfam database (v.12.3, June 2017) (58). To investigate the co-occurrence of *gifA* and *gifB* genes a clustering of homologous proteins for the same 60 cyanobacteria was performed at MBGD (<http://mbgd.genome.ad.jp/>, release 2016/05/19) (59) with the parameters set to -o1 -HO -S -c60 -p0.5 -V0.6 -C80 -ao0.8 -ai0.95 -ne1 -EVAL = 0.00001 -SCORE = 60.

Predicted peptides downstream of retrieved RNA motifs were analyzed with PfamScan (v.1.6) against Pfam (v31.0, March 2017) (60). For this, 600 nucleotides downstream of the detected RNA motifs were extracted and translated to

peptide sequences with transeq (EMBOSS v.6.5.7.0) (61). Because strand information is returned by cmsearch, the 600 nucleotides were translated only using the three reading frames corresponding to the strand where the RNA motifs are located.

The source code for the bioinformatics analyses of cyanobacterial genomes is available on github: [https://github.com/PatrickRWright/gifA\\_gifB\\_RF01739\\_RF01704](https://github.com/PatrickRWright/gifA_gifB_RF01739_RF01704).

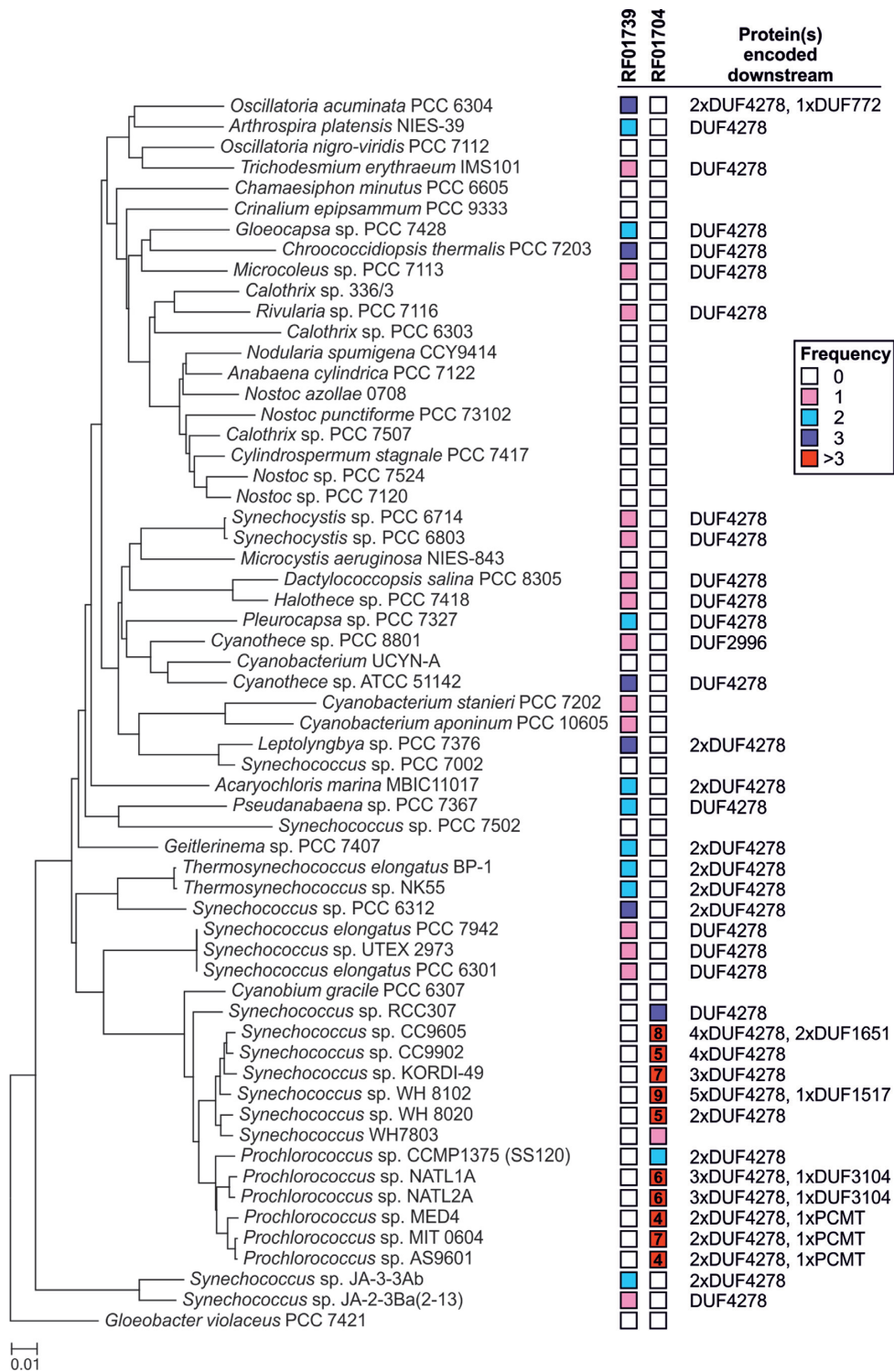
In a comprehensive follow-up search, all bacterial and archaeal sequences from RefSeq version 80 (62) were searched for representatives of RF01739 and RF01704 with Infernal (v.1.1.2) using the Rfam filtering parameter. Predicted downstream proteins were annotated by searching Conserved Domain Database version 3.16 (63). A collection of metagenomic sequences elsewhere described (64) was also searched for the aptamer motifs and annotated in a similar fashion.

## RESULTS

Genome sequences of 60 cyanobacterial strains representative for the major taxonomic and morphological clades were scanned for the presence of the *glnA* aptamer and the related DP aptamer. The *glnA* aptamer was detected in 28 of the 60 analyzed genomes (Figure 1, Supplementary Table S1) and hence is widely distributed throughout the cyanobacterial phylum. Whereas most of these genomes contain a single instance of the *glnA* aptamer, a few strains possess two or three distinct copies. Conspicuously, no homologs were found in the clade of filamentous, heterocyst-forming and N<sub>2</sub>-fixing cyanobacteria including *Anabaena* (*Nostoc*) sp. PCC 7120, *Nostoc punctiforme* PCC 73102 and *Nodularia spumigena* CCY9414. The DP aptamer, in contrast, is restricted to marine picocyanobacteria of the genera *Synechococcus* and *Prochlorococcus*, where it occurs frequently in higher numbers, e.g. nine copies in *Synechococcus* sp. WH8102 (Figure 1). Both aptamers are mutually exclusive, as we did not observe a single case of co-occurrence. Potentially, the DP aptamer is a derivative of the *glnA* aptamer, which has evolved in the monophyletic group of marine picocyanobacteria. Altogether, we found the RNA aptamers in 41/60 studied genomes. We conclude that the *glnA* and DP aptamers are present in the majority of cyanobacteria.

### The RNA aptamers are frequently linked to a gene encoding a GS inhibitory protein

To elucidate the genes that are associated with the RNA aptamers, we compared the immediate downstream sequences against the Pfam database. In 25/28 genomes the *glnA* aptamer was located upstream of an ORF encoding a protein with a domain of unknown function (DUF4278) (Figure 1). In strains harboring more than one *glnA* aptamer, such as *Acaryochloris marina* MBIC11017 and *Thermosynechococcus elongatus* BP-1, it was frequently associated with a second DUF4278-encoding gene. With only a few exceptions, the DP aptamer was also associated with DUF4278-encoding genes in marine picocyanobacteria (Figure 1). However, in some cases, the aptamers were linked to other genes encoding proteins without matches in the Pfam database.



**Figure 1.** Occurrence of the RF01739 (*glnA* aptamer) and the RF01704 (DP aptamer) motifs among cyanobacterial genomes. The phylogenetic tree was generated using the Neighbor-Joining method (55) and the 16S rDNA sequences of 60 representative cyanobacteria. Evolutionary analyses were conducted in MEGA7 (56). The RNA motif frequency within a particular genome is color coded. If the frequency was >3 the exact number of motifs was specified. Furthermore, the proteins encoded downstream of both RNA motifs were identified using the Pfam database. Note that DUF4278 is part of the GS inactivating factor IF17. Thus, the Pfam hits referring to DUF4278 most likely represent IF17 homologs or IF17-like proteins. If the number of Pfam hits was lower than the number of reported RNA motif hits, then not every downstream query returned a hit in the Pfam database. A detailed description is provided in the Material and Methods section. The complete dataset, i.e. the exact loci of the individual RNA motifs are given in Supplementary Table S1.

Weinberg et al. (41) reported a few instances in which the *glnA* aptamer was associated with N transporter or glutamine synthetase (*glnA*) genes. Indeed, we could confirm those associations in environmental DNA sequence databases, but found not a single case within the available cyanobacterial genome sequences (Supplementary Table S2). We conclude that the aptamers are mainly associated with genes encoding DUF4278-containing proteins and the link to N related transporters and the *glnA* gene is an exception rather than the rule.

In *Synechocystis*, DUF4278 characterizes the GS inactivating factor IF17 encoded by *gifB*. This protein has been verified as major regulatory factor of cyanobacterial N assimilation because it inhibits GS (20). Consistently, there is a good correlation between the occurrence of *gifB* homologs and the *glnA* aptamer. In 32 out of 60 analyzed genomes we found *gifB* homologs and in 28 of these 32 genomes the *glnA* aptamer was upstream. However, it should be noted that several cyanobacterial strains only harbor a *gifA* and no *gifB* homolog such as *Anabaena* (*Nostoc*) sp. PCC 7120 (65) and closely related strains (Supplementary Table S1). As mentioned above, none of the strains clustering with *Anabaena* sp. PCC 7120 possess the *glnA* aptamer, indicating that it is indeed functionally associated with *gifB* (Figure 1).

However, previous reports indicated that marine picocyanobacteria of the genus *Prochlorococcus* which exclusively harbor the DP aptamer lack full length homologs of GS inactivating factors (66). Consistently, we found no homologs to the *Synechocystis* IF17 in *Prochlorococcus* strains by using the BLASTP algorithm. Nevertheless, our analyses revealed that the ORFs downstream of the DP aptamer also frequently encode DUF4278-containing proteins. Although shorter, these proteins share partial sequence similarity with IF17 proteins from other cyanobacteria (Supplementary Figure S1). This points towards a similar function and hence they should be regarded as IF17-like proteins. Altogether, we found a tight association of the *glnA* and DP aptamers with genes encoding IF17 or IF17-like proteins. Hence, we hypothesized whether IF17 expression in cyanobacteria could be commonly regulated by glutamine riboswitches.

### The 5' UTR of the *gifB* gene from *Synechocystis* binds glutamine

For the functional analysis of a glutamine-binding aptamer we focused on the model *Synechocystis*, in which the mechanism of GS inactivation via inhibitory proteins is well investigated (20). In this organism, the *gifB* (*sll1515*) gene is transcribed together with a 104 nt long 5' UTR, which contains the predicted *glnA* aptamer (Figure 2A). To verify glutamine binding, in-line probing assays (54) were performed. Indeed, we observed that certain parts of the RNA construct undergo changes in the extent of spontaneous chemical degradation in presence of glutamine, which indicates that glutamine induces structural modulation (Figure 2B). The RNA exhibited an apparent dissociation constant ( $K_D$ ) for glutamine of  $\sim 2.3$  mM (Figure 2C). To ensure that glutamine specifically binds to the aptamer, we introduced mutations disrupting the predicted secondary structure (Figure

2A). Indeed, glutamine-dependent RNA structural changes were prevented by a mutation that disrupts conserved base-pairing (M1) and were restored by a compensatory mutation (M3, Figure 2D). The data clearly show that the 5' UTR of the *gifB* gene harbors an RNA aptamer that binds and changes conformation in response to glutamine.

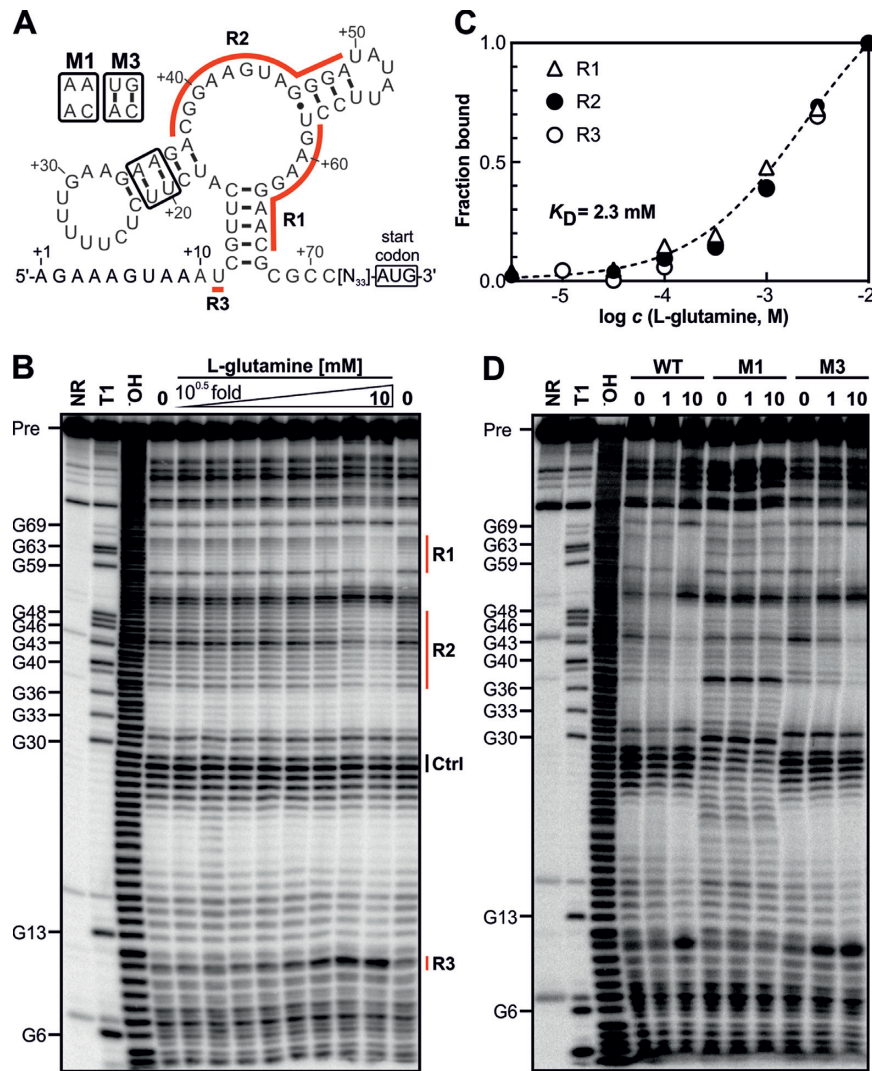
### The glutamine-binding aptamers are part of functional riboswitches that regulate gene expression

To analyze its regulatory impact, the glutamine-binding aptamer of *Synechocystis* was characterized *in vivo*. First, we performed reporter gene assays in *Synechocystis* using the *superfolder gfp* (*sfgfp*) gene, which was translationally fused to a truncated *gifB* gene and its entire 5' UTR (Figure 3A). Transcription was driven by the synthetic BBa\_J23101 promoter (iGEM Registry) which acts as a strong, constitutive promoter in *Synechocystis* (49). Compared to a WT strain, which did not harbor a *sfgfp* gene, substantial GFP fluorescence was detectable in a reporter strain carrying the J23101::*gifB*::*sfgfp* construct on a replicative plasmid (Figure 3B). These observations confirmed the activity of the synthetic promoter and sufficient translation initiation from the *gifB* 5' UTR.

Sudden upshifts in  $\text{NH}_4^+$  availability lead to rapid increases in intracellular glutamine levels in *Synechocystis* (22,36). Because a rapid increase of glutamine is accompanied by IF17 accumulation (67) in turn inactivating GS, it is reasonable to assume that the glutamine-binding aptamer upstream of *gifB* has a positive effect on IF17 production. To test this hypothesis we added 10 mM  $\text{NH}_4\text{Cl}$  to cultures of the reporter strains. As expected, the GFP fluorescence rapidly increased after  $\text{NH}_4^+$  upshift (Figure 3C). Because we used a constitutive, NtcA-independent promoter, the rise in GFP fluorescence most likely resulted from increased translation of the *gifB*::*sfgfp* mRNA mediated by the glutamine riboswitch.

To exclude the possibility that  $\text{NH}_4^+$  is an additional ligand of the *glnA* aptamer and directly responsible for the observed changes in GFP fluorescence, we performed further in-line probing analyses using increasing concentrations of  $\text{NH}_4\text{Cl}$  (Supplementary Figure S2). However, the RNA degradation patterns did not change, excluding the possibility that  $\text{NH}_4^+$  is a ligand of the *glnA* aptamer. Hence,  $\text{NH}_4^+$  cannot be directly responsible for the changes in GFP fluorescence. Moreover, because internal glutamate levels exhibit a transient negative response to  $\text{NH}_4^+$  upshifts (22,36), we also tested L-glutamate as possible ligand. Again, no binding was evident from in-line probing assays (Supplementary Figure S2).

To additionally exclude the possibility of transcriptional effects that might cause the increased GFP fluorescence (e.g. due to changes in promoter activity), we introduced the same mutations used to evaluate glutamine binding by in-line probing (Figure 2). For example, the mutation M1 abolished the observed rise in GFP fluorescence after  $\text{NH}_4^+$  addition (Figure 3D). Nevertheless, despite being unaffected by  $\text{NH}_4^+$  upshift measurable GFP fluorescence was observed compared to the negative control (Figure 3F). Consistent with the RNA structure prediction and the in-line probing assays, the mutation M3, which restored base-

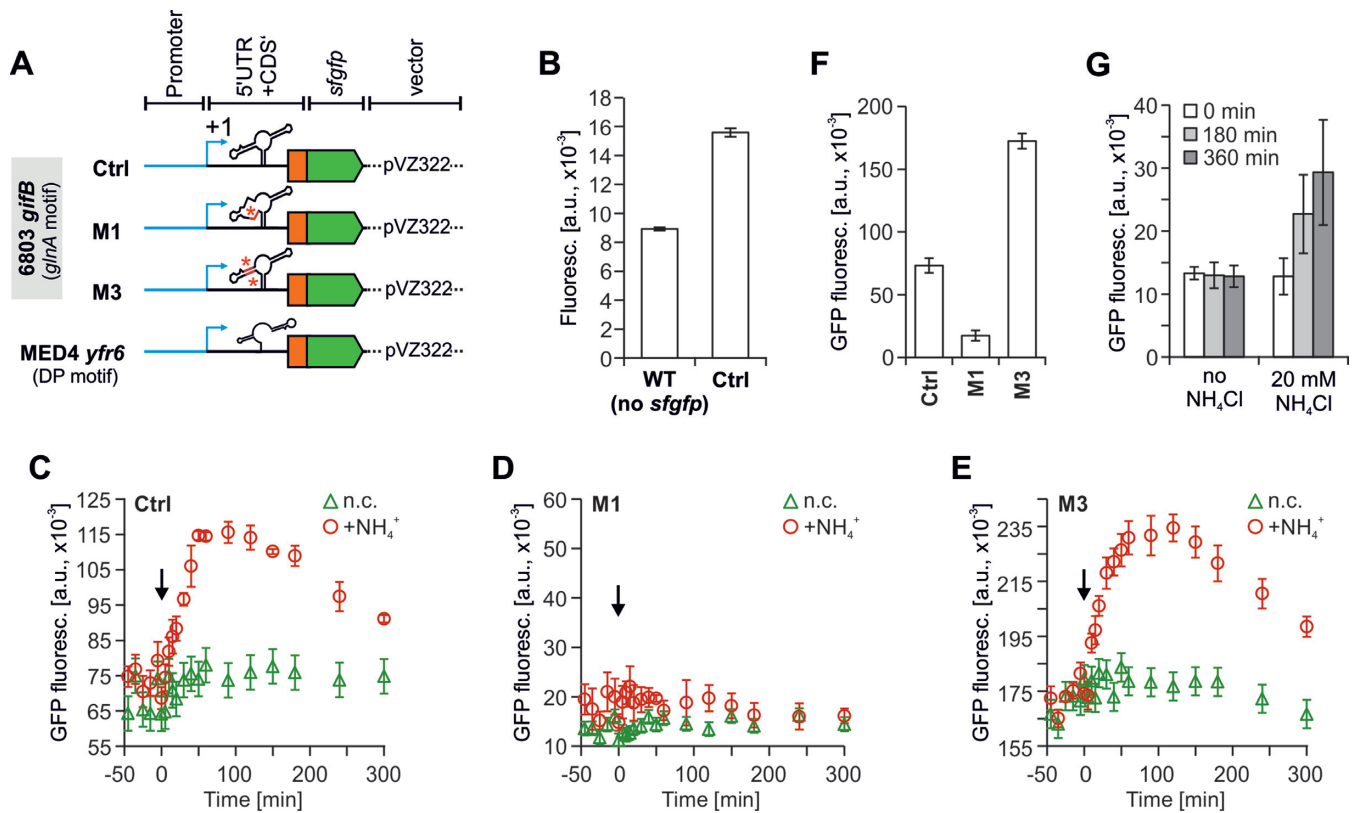


**Figure 2.** The *gifB* 5' UTR of *Synechocystis* harbors a glutamine-binding aptamer. (A) Predicted secondary structure of the *glnA* aptamer upstream of the *gifB* gene. The nucleotide positions refer to the transcriptional start site of the *gifB* gene (+1, ref. (21)). R1–3 label the regions for which structural modulation was observed (see panel B). The secondary structure is based on the secondary structure model of *glnA* motif RNAs published previously (42). (B) In-line probing analysis using a 5' <sup>32</sup>P-labeled *gifB* 5' UTR. Precursor RNA (Pre) was incubated for 2 days at 23°C without a ligand (0) or in presence of increasing concentrations of L-glutamine (3.2 μM to 10 mM, half-log dilutions). The same RNA was also partially treated with Na<sub>2</sub>CO<sub>3</sub>, which mediates alkaline degradation (-OH) or with RNase T1, which cleaves after G residues (T1). A non-treated RNA served as control (NR, no reaction). (C) Densitometric evaluation of the bands corresponding to regions R1–3 whose intensities changed due to the presence of glutamine (marked by red lines in panel B). The intensities were normalized to bands which did not show glutamine-dependent intensity changes (Ctrl). (D) In-line probing using mutated RNAs. The NR, T1 and -OH samples are shown for the WT RNA.

pairing disrupted by M1 also restored the NH<sub>4</sub><sup>+</sup>-induced rise in GFP fluorescence (Figure 3E). The data indicate that the structural change of the *glnA* aptamer strongly enhances *gifB* expression and adds an additional layer of control, which is glutamine dependent.

Strains harboring the DP aptamer are currently not amenable to genetic manipulation. Nevertheless, to examine its function we also fused a DP aptamer of *Prochlorococcus* MED4 to the *sfgfp* gene and the BBa\_J23101 promoter. After introducing the construct into *Synechocystis* we performed measurements as described above. Initially, the here chosen sequence harbouring the DP aptamer was regarded as a non-coding RNA (Yfr6) (47). Later it was found that Yfr6 contains an ORF of 33 codons (68) as well

as the DP aptamer in the putative 5' UTR (41). Consistently, substantial GFP fluorescence was detected in a *Synechocystis* strain that transcribes this 5' UTR in fusion with *sfgfp* (Figure 3G). This indicates that it indeed contains elements for translation initiation and that the corresponding IF17-like protein exists in *Prochlorococcus* (Figure S1). Although not as fast and sensitive as the *glnA* aptamer, the DP aptamer also mediates N-dependent expression regulation because an increased GFP fluorescence was observed upon NH<sub>4</sub><sup>+</sup> addition (Figure 3G). Altogether, the data verify that the *glnA* and DP aptamers are indeed part of functional riboswitches that control gene expression in a glutamine-dependent manner.



**Figure 3.** GFP fluorescence measurements of *Synechocystis* strains carrying translational fusions of DUF4278-encoding genes from *Synechocystis* or *Prochlorococcus* sp. MED4 with the *superfolder gfp* gene. (A) Schematic illustration of the reporter constructs on a pVZ322 plasmid. The construct representing the *glnA* aptamer contained the entire 5' UTR of *gfpB* and 54 nt of its coding region in frame with the *sfgfp* gene. Transcription was driven by the BioBrick BBa\_J23101 promoter (iGEM Registry, (48)) which was found to be constitutively active at a high level in *Synechocystis* (49). (B) Raw fluorescence of a WT (no *sfgfp* gene present) and a reporter strain carrying the Ctrl plasmid shown in panel A. Both strains had the same  $\text{OD}_{750}$ . For each following measurement the signals measured from a simultaneously cultivated WT was subtracted from the measured fluorescence values (background correction). (C–E) Background corrected GFP fluorescence of strains carrying the control construct (Ctrl, C) or point mutated versions of the *glnA* aptamer (D, E). Please note that M3 is the complementary mutation to M1 (see Figure 2A). At time point 0, 10 mM  $\text{NH}_4\text{Cl}$  (f.c.) were added to the cultures (indicated by arrow). In parallel, the same number of independent cultures remained untreated (negative control, n.c.). (F) Background corrected GFP fluorescence of reporter strains under standard (non-induced) growth conditions. (G) Background corrected GFP fluorescence of a *Synechocystis* strain carrying a DP aptamer within the 5' UTR of the gene WP\_071812974 of *Prochlorococcus* sp. MED4 in fusion with *sfgfp* (as shown in panel A). The measurement was performed under standard growth conditions and after adding 20 mM  $\text{NH}_4\text{Cl}$ . Data are the mean  $\pm$  SD of three biological replicates.

### Glutamine riboswitches are important for IF17 production and GS inactivation

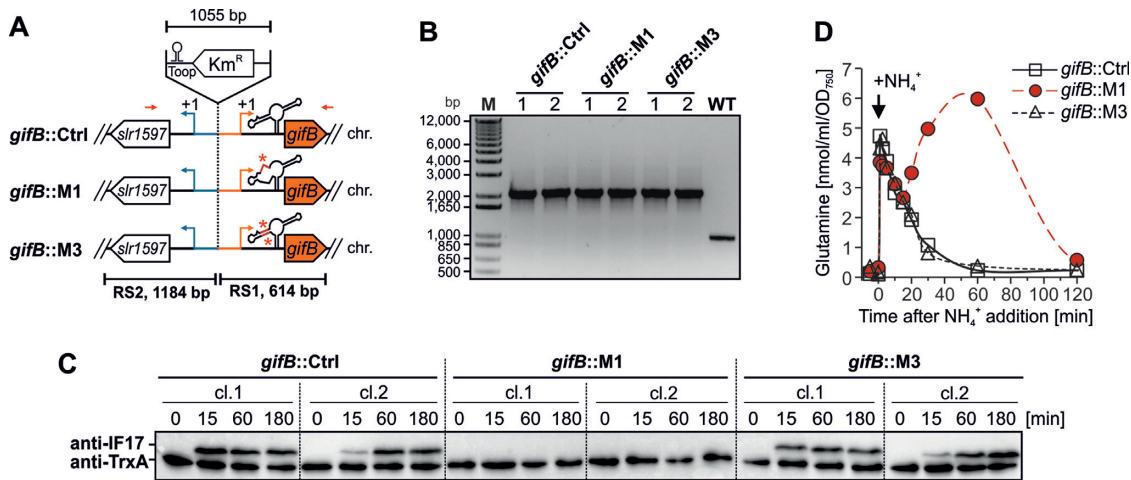
Our findings provide evidence that the *gfpB* gene and IF17 production is regulated by a glutamine riboswitch. However, the expression of *gfpB* is also controlled by the transcription factor NtcA (21). To directly address the impact of the riboswitch regulation on IF17 production *in vivo*, we introduced point mutations into the genomic locus upstream of *gfpB*. For this, we inserted a kanamycin resistance cassette ( $\text{Km}^R$ ) in the intergenic space between *gfpB* and the upstream gene *slr1597* by homologous recombination. In addition, we generated constructs carrying the mutations M1 and M3 in the *gfpB* 5' UTR which were also used in the other experiments. By this strategy we obtained the strains *gfpB::Ctrl*, *gfpB::M1* and *gfpB::M3* (Figure 4A, B).

Using an IF17-specific antibody, two clones of each strain were analyzed for *gfpB* expression and IF17 accumulation after adding  $\text{NH}_4\text{Cl}$ . Rapid IF17 accumulation was observed in the *gfpB::Ctrl* strain (Figure 4C), matching the WT situation. However, no IF17 accumulation was observed in

*gfpB::M1*, which carries a mutation in the *gfpB* 5' UTR that disrupts the glutamine-binding aptamer. To verify that this did not result from disrupting a sequence that is required for expression in general, we also examined the complementary point mutation M3 in which mutation M1 is still present but the structure of the RNA aptamer was restored. Indeed, IF17 accumulation was similar between the *gfpB::M3* and the *gfpB::Ctrl* strains (Figure 4C), verifying that the RNA structure itself is required for efficient IF17 production. Because we used the same mutations as in the in-line probing experiments, in which glutamine binding was abolished by M1 but restored by M3, it is very likely that the rapid IF17 accumulation in response to  $\text{NH}_4^+$  upshifts is a result of glutamine-dependent RNA structural modulation.

IF17 accumulation was lacking in *gfpB::M1*, even though the NtcA-dependent promoter was intact. Therefore, the glutamine riboswitch appears to exert the major control mechanism for IF17 production. Moreover, IF17 was reported as the functionally dominant of the two GS inactivating factors (20). Hence, the *gfpB*-associated glutamine riboswitch might also be the most critical part for the regu-





**Figure 4.** Mutagenesis of the glutamine riboswitch reveals its importance for *gifB* expression and GS regulation. (A) Schematic overview of the mutated genomic locus in *Synechocystis* strains carrying chromosomal point mutations in the glutamine riboswitch upstream of *gifB*. The kanamycin resistance ( $\text{Km}^R$ ) gene was introduced between the promoters of *gifB* and *slr1597*. The *gifB::Ctrl* strain harbors the WT sequences whereas in *gifB::M1* and *gifB::M3* the glutamine-binding aptamer carries point mutations M1 or M3 as shown in Figure 2A. Red arrows indicate the relative binding sites of primers used for PCR (panel B). RS - recombination site, chr. - chromosome. (B) PCR verification of the mutant strains using two biological replicates. Using WT gDNA a 977 bp fragment was amplified whereas a fragment of 2032 bp was amplified in the mutants. The point mutations M1 and M3 were verified by sequencing the PCR product. No WT allele was amplified in the mutants indicating that they are fully segregated. (C) Immunoblots showing the  $\text{NH}_4^+$  induced accumulation of IF17 (upper band). At time point 0, 10 mM  $\text{NH}_4\text{Cl}$  (f.c.) were added to cells grown in BG11 containing nitrate as sole N source. The TrxA protein level is shown as loading control (lower band). (D) Kinetics of intracellular glutamine levels in response to an upshift in  $\text{NH}_4^+$  availability (same conditions and treatment as used for immunoblots). Data are the mean of the two biological replicates.

lation of GS activity in cyanobacteria. To test this hypothesis, we determined the glutamine levels in response to  $\text{NH}_4^+$  upshifts in the strains *gifB::Ctrl*, *gifB::M1* and *gifB::M3*. As expected, cells of the *gifB::Ctrl* strain showed rapid and high accumulation of glutamine because this amino acid is the primary product of  $\text{NH}_4^+$  assimilation by GS (Figure 4D). Consistent with fast IF17 accumulation the glutamine levels started to decline immediately and reached the initial levels after about 60 min. Similar kinetics were observed in the *gifB::M3* strain. In *gifB::M1*, however, the levels declined only transiently and increased again after 20 min pointing towards an insufficient GS regulation in that time span (Figure 4D). After 2 h also in the *gifB::M1* strain similar glutamine levels were detected as observed prior to  $\text{NH}_4^+$  addition. Albeit other regulatory mechanisms involved, GS activity and glutamine synthesis are also significantly determined by riboswitch-controlled IF17 synthesis.

## DISCUSSION

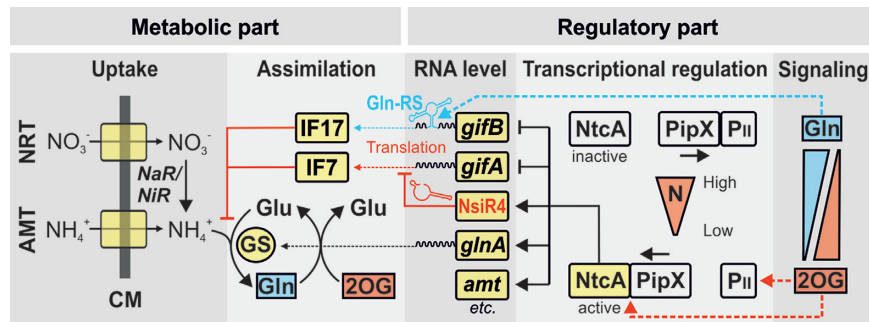
Our findings demonstrate that the previously reported *glnA* and DP aptamers (41,42) function as critical elements of glutamine riboswitches *in vivo*. Given their secondary structure differences, we suggest renaming these elements as type 1 and type 2 glutamine riboswitches.

### A type 1 glutamine riboswitch regulates the major GS inactivating factor in the majority of cyanobacteria

Here, we show that a type 1 glutamine riboswitch is part of the N-regulatory network in *Synechocystis* (Figure 5). Thereby, the riboswitch is a crucial regulatory element for the major GS inactivating factor IF17. In *Synechocystis*, GS activity is exclusively determined by the concentration

of its inactivating factors because both IF7 and IF17 can bind and inactivate GS without further modification (20). Although both factors seem to work cumulatively *in vivo*, IF17 appears to be more important for GS inactivation due to two reasons. First, IF17 has a higher affinity to GS compared to IF7. Second, upon  $\text{NH}_4^+$  upshift GS was hardly inactivated in a mutant strain lacking IF17 ( $\Delta gifB$ ) whereas GS was still significantly inactivated in a mutant strain lacking IF7 ( $\Delta gifA$ ) (20). A mutant strain carrying the dysfunctional riboswitch showed no IF17 accumulation, albeit the NtcA-regulated promoter mediating N-dependent *gifB* transcription was still intact. Consistently, the same mutant was strongly deficient in restoring low glutamine levels after sudden increases in  $\text{NH}_4^+$  availability pointing towards a partial loss of GS regulation. Nevertheless, after reaching the maximum of glutamine accumulation the levels started to decline in the riboswitch mutant as well (Figure 4D). This could be explained by the accumulation of IF7, the second GS inactivating factor which was still intact in *gifB::M1*. However, GS inhibition by IF7 alone is obviously not sufficient to reach the initial glutamine levels in the same time span as in the WT which is also consistent with literature data (20).

The major impact of the type 1 glutamine riboswitch was somewhat surprising because transcriptional control by NtcA was regarded as the major layer of control in the expression of both *gifA* and *gifB*, as well as the regulation of GS in cyanobacteria (21). However, our data clearly show that in addition to the NtcA-mediated transcriptional control, the regulation by a type 1 glutamine riboswitch constitutes another major control mechanism for IF17 production and GS activity, particularly after sudden changes in N availability. Similar type 1 glutamine riboswitches are fre-



**Figure 5.** Simplified overview of N control and GS regulation in the cyanobacterial model strain *Synechocystis* involving two different types of non-coding RNAs. AMT - ammonium transport system, NRT - nitrate transport system, CM - cell membrane, NaR - nitrate reductase, NiR - nitrite reductase, GS - glutamine synthetase, Gln - glutamine, Glu - glutamate, 2OG - 2-oxoglutarate, Gln-RS - glutamine riboswitch.

quently located upstream the ORFs of *gifB* homologs in other strains. Hence, this crucial regulatory mechanism appears to be common among cyanobacteria.

Nevertheless, its mode of action remains unclear to date. Structural analysis of a representative from *S. elongatus* PCC 7942 in a ligand-free as well as in the glutamine-bound state indicates that it might regulate translation. Ren et al. reported that high selectivity but low affinity binding of L-glutamine induces a conformational transition which includes a large change in the orientation of the P3 stem relative to P2 and P1 (43). This promotes long-range interactions, including a critical Watson-Crick base-pair interaction between G23-C60 (see Figure 4A of (43)). These conserved positions correspond to G40 and C68 in the *Synechocystis* riboswitch studied here (see Figure 2 and Supplementary Figure S3). Ren et al. also reported that the conformational transition and the long-range interaction melt a short helix attached to P1 which might modulate downstream events in gene expression (43). In *Synechocystis*, C68 and the downstream adjacent nucleotides are part of a short sequence that allows base-pairing with a putative ribosomal binding site (RBS) and might block the access of ribosomes (Supplementary Figure S3). Based on the structural data (43), we postulate that glutamine binding melts this inhibitory double strand and hence allows translation initiation (Supplementary Figure S3).

### Type 2 glutamine riboswitches are associated with IF17-like proteins in marine picocyanobacteria

The type 2 glutamine riboswitch was found in the monophyletic and specialized group of marine picocyanobacteria and regulates genes encoding proteins that share the DUF4278 with IF17. Therefore, these proteins can be regarded as IF17-like, which might also function as GS inactivating factors. However, it should be noted that the IF17-like proteins in *Prochlorococcus* strains lack the C-terminal amino acid sequences shown to be crucial for the interaction of IF17 with GS in *Synechocystis* (66). Nevertheless, it is possible that the *Prochlorococcus* GS co-evolved structural differences allowing interaction with these truncated proteins. From an evolutionary perspective, there must be an important reason for the presence and the high copy numbers of these IF17-like proteins and their pronounced regulation by a type 2 glutamine riboswitch.

*Prochlorococcus* is highly abundant in ultraoligotrophic marine waters and hence plays a major role in the nutrient cycles of those habitats. It is thought that its adaptation to these nutrient-poor conditions involved strong genome streamlining (69,70) and the replacement of protein regulators by non-coding RNAs (68). The presence and high frequency of glutamine riboswitches and of shortened versions of IF17 proteins is highly consistent with these ideas. Further evidence that these IF17-like proteins might have a function in N assimilation in marine picocyanobacteria is provided by the fact that they are most likely regulated by NtcA. A perfect NtcA-binding site overlapping the transcriptional start site was found upstream of the gene WP\_071812974, which encodes an IF17-like protein in *Prochlorococcus* sp. MED4 (Supplementary Figure S4). NtcA binding sites usually overlap the -35 element in promoters that are activated by it (19). In contrast, NtcA binding at positions close to or overlapping with the -10 element represses transcription (21,65). Hence, it can be assumed that IF17-like proteins are also under negative NtcA control in *Prochlorococcus*, similar to *gifB* in *Synechocystis* and other cyanobacteria.

Altogether, we conclude that the regulation of the major GS inactivating factor by glutamine riboswitches as described in this study is crucial for the regulation of GS activity in the majority of cyanobacteria.

### GS regulation in cyanobacteria involves different classes of non-coding RNAs

Controlling GS activity via inhibitory proteins is unique to cyanobacteria because other major bacterial groups use covalent modification to regulate this enzyme (9,20). Consistently, the glutamine riboswitches only occur in cyanobacteria and/or marine metagenome sequences. It should also be noted that, in metagenomic sequences of several marine samples, the type 1 glutamine riboswitch was also found upstream of *glnA* genes. Hence, in these organisms GS expression appears to be directly coupled to glutamine levels. Although negative regulation is reasonable, it is not known so far which effect glutamine-binding has on *glnA* expression in these organisms, nor what their taxonomic affiliation was.

The regulation of IF17 expression by a glutamine riboswitch adds another class of non-coding RNAs to the complex network of GS regulation in cyanobacteria. As

reported previously, the regulatory sRNA NsiR4 interacts with the *gifA* mRNA encoding IF7, the second GS inactivating factor (36). This interaction interferes with *gifA* translation and thus fine-tunes IF7 accumulation kinetics. Nevertheless, transcriptional regulation remains the major level of control in this special case because the transcription of both *gifA* and NsiR4 is controlled by NtcA (Figure 5). Whereas NsiR4 expression is activated by NtcA under N limitation, *gifA* is repressed under N limitation and de-repressed under N excess (21,36). Hence, NtcA and NsiR4 jointly suppress IF7 expression when the synthesis of a GS-inactivating factor is becoming disadvantageous under N-limitation. Altogether, RNA-mediated regulation can be regarded as crucially important for GS regulation in cyanobacteria because the expression control of both IF7 and IF17 involves non-coding RNAs which are widely distributed among this bacterial group.

### Glutamine is a signaling molecule in cyanobacteria

Several representatives of glutamine riboswitch aptamers have now been tested *in vitro*. Except for the aptamer of *Synechococcus elongatus* sp. PCC 7942, which also binds the non-natural D-glutamine with very poor affinity, all other representatives were verified to bind L-glutamine and none of the other tested molecules (42). The molecules tested include structural analogs of glutamine, other amino acids and also 2OG. Here, we confirmed glutamine binding by the aptamer present in the 5' UTR of the *Synechocystis gifB* gene. In addition to the previously examined ligand candidates, we demonstrate that the corresponding aptamer also rejects L-glutamate as well as  $\text{NH}_4\text{Cl}$ . Hence, we conclude that the regulatory effects observed *in vivo* after adding  $\text{NH}_4^+$  result from glutamine induced structural modulation. Altogether, the data provide strong evidence that glutamine is an additional signaling molecule in cyanobacteria, which is specifically sensed by an RNA aptamer that processes the signal directly to regulate gene expression.

Our findings contribute to the general understanding of sensing the N status in bacteria. Albeit the specific regulatory mechanisms are diverse, it is widely accepted that bacteria perceive the cellular N status via the intracellular concentration of 2OG (7). Nevertheless, glutamine is also central for N-signaling because several bacterial groups possess a second mode of signal perception via the glutamine sensing and  $\text{P}_{\text{II}}$ -modifying enzyme GlnD (for a review of glutamine signaling see ref. (6)). Moreover, in those bacteria that lack homologs of GlnD, independent glutamine signaling cascades evolved. For instance, in *Bacillus subtilis* the GS itself is the major glutamine sensor that transduces the signal to a transcriptional regulator (71). Accordingly, the *Bacillus* GS shows feedback inhibition by glutamine which is in contrast to the GS of proteobacteria that is inhibited by various metabolites but not by glutamine (72). In cyanobacteria, GS is neither inhibited by glutamine (73) nor regulated via a glutamine-dependent  $\text{P}_{\text{II}}$  signaling pathway due to the lack of GlnD homologs (6). Accordingly, for a long time it was believed that glutamine does not contribute to the perception of the N status in cyanobacteria. However, our data confute that assumption as we show that cyanobacteria evolved an alternative mechanism of sensing

and processing the glutamine signal by using a riboswitch. This sensing mechanism establishes a feedback loop by inducing the accumulation of IF17, which in turn inactivates GS and glutamine synthesis (Figure 5).

The glutamine riboswitch aptamer examined in this study exhibited poor ligand affinity compared to the aptamers of other riboswitch classes. This is consistent with  $K_{\text{D}}$  values between 0.5 and 5 mM previously reported for both types of glutamine aptamers (42). Much lower  $K_{\text{D}}$  values (i.e. higher affinities) have been reported, for instance, for glycine ( $K_{\text{D}} \sim 10 \mu\text{M}$ ) or lysine ( $K_{\text{D}} \sim 1 \mu\text{M}$ ) riboswitch aptamers (39,40). However, it should be noted that the intracellular glutamine level is rather high compared to other amino acids and highly dynamic in response to changes in N availability. In our experiments, the glutamine levels increased ~40 fold when cells grown on nitrate as the sole N source were suddenly exposed to  $\text{NH}_4^+$ . Similar dynamics have been reported for other bacteria such as *E. coli* (74) or *Salmonella typhimurium* (75). Moreover, the absolute glutamine concentration in glucose-fed, exponentially growing *E. coli* cells was measured to be 3.8 mM (76). Assuming a similar concentration range in cyanobacteria and taking the dramatic increases up to 40-fold into account, the poor ligand affinity of the glutamine riboswitch seems to be perfectly tuned to the physiologically relevant concentration of its ligand, perhaps to avoid continuous glutamine binding and switching into the ON state conformation.

Interestingly, similar affinities have been reported for other glutamine-sensing systems. Biochemical analyses of the bifunctional enzyme GlnD from *E. coli* which reversibly uridylylates/deuridylylates the  $\text{P}_{\text{II}}$  protein revealed that the reaction responded strongly to changes in the glutamine levels in the range between 0.1 and 4 mM (77), which is also within the physiological range of the reported cellular glutamine concentrations (76). Moreover, in green algae, mosses and higher plants, glutamine is sensed by a C-terminal extension of the  $\text{P}_{\text{II}}$  protein, the Q loop (78). For several representatives,  $K_{\text{D}}$  values for glutamine were estimated ranging from 2.4 to 9.2 mM (78) which are again within the range of glutamine concentrations reported in several plant species (79–81). This emphasizes how the independent and diverse glutamine-sensing elements have evolved to perfectly match the cellular levels of glutamine.

### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

### ACKNOWLEDGEMENTS

We thank Klaudia Michl for technical assistance during amino acid measurements and M.I. Muro-Pastor and F.J. Florencio (University of Seville, Spain) for providing the antisera used in this study. Moreover, we thank Karl Forchhammer (University of Tübingen, Germany) for critical reading.

*Author Contributions:* S.K. designed the study. S.K., P.B. and R.M.A. performed experiments. P.R.W. and K.I.B. performed bioinformatics. M.H. contributed the amino acid analysis. S.K., P.R.W., R.M.A., K.I.B., R.R.B. and W.R.H. analyzed data. S.K. wrote the manuscript with contributions from all authors.

## FUNDING

Wissenschaftliche Gesellschaft Freiburg im Breisgau and the Grünewald-Zuberbier-Stiftung [to S.K.]; National Science Foundation Graduate Research Fellowship Program [DGE1122492 to R.M.A.]; Howard Hughes Medical Institute; National Institutes of Health [GM022778 to R.R.B.]. Funding for open access charge: Helmholtz-Centre for Environmental Research.

*Conflict of interest statement.* None declared.

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