The sRNA NsiR4 is involved in nitrogen assimilation control in cyanobacteria by targeting glutamine synthetase inactivating factor IF7

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Glutamine synthetase (GS), a key enzyme in biological nitrogen assimilation, is regulated in multiple ways in response to varying nitrogen sources and levels. Here we show a small regulatory RNA, NsiR4 (nitrogen stress-induced RNA 4), which plays an important role in the regulation of GS in cyanobacteria. NsiR4 expression in the unicellular *Synechocystis* sp. PCC 6803 and in the filamentous, nitrogen-fixing *Anabaena* sp. PCC 7120 is stimulated through nitrogen limitation via NtcA, the global transcriptional regulator of genes involved in nitrogen metabolism. NsiR4 is widely conserved throughout the cyanobacterial phylum, suggesting a conserved function. In silico target prediction, transcriptome profiling on pulse overexpression, and site-directed mutagenesis experiments using a heterologous reporter system showed that NsiR4 interacts with the 5'UTR of *gifA* mRNA, which encodes glutamine synthetase inactivating factor (IF7). In *Synechocystis*, we observed an inverse relationship between the levels of NsiR4 and the accumulation of IF7 in vivo. This NsiR4-dependent modulation of *gifA* (IF7) mRNA accumulation influenced the glutamine pool and thus NH_4^+ assimilation via GS. As a second target, we identified *ssr1528*, a hitherto uncharacterized nitrogen-regulated gene. Competition experiments between WT and an ΔnsiR4 KO mutant showed that the lack of NsiR4 led to decreased acclimation capabilities of *Synechocystis* toward oscillating nitrogen levels. These results suggest a role for NsiR4 in the regulation of nitrogen metabolism in cyanobacteria, especially for the adaptation to rapid changes in available nitrogen sources and concentrations. NsiR4 is, to our knowledge, the first identified bacterial sRNA regulating the primary assimilation of a macronutrient.

regulatory RNA | *Synechocystis* | nitrogen assimilation | glutamine synthetase inactivating factors | NtcA

Cyanobacteria originated oxygenic photosynthesis and are of substantial morphological and physiological diversity. In addition to the photosynthetic fixation of inorganic carbon, some cyanobacteria also fix atmospheric dinitrogen, hence injecting combined nitrogen sources into the biogeochemical cycles. Therefore, cyanobacteria play an important role in global carbon and nitrogen cycles (1, 2). Unicellular strains, such as the non-diazotrophic *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803), and multicellular strains with differentiated cells, such as the diazotrophic *Anabaena* sp. PCC 7120 (hereafter *Anabaena* 7120), have been established as widely accepted model organisms.

Nitrogen Assimilation in Cyanobacteria

Cyanobacteria use different inorganic and organic sources of combined nitrogen, such as ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and urea, or some amino acids (3–6). Intracellularly, NO_3^- , NO_2^- , and urea are converted to NH_4^+ . The availability of NH_4^+ , the energetically most favorable nitrogen source, represses the uptake of other nitrogen sources (7). As in eukaryotic algae and plants, the key enzyme of nitrogen assimilation is glutamine synthetase (GS, encoded by *glnA*), which incorporates NH_4^+ into

metabolism via the amidation of glutamate to glutamine. Subsequently, glutamate synthase [glutamine oxoglutarate amidotransferase (GOGAT)] catalyzes the transfer of the amide group from glutamine to 2-oxoglutarate (2-OG), producing two molecules of glutamate. The posttranslational inactivation of GS is a major regulatory mechanism to limit nitrogen assimilation and maintain C/N balance. Whereas in *Escherichia coli* and most other gram-negative bacteria, GS activity is modulated through adenylation/deadenylation (8), cyanobacteria have evolved a unique mechanism of GS inactivation mediated through the small inhibitory proteins (inactivating factors) IF7 and IF17, encoded by the genes *gifA* and *gifB*, respectively (9). In addition to its function as substrate of the GS-GOGAT cycle, 2-OG is also a metabolite of the tricarboxylic acid (TCA) cycle, connecting nitrogen and carbon metabolism at a central point. Depending on the nitrogen or carbon availability, the level of 2-OG varies, making this metabolite an excellent indicator of nitrogen status and the C/N ratio (10, 11).

The Nitrogen Regulatory Network in Cyanobacteria

At the molecular level, 2-OG regulates the activity of the main transcriptional regulators of nitrogen assimilation (NtcA) (12, 13) and carbon metabolism (NdhR/CcmR) (14). Moreover, 2-OG also modulates the activity of two other regulators of cyanobacterial nitrogen metabolism: the signal transduction protein P_{II} and the

Significance

A key enzyme in inorganic nitrogen assimilation, glutamine synthetase, is the target of multiple regulatory mechanisms. Here we describe the nitrogen stress-induced RNA 4 (NsiR4), a small regulatory RNA that reduces the expression of inactivating factor 7 (IF7), an inhibitory factor of glutamine synthetase in cyanobacteria. The expression of NsiR4 is under positive control through the nitrogen control transcription factor (NtcA). NtcA also induces the transcription of the glutamine synthetase gene and represses the gene encoding IF7. Therefore, NsiR4 is a new player in the NtcA-mediated regulation of nitrogen assimilation, which is important for adaptations to rapid changes in available nitrogen sources and concentrations.

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regulatory factor PipX. Depending on the 2-OG level, which reflects the nitrogen status, PipX interacts with either NtcA or P_{II} (15, 16). For a schematic overview of the nitrogen regulatory network, see *SI Appendix, Fig. S1*.

The nitrogen control transcription factor NtcA binds as a homodimer to the conserved palindromic sequence GTA-N₈-TAC (17–20). Under nitrogen excess (e.g., in presence of NH₄⁺), the 2-OG level is low and NtcA is present in an inactive form, which has low affinity to its target promoters (21). Under nitrogen depletion, however, the 2-OG level increases and stimulates the complex formation between PipX and NtcA and the affinity of the binding of this complex to target promoters (22, 23). Depending on the location of the binding motif relative to the transcriptional start site (TSS), NtcA can act as either an activator or repressor (24). NtcA activates the expression of genes that are important when nitrogen is limiting, such as nitrogen uptake systems (e.g., *amt1*) and components for NO₃[−] (e.g., *nirA*) and NH₄⁺ assimilation (e.g., *glnA*) (18). In contrast, on nitrogen depletion, NtcA acts as a repressor of *gifA* and *gifB*, which encode the GS IFs (24).

Bacterial Small RNAs with Regulatory Potential

Bacterial small RNAs (sRNAs) can posttranscriptionally activate or repress gene expression (25). Genome-wide mapping of TSSs revealed a high number of potentially regulatory sRNAs in cyanobacteria (19, 26, 27). One of these sRNAs, the light-regulated and widely conserved PsrR1, is a key regulator of photosynthesis (28).

The expression of some sRNAs increases in nitrogen-limited cells of *Anabaena* 7120 or *Synechocystis* 6803, qualifying these molecules as potential regulators within the nitrogen regulatory network. Accordingly, these sRNAs were named nitrogen stress-induced RNAs (NsiR). The expression of NsiR1 is induced on nitrogen depletion, specifically in cells of *Anabaena* 7120 that are differentiating as nitrogen-fixing heterocysts (29, 30). NsiR2 and NsiR3 have also been identified in *Anabaena* 7120 (19), but the functions of these molecules have remained enigmatic. NsiR4 was first computationally predicted in *Synechocystis* 6803 (31) and subsequently verified as an expressed sRNA (called SyR12) in the genome-wide mapping of TSSs (26). NsiR4 is a 70-nt transcript that becomes strongly induced under nitrogen depletion in *Synechocystis* 6803 (27).

Here we elucidated the molecular functions of NsiR4 through the identification of two different targets, the mRNAs of *gifA* and *ssr1528*, the latter encoding a conserved but uncharacterized protein. We present evidence for an additional layer of GS regulation via the direct interaction of NsiR4 with the 5'UTR of the mRNA of the GS inactivating factor IF7, affecting the expression of this molecule and thereby impacting GS activity and NH₄⁺ assimilation. Thus, NsiR4 is the first example of an sRNA controlling the assimilation of a macronutrient.

Results

NsiR4 Is a Widely Conserved sRNA in Cyanobacteria. The phylogenetic conservation of an sRNA is an indicator of functionality. Therefore, we investigated whether NsiR4 homologs are also detected within the more than 200 cyanobacterial genomes publicly available through the Joint Genome Institute (JGI) database in January 2015. Using BlastN, putative *nsiR4* homologs were identified in at least 38 additional genomes, including the closely related *Synechocystis* sp. PCC 6714 (hereafter referred to as *Synechocystis* 6714) (32, 33), more distantly related strains, such as *Synechococcus* sp. PCC 7002, or several *Anabaena* and *Nostoc* species (Fig. 1A). Homologs of NsiR4 were not found in α -cyanobacteria (mainly marine *Prochlorococcus* and *Synechococcus*), *Thermosynechococcus*, and *Gloeobacter*, as well as some *Oscillatoria* (*SI Appendix, Fig. S2*). We concluded that *nsiR4* homologs exist in representatives from all five morphological subsections (34) but not in all cyanobacteria, suggesting a widely conserved function.

In addition to *Synechocystis* 6803, the expression of *nsiR4* was observed in the transcriptomic datasets available for *Synechocystis* 6714, *Anabaena* 7120, and *Synechococcus* sp. PCC 7002 (19, 35, 36). The genomic information and mapped TSSs indicated that two distinct NsiR4 forms of different lengths exist in different cyanobacteria. NsiR4 in strains with the longer form (e.g., *Synechocystis*, *Microcystis*, *Cyanothece*) possesses an additional 19- to 20-nt domain at the 5' end, capable to form a short hairpin (Fig. 1A and B). Otherwise, the predicted secondary structures appeared similar for both NsiR4 types, suggesting a potential role for the 16-nt single-stranded region, which is nearly identical in all NsiR4 sequences (Fig. 1A and B). Such regions usually facilitate interactions with the respective target mRNAs.

NsiR4 Expression Is Associated with the Nitrogen Status and Positively Regulated Through NtcA.

NsiR4 is one of the most highly induced sRNAs on nitrogen depletion in *Synechocystis* 6803 (27) and is the second-most abundant sRNA in the primary transcriptome (37). To determine the time course of induction and potential effects of different nitrogen sources, we measured NsiR4 expression in WT *Synechocystis* 6803 grown under standard conditions (nitrogen replete, 17.6 mM NO₃[−]) and after transfer into nitrogen-free medium. NsiR4 was detected at a low level in cells grown in the presence of NO₃[−], and started to accumulate at a higher level 12 h after removing NO₃[−]. After 48–72 h, the expression was ~10-fold higher than at the start of the experiment (Fig. 1C, Left). To examine the influence of the nitrogen source, three WT cultures were initially grown under standard conditions and then transferred to nitrogen-free medium. Into two of these cultures, we added 10 mM NH₄⁺ or 17.6 mM NO₃[−], cultivation was continued for 24 h, and the relative NsiR4 abundance was analyzed. Approximately 10-fold higher NsiR4 expression was observed in nitrogen-depleted cells compared with cells grown in the presence of NO₃[−]. In contrast, in the presence of NH₄⁺, NsiR4 levels remained below the detection limit (Fig. 1C, Right). We concluded that the expression of NsiR4 is influenced by the nitrogen status of the cell. Moreover, NsiR4 expression is also regulated through nitrogen in *Synechocystis* 6714 and *Anabaena* 7120 (19, 35), suggesting a conserved function associated with nitrogen metabolism.

To examine whether the nitrogen-regulated expression of NsiR4 is under transcriptional control, we conducted reporter gene assays. The upstream sequence of *nsiR4* from *Synechocystis* 6803 was fused to *luxAB* genes encoding luciferase, and expression was measured as bioluminescence in vivo. Indeed, the promoter activity showed similar kinetics in response to nitrogen depletion, as observed for the sRNA accumulation (Fig. 2A). In NtcA-activated promoters, NtcA-binding sites frequently overlap the −35 region and are centered close to position −41.5 with respect to the TSS (18, 19). Indeed, the sequence 5'-GTCAAATAGGCTAC-3', similar to the consensus for NtcA binding sites (17), is located 48–35 nt upstream the TSS of *nsiR4* (at position 1289326 on the complementary strand). Moreover, potential NtcA binding sites exist upstream of the putative *nsiR4* homologs in all other strains (Fig. 2C). We examined the functionality of this site in a reporter gene assay comparing the WT promoter with a mutated version in which three nucleotides were substituted (noted with underlining; GTCAAATAGGCTAC to CTCAAATAGGCATC). Indeed, the activation of *luxAB* expression through nitrogen depletion was lost in the strain carrying the mutated promoter (Fig. 2B). Moreover, NsiR4 expression could not be activated in the nitrogen-depleted cells of an *Anabaena* 7120 *ntcA* mutant (Fig. 2D). These data demonstrate that the NsiR4 promoter is under NtcA control in both *Synechocystis* 6803 and *Anabaena* 7120.

Advanced Target Prediction and Transcriptome Analyses Suggest *gifA* and *ssr1528* as NsiR4 Targets. To predict potential targets of NsiR4, we used the CopraRNA algorithm, considering the folding,

Fig. 1. NsiR4 is broadly conserved in cyanobacteria. (A) Alignment of genome loci putatively encoding NsiR4. Verified TSSs are boxed in red (19, 26, 35). Roman numerals indicate the respective morphological subsections (34). (B) Conservation of the NsiR4 structure. The consensus structure was predicted using RNAalifold (61) and 30 randomly selected NsiR4 homologs of both types. The short hairpin was predicted for the 5' extension of long NsiR4 forms. The color indicates the number of different interacting pairs (C-G, G-C, A-U, U-A, G-U, or U-G) and thereby conservation of these base pairs. The saturation increases with the number of compatible base pairs, thus indicating the structural conservation. (C) Nitrogen-responsive expression of NsiR4 in *Synechocystis* 6803. Cells were transferred from standard conditions (17.6 mM NO₃⁻) to NO₃⁻-free BG11, total RNA was separated, blotted and hybridized with ³²P-labeled, single-stranded RNA probes. (Left) Expression kinetics of NsiR4 in response to N depletion. (Right) Steady-state levels in response to the nitrogen status mediated through different nitrogen sources or the depletion of N. Cells grown in the presence of NO₃⁻ were washed and resuspended in media containing 17.6 mM NO₃⁻, 10 mM NH₄⁺, or no nitrogen source. The cells were grown for additional 24 h before RNA extraction.

hybridization, and conservation of a particular sRNA (38). The highest interaction probability was predicted for the *gifA* (*ssl1911*) mRNA (Table 1). This gene encodes the GS inactivating factor IF7 and is also, but negatively, regulated through NtcA.

To identify potential target genes experimentally, we generated *Synechocystis* strains with altered NsiR4 expression, i.e., overexpression (NsiR4oex), KO (Δ nsiR4), and compensatory (Δ nsiR4::oex) strains. To achieve inducible expression, we fused the NsiR4 sequence to the *petE* promoter, which is specifically activated in the presence of Cu²⁺. The absence of NsiR4 in the KO strain and the Cu²⁺-dependent inducibility of NsiR4 in the other two strains was experimentally verified (SI Appendix, Fig. S3).

The target-sRNA interaction frequently reduces mRNA stability (39). Thus, a microarray experiment was performed with

RNA extracted from cells in which NsiR4 was pulse-expressed through the addition of Cu²⁺ for 12 h. Compared with WT, in NsiR4oex reduced *gifA* mRNA abundance was observed, for which an interaction was also predicted by the CopraRNA algorithm (compare Table 1 and SI Appendix, Table S1). Additionally, *gifA* expression was increased in Δ nsiR4, but decreased on introduction of the compensatory construct (Δ nsiR4::oex; (SI Appendix, Table S1). Another gene, *ssr1528*, exhibited a similar pattern, suggesting that this gene is a second target of NsiR4. The product of *ssr1528* is a hypothetical protein, with a domain (DUF4090) widely conserved among cyanobacteria (SI Appendix, Fig. S2). Moreover, similar to *gifA*, the expression of this gene is repressed under nitrogen depletion (40), suggesting a function related to the cellular nitrogen status.

Fig. 2. NsiR4 expression is mediated through an NtcA-activated promoter. (A) Bioluminescence of a *Synechocystis* reporter strain harboring a transcriptional fusion of *PnsiR4* (−130 to +49, TSS at +1) and *luxAB* genes in response to N depletion. Initially, cells were grown under standard conditions (17.6 mM NO_3^-) and subsequently transferred to NO_3^- -free BG11 medium. (B) Bioluminescence of a *Synechocystis* *PnsiR4::luxAB* reporter strain bearing a mutated NtcA motif in the presence of 17.6 mM NO_3^- (+N) or under N depletion for 24 h (−N). To improve and compare bioluminescence mediated through both promoters under +N, 10 mM glucose was added to the cultures. Thus, the values are higher than in A. Bioluminescence data are presented as the means $\pm$ SD of n independent measurements in at least two independent experiments, including two biological replicates (= independent transformants). In each experiment, a strain carrying a promoterless *luxAB* was used as a negative control (in each case measured in two independent cultures, $n = 2$). (C) The sequences upstream of *nsiR4* from different cyanobacteria. Putative NtcA binding sites and −10 elements are highlighted. The verified TSSs are shown in red. (D) Verification of N-regulated and NtcA-dependent NsiR4 expression in *Anabaena* 7120 WT and an *ntcA* insertion mutant through Northern blot analysis.

We speculated that the modulation of NsiR4 expression and its potential impact on nitrogen-associated target genes might also affect growth or the tolerance to high concentrations of NH_4^+ . In short-term growth experiments strains with altered levels of NsiR4 showed no noticeable phenotype (SI Appendix, Fig. S4). However, in long-term growth competition experiments, especially when the availability of nitrogen is fluctuating, the ΔnsiR4 mutant strain showed reduced growth rates compared with WT (Fig. 3). These findings directly support the physiological importance of NsiR4 in addition to its evolutionary conservation, suggesting that the presence of NsiR4 has been under positive selection in cyanobacteria.

The *ssr1528* Gene Is Directly and Posttranscriptionally Regulated Through NsiR4. The changes observed in the microarray experiment were verified through a detailed expression analysis. The expression of *ssr1528* was detected in WT under standard conditions (17.6 mM NO_3^-), which was not significantly affected by the addition of Cu^{2+} (Fig. 4A). However, in strain NsiR4oex carrying an additional, *PpetE*-regulated *nsiR4* copy, *ssr1528* mRNA abundance was diminished on NsiR4 induction. Consistently, *ssr1528* mRNA abundance increased in strain ΔnsiR4 compared with WT. In response to nitrogen depletion, the *ssr1528* mRNA levels decreased in WT and NsiR4oex, confirming the N-regulated expression. However, whereas the *ssr1528* mRNA disappeared in WT after 12-h N depletion, the expression of this gene remained at a high level in ΔnsiR4 (Fig. 4A). Moreover, *ssr1528* expression did not increase when 10 mM NH_4^+ was added to the medium (SI Appendix, Fig. S5), indicating that the decrease under N depletion is not directly NtcA dependent and might be exclusively regulated through NsiR4 at the posttranscriptional level.

To examine whether a direct NsiR4:*ssr1528* interaction caused the observed changes in mRNA expression, the *ssr1528* 5'UTR was fused to the gene for the superfolder green fluorescent pro-

tein (*sgfp*) and coexpressed with NsiR4 in *E. coli*. GFP fluorescence was measured in strains carrying various combinations of plasmids (41). Compared with the control (pXG-0+pJV300), the strain carrying the *ssr1528-sgfp* fusion showed significant GFP fluorescence, demonstrating that the translation initiation from the *ssr1528* 5'UTR of *Synechocystis* is also functional in *E. coli* (Fig. 4B). In the presence of the NsiR4-expressing plasmid, the GFP fluorescence decreased approximately twofold, indicating a direct interaction between NsiR4 and the *ssr1528*-5'UTR, which affects translation. To verify the interaction at the predicted site, point mutations were introduced in *ssr1528* (positions +22 and +23: UA > AU, +1 = TSS) or NsiR4 (positions +16 and +17: UA > AU; Fig. 4D). Indeed, the mutation of either one of these sequences diminished the interaction, indicated as reduced repression (Fig. 4C). However, the combination of both mutations restored repression (both mutations are complementary; Fig. 4C). These data confirmed the direct interaction of NsiR4 with *ssr1528* mRNA, which also potentially affects the translation of this protein in *Synechocystis*.

The *gifA* Gene Is Directly and Posttranscriptionally Regulated Through NsiR4. The *gifA* mRNA was suggested as a direct NsiR4 target through computational prediction and microarray analysis. Similar to *ssr1528*, we performed detailed expression analysis for *gifA* in response to Cu^{2+} (for overproduction in NsiR4oex) and N depletion (induction of native *nsiR4* expression) or increased NH_4^+ availability (for repressing native *nsiR4* and de-repressing *gifA*). As observed for *ssr1528*, *gifA* was expressed in WT strains under standard conditions (17.6 mM NO_3^-) and was not significantly affected when adding Cu^{2+} (Fig. 5A). However, compared with WT, the *gifA* mRNA level was clearly diminished in NsiR4oex (Fig. 5A) and increased in strain ΔnsiR4 . In response to N depletion and due to negative regulation through NtcA, the *gifA* mRNA levels decreased in a similar manner in all strains. However, in response to

Table 1. List of predicted interaction partners for NsiR4 based on the CopraRNA algorithm

Rank	P value	Locus tag	Gene	Annotation
1	5.31E-08	<i>ssl1911</i>	<i>gifA</i>	Glutamine synthetase inactivating factor IF7
2	4.59E-06	<i>slr0701</i>	<i>merR</i>	Mercuric resistance operon regulator
3	6.17E-05	<i>slr0394</i>	<i>pgk</i>	Phosphoglycerate kinase
4	1.31E-04	<i>slr0869</i>	<i>aat</i>	Leucyl/phenylalanyl-tRNA transferase
5	2.54E-04	<i>slr0376</i>	NA	Hypothetical protein
6	3.99E-04	<i>slr5083</i>	NA	Hypothetical protein
7	4.30E-04	<i>slr0572</i>	NA	Hypothetical protein
8	6.14E-04	<i>slr1176</i>	<i>glgC</i>	Glucose-1-phosphate adenylyltransferase
9	6.97E-04	<i>slr0162</i>	NA	Hypothetical protein
10	7.80E-04	<i>slr1543</i>	NA	Hypothetical protein

NsiR4 sequences from the following strains were used: *Synechocystis* 6803, *Microcystis aeruginosa* NIES-843, *Cyanothece* sp. ATCC 51142, *Cyanothece* sp. PCC 7424, *Cyanothece* sp. PCC 7822, *Synechococcus* sp. PCC 7002, *Pleurocapsa* sp. PCC 7327, *Stanieria cyanosphaera* PCC 7437. NA, not available.

elevated NH_4^+ concentrations, the differences between the strains became more evident (Fig. 5B). The strong accumulation of *gifA* mRNA, which is typical for WT, was not observed in NsiR4oex, indicating the suppression by high levels of NsiR4. Consistent with previous studies (9), the mRNA induction in WT was rather

transient, peaking after 3–6 h. Compared with WT, the maximum *gifA* mRNA level was approximately sixfold lower in NsiR4oex and ~40% higher in ΔnsiR4 . In all cases, the increased levels in ΔnsiR4 were abolished when NsiR4 was ectopically expressed (compensatory strain $\Delta\text{nsiR4}::\text{oex}$).

Fig. 3. Competitive growth analysis of WT and ΔnsiR4 . (A) Experimental setup. Three independent, exponentially growing cultures of WT and ΔnsiR4 were diluted to an OD_{750} of 0.1 and mixed 1:1. (B) Growth performance during long-term cultivation. Cultures were rediluted every 3 or 4 d to an OD_{750} of 0.1 and 25 μL of a 1:1,000 dilution were dropped on BG11 agar plates with and without 40 $\mu\text{g}/\text{mL}$ kanamycin. Data are the mean $\pm$ SD of WT and ΔnsiR4 , as well as three mixed WT/ ΔnsiR4 cocultures. Due to the lowered NO_3^- content (1 mM instead of 17.6 mM NO_3^-), a strong fluctuation in the cellular N status could be assumed, which was also indicated by the cultures switching between green or bleached appearance at lower (0.1–0.8) and higher (>0.8) ODs (see also C). (C) Whole cell absorption spectra of the WT culture indicating cellular bleaching, which was due to pigment degradation as response to initiated internal N limitation. (D) Number of generations that were observed over 50 d at average growth rates of $\mu = 0.751 \pm 0.065$. (E) Evolution of the ratio between kanamycin-resistant (ΔnsiR4) and total (WT + ΔnsiR4) colony forming units (CFUs) during consecutive cultivation and redilution. (F) Photographs of CFUs at the beginning of the experiment and after 33 and 48 generations (g). (G) Verification of the ΔnsiR4 mutant allele reduction (upper band) compared with the WT allele (lower band) by PCR. *To one of the WT/ ΔnsiR4 cocultures once per day 100 μM NH_4Cl (f.c.) was added to intensify the fluctuation in the N availability. However, no change in growth performance compared with the other two cocultures was observed. Thus, all three cocultures were averaged as shown in E.

Fig. 4. Verification of the posttranscriptional regulation of *ssr1528* through direct NsiR4 interaction. (A) Changes in the mRNA abundance of *ssr1528* in response to altered NsiR4 expression detected by hybridization with a single-stranded ^{32}P -labeled transcript probe. Nitrogen depletion was induced in cultures grown in the presence of $2\ \mu\text{M}\ \text{Cu}^{2+}$ for 48 h (= 0 h –N). A representative 5S rRNA loading control hybridization (for WT) is shown. WT, *Synechocystis* 6803 WT; NsiR4oex, strain carrying pVZ322-PpetE::nsiR4::oop plasmid (overexpressor); ΔnsiR4 , deletion mutant; $\Delta\text{nsiR4::oex}$, deletion strain in which NsiR4 expression was restored by the pVZ322-PpetE::nsiR4::oop plasmid. (B) GFP fluorescence measurements of *E. coli* TOP10 strains with various combinations of plasmids expressing NsiR4 or *ssr1528*-sgfp fusions. The plasmids pXG-0 (encoding luciferase) and pJV300 (encoding a control RNA) were used as negative controls (for experimental details, see ref. 41). (C) Repression was calculated by dividing the values measured for *ssr1528*-sgfp in the presence of pJV300 with the corresponding value when NsiR4 was present. Autofluorescence measured for negative control cells was subtracted from every measurement before the calculation. The data are presented as the means $\pm$ SD of six independent colonies. (D) Predicted interaction site between NsiR4 and *ssr1528* and the respective hybridization energies of the native and mutated sequences at $30\ ^\circ\text{C}$ (the respective nucleotides are boxed in red). The gray box highlights the *ssr1528* start codon. In the RNA sequences, the numbers refer to the TSS at position +1. Predictions were made using IntaRNA (42).

To verify the interaction, the *gifA* 5'UTR was fused to the *sgfp* gene and coexpressed with NsiR4, as done before for *ssr1528*. In the presence of NsiR4, GFP fluorescence decreased two- to threefold, indicating a direct interaction between NsiR4 and the *gifA*-5'UTR, which affects protein synthesis (Fig. 5C). The IntaRNA algorithm (42) predicted the interaction site between NsiR4 and the *gifA* mRNA at positions +28 to +57 with respect to the TSS. This site directly overlaps with the *gifA* start codon at positions +52 to +54 (Fig. 5E). To verify the interaction at this site, we introduced point mutations in either *gifA* (position +30: C > T) or NsiR4 (position +31: G > A; Fig. 5E). The mutation of either one of these sequences prevented the interaction, whereas the combination of these two mutations, which are complementary, restored repression (Fig. 5D). Thus, the direct interaction between both RNAs at the predicted site was confirmed.

The results of the *E. coli* reporter assays suggested a negative impact of NsiR4 on the protein level. To investigate whether the level of IF7 is affected through ectopic NsiR4 expression also in *Synechocystis* 6803, we performed Western blot analysis using antibodies specific for IF7 (and IF17, which should not be influenced through NsiR4). The cells were supplemented with $10\ \text{mM}\ \text{NH}_4^+$ to de-repress *gifA* expression and induce IF7 accumulation, which was observed in WT as expected (Fig. 6A). However, in strain NsiR4oex, the IF7 protein level was lower compared with WT

and was not stimulated through NH_4^+ addition. The IF17 accumulation was barely affected in this strain. In contrast, in ΔnsiR4 , strong IF7 accumulation was observed after adding NH_4^+ , and the steady-state level was $\sim 60\%$ higher than in WT, whereas the levels of IF17 were similar to those in the WT experiment. Consistently, this effect was compensated in strain $\Delta\text{nsiR4::NsiR4oex}$, yielding a similar pattern as observed in WT (Fig. 6B).

The Kinetics of Glutamine/Glutamate Levels Suggest an Indirect Impact of NsiR4 on GS Activity and NH_4^+ Assimilation. The inactivation of GS on increased nitrogen supply correlates with the presence of inactivating factors IF7 and IF17 (9). Therefore, the posttranscriptional regulation of *gifA* through NsiR4 could influence GS activity and modulate the rate of nitrogen entry into metabolism. To examine whether the modified *gifA* expression in the *nsiR4* mutant strains also affects GS activity, we examined the substrate and product kinetics after a sudden upshift of the external NH_4^+ concentration. In cyanobacteria, a rapid but transient change in the intracellular amino acid and 2-OG pools occurs on changes in nitrogen availability (10).

The primary reaction of NH_4^+ assimilation is consuming glutamate and producing glutamine. In all strains used here the internal pools of both amino acids strongly changed within minutes after adding $10\ \text{mM}\ \text{NH}_4^+$, i.e., the levels of glutamine (produced by GS) increased and glutamate (consumed by GS) decreased. Moreover,

Fig. 5. Changes in the mRNA abundance of *gifA* in response to altered NsiR4 expression. (A) Expression kinetics was measured after Cu^{2+} addition and nitrogen depletion. Nitrogen depletion was induced in cultures grown in presence of $2 \mu\text{M}$ Cu^{2+} for 48 h (=0 h –N). (B) Expression of *gifA* after adding 10 mM NH_4^+ . Before NH_4^+ addition, the strains were precultivated for 6 h in the presence of $2 \mu\text{M}$ Cu^{2+} . The order of blots is the same as in A. For clarity, only a representative 5S rRNA loading control hybridization for NsiR4oex is shown. WT, *Synechocystis* 6803 WT; NsiR4oex, WT strain carrying pVZ322-PpetE::nsiR4::oop plasmid (overexpression strain); ΔnsiR4 , deletion mutant; $\Delta\text{nsiR4}::\text{oex}$, deletion strain in which NsiR4 expression was restored by the pVZ322-PpetE::nsiR4::oop plasmid. (C) Verification of the direct interaction between NsiR4 and *gifA* using an in vivo reporter system. GFP fluorescence measurements of *E. coli* TOP10 strains with various combinations of plasmids expressing NsiR4 or *gifA*-sgfp-fusions. The plasmids pXG-0 and pJV300 were used as negative controls (for experimental details, see ref. 41). (D) Repression calculated by dividing the values measured for *gifA*-sgfp with the corresponding value when NsiR4 was present. Autofluorescence correction and number of biological replicates as in the legend to Fig. 4C. (E) Predicted interaction site between NsiR4 and *gifA* and the respective hybridization energies of the native and mutated sequences at 30 °C (the respective nucleotides are boxed in red). The positions are indicated with respect to the TSS +1 for *nsiR4* (26) and *gifA* (24). The gray box highlights the *gifA* start codon. Predictions were made using the IntaRNA webserver (42).

the typical restoration of the glutamine/glutamate pool could also be observed, and the steady-state levels at 24 h after NH_4^+ addition were similar in all examined strains. However, in ΔnsiR4 , which showed the rapid accumulation of IF7 protein compared with WT (Fig. 6), the glutamine level also declined more rapidly (Fig. 7). Thus, the higher IF7 level in this strain indeed significantly contributed to GS inactivation. Additionally, an inverse tendency was observed for the glutamate pool, again with differences between WT and ΔnsiR4 . However, the kinetics of both strains harboring the plasmid for ectopic Cu^{2+} -regulated expression was similar to WT. This result indicates that the altered rate of glutamine/glutamate pool restoration in ΔnsiR4 is compensated through NsiR4 expression. However, the overexpression of NsiR4 and delayed accumulation of IF7 (Fig. 6) only slightly impacted GS inactivation, which is additionally achieved through the accumulation of the other inactivating factor IF17 (not affected through altered NsiR4 expression). Interestingly, in addition to the effects on GS and the glutamine/glutamate pool, we also observed differences in response to NH_4^+ for another amino acid, arginine (Fig. 7 C and D), when NsiR4 was overexpressed. The changes in the arginine pool appeared delayed compared with the changes in glutamine levels. Moreover, in contrast to glutamine and glutamate, the arginine steady-state levels (24 h after NH_4^+ addition) were also enhanced in the strains expressing NsiR4 ectopically.

Discussion

Nitrogen-Regulated sRNAs in Bacteria. To date, little is known about nitrogen-regulated sRNAs in bacteria. In *Pseudomonas aeruginosa*, the nitrogen-regulated sRNA NrsZ controls the production of rhamnolipid surfactants, as virulence factors required for

swarming motility, via the posttranscriptional control of the rhamnolipid synthesis gene *rhlA* (43). The expression of NrsZ is induced through the NtrB/C two-component system and is highly conserved among *Pseudomonads* (43). Other sRNAs are indirectly associated with nitrogen metabolism. In many γ -proteobacteria, GcvB acts as a distinct regulatory factor in nitrogen metabolism through the repression of transporters involved in amino acid uptake (44). In cyanobacteria, several nitrogen stress-induced RNAs (NsiR1, NsiR2, and NsiR3) have been described in *Anabaena* 7120 (19, 29), but their functions remain elusive. In the present study, we demonstrate NsiR4 to be directly involved in the regulation of primary nitrogen assimilation.

NsiR4 Executes the Posttranscriptional Regulation of Glutamine Synthetase Inactivating Factor IF7 and Interferes with NH_4^+ Assimilation.

The expression and activity of GS is controlled through the nitrogen status in various ways. The main physiological function of the GS inactivation system in cyanobacteria is to restore amino acid homeostasis on strong changes in the availability of nitrogen (10). Due to the central role of GS, similar responses to nitrogen changes, such as a decrease in the glutamine pool under nitrogen starvation, were observed, even in microorganisms as different as *E. coli* and yeast (45). Consistently, a transient increase in glutamine was observed in *E. coli* after the addition of 10 mM NH_4^+ (46). Interestingly, a $\Delta\text{gifA}\Delta\text{gifB}$ double mutant of *Synechocystis* 6803 showed no GS inactivation and lacked the ability to rapidly restore the glutamine pool to initial levels on NH_4^+ addition (10). Similarly, in strains bearing a mutant GS protein not susceptible to inactivation through IFs, the same phenotype was observed concerning the restoration of the glutamine pool (47). Because the inactivation of GS requires adequate levels of IF7 and/or IF17 (9), the posttranscriptional regulation

Fig. 6. Changes in the IF7 protein levels in response to altered NsiR4 expression. (A) Immunoblots using antibodies specific for inactivating factors IF7 and IF17 (and TrxA, which was used as a loading control). (B) Densitometric evaluation of the signals for IF7 after normalization to the TrxA signal intensity. The data are given as the relative expression compared with the value for strain $\Delta nsiR4$ at 180 min (=100%). WT, *Synechocystis* 6803 WT; NsiR4oex, WT strain carrying pVZ322-PpetE::nsiR4::oop plasmid (overexpression strain); $\Delta nsiR4$, deletion mutant; $\Delta nsiR4::oex$, deletion strain in which NsiR4 expression was restored by the pVZ322-PpetE::NsiR4::oop plasmid.

of *gifA* through NsiR4 impacts GS activity and hence should code-terminate the rate of nitrogen entry into metabolism. Indeed, this assumption is consistent with the rate of glutamine decrease in $\Delta nsiR4$ observed in the present study (Fig. 7). However, IF7 is assumed to have a lower impact on GS inactivation compared with IF17, as GS activity was still reduced to 50% in a $\Delta gifA$ single mutant in response to excess NH_4^+ , whereas in the $\Delta gifB$ single mutant, GS maintained ~90% activity (9). These data are consistent with the findings of the present study, as the strain overexpressing NsiR4 (showing reduced IF7 levels) did not show altered glutamine/glutamate kinetics compared with WT because GS inactivation was primarily influenced through the remaining IF7 protein, and IF17 protein levels were not affected. Nevertheless, the evolutionary conservation of NsiR4 and *gifA* indicates that the regulatory interaction of these two molecules is relevant. The changed glutamine kinetics in response to NH_4^+ excess in the $\Delta nsiR4$ KO strain highlights the physiological significance of NsiR4-mediated modulation of the IF7 expression level on GS activity. Assuming that the interaction with NsiR4 reduces the half-life of the *gifA* mRNA, this posttranscriptional mechanism might be faster and more effective for reducing the IF7 protein content and increasing GS activity under

nitrogen limitations. Moreover, even subtle changes in GS regulation have a significant impact on the fitness of *Synechocystis* 6803 cells exposed to nitrogen pulses, because the WT with intact IF7-dependent GS regulation outcompeted the $\Delta nsiR4$ mutant under fluctuating nitrogen conditions, which are characteristic for natural environments.

Interestingly, in addition to the effects on GS and the glutamine/glutamate pool, we also observed differences in response to NH_4^+ for another amino acid, arginine. However, it is unclear whether this effect is secondary in response to the changed GS activity or highlights a role for Ssr1528 or additional as-yet unidentified targets of NsiR4 (see below). Nonetheless, the changed arginine pools support a relevant role for NsiR4 in the nitrogen regulatory network, as the *N*-acetylglutamate kinase, involved in arginine synthesis, is another key enzyme in the nitrogen assimilation network controlled through the regulatory P_{II} protein (48).

Distribution of NsiR4 Among Cyanobacteria and Evolution of Target Sets. In addition to *gifA*, NsiR4 might regulate additional targets (Table 1), similar to many other sRNAs, e.g., the cyanobacterial sRNA PsrR1, which regulates a set of photosynthesis genes, thereby executing a global regulatory function (28). In the NsiR4oex strain, decreased *ssr1528* gene expression was observed, whereas increased *ssr1528* gene expression was observed in the $\Delta nsiR4$ KO strain. The changed expression and the direct interaction between NsiR4 and *ssr1528* were verified in the present study, extending the NsiR4 target set to at least two genes. Unfortunately, the function of *ssr1528* is not known. This gene encodes a small protein of 94 amino acids with a widely conserved domain (DUF4090) among cyanobacteria (*SI Appendix*, Fig. S2).

Fig. 7. Kinetics of intracellular amino acid pools in response to a sudden increase in NH_4^+ availability. Kinetics of glutamine (A) and glutamate (B) are shown for WT and the $\Delta nsiR4$ KO strain. Additionally, arginine kinetics (C) and steady-state levels before and 24 h after NH_4^+ upshift (D) are given for $\Delta nsiR4$, NsiR4oex, and the compensatory strain $\Delta nsiR4::oex$ compared with WT. The cells were grown in 250-mL Erlenmeyer flasks containing 100 mL BG11 and 17.6 mM NO_3^- . After reaching an OD_{750} of ~0.8, 2 μ M $CuSO_4$ was added, and after further incubation for 24 h, 10 mM NH_4Cl was added to the cultures (indicated with an arrow). Amino acids were extracted from cells before and after NH_4^+ addition and quantified through HPLC. The values for each strain are indicated as the relative levels referring to the maximum/minimum amount at 2.5 min (glutamine/glutamate) or at 30 min (arginine) after NH_4^+ addition (=1). The data are presented as the means $\pm$ SD of two biological replicates, measured in two technical replicates. Before the calculation of the relative amino acid levels, the absolute amounts were normalized to OD_{750} and the total concentration of free amino acids.

Fig. 8. NsiR4 is involved in regulating NH_4^+ assimilation. Schema illustrating the N-regulated feed-forward mechanism for the direct regulation of the glutamine synthetase inactivating factor IF7, thereby also affecting GS activity. AMT, ammonium transporter; NRT, nitrate transporter; GS, glutamine synthetase; GOGAT, glutamine oxoglutarate amidotransferase (glutamate synthase); NR, nitrate reductase; NIR, nitrite reductase; CM, cytoplasmic membrane.

Moreover, *ssr1528* expression was repressed under N depletion, similar to *gifA*, suggesting a function associated with the cellular nitrogen status.

Although the microarray analysis of strains with altered sRNA expression has proven valuable for functional studies of sRNA regulators in enterobacteria (39) and cyanobacteria (28), additional targets might have been missed. This approach is limited when a gene is not expressed under the investigated growth conditions, i.e., some targets might be only expressed in N-depleted cells. Furthermore, the regulation through sRNAs at the posttranscriptional level does not necessarily lead to a significant change in the accumulation of the target mRNAs. Therefore, some of the other predicted targets might also represent interaction partners for NsiR4.

NsiR4 is present only in cyanobacteria, suggesting that its target set might also be restricted to these organisms. Indeed, GS inactivation via inhibitory proteins is specific for cyanobacteria, and thus, *gifA*, the main NsiR4 target, is only present in this phylum. Moreover, the presence of *ssr1528* homologs is also restricted to cyanobacteria. However, a direct correlation between the occurrence of NsiR4 and *gifA* or *ssr1528* was not observed (SI Appendix, Fig. S2). Moreover, in many strains, *nsiR4* is associated with a gene encoding a conserved but unknown protein (DUF362). The DUF362 domain has been associated with iron-sulfur clusters. However, the functional association of this domain with NsiR4 remains elusive.

NsiR4 Constitutes a Previously Unidentified Regulatory Element Within the NtcA-Controlled Network. The assimilation of NH_4^+ through GS is the port of entry for inorganic nitrogen as NH_4^+ into cellular metabolism. Many of the components involved in nitrogen assimilation including the GS encoding gene *glnA* are controlled through NtcA (18, 24). In the present study, we show strong evidence that NsiR4 expression is activated through NtcA, and accordingly, the expression of NsiR4 increases when nitrogen

sources are limiting. The dependence on NtcA underlines the importance of NsiR4, consistent with the impact NsiR4 has on NH_4^+ assimilation and the amino acid pool. The joint regulation of *gifA* through NtcA and NsiR4 establishes a coherent negative feed-forward loop (Fig. 8). This type of regulatory motif results in a delayed response to the transcription factor offset (49). Mixed feed-forward loops involving transcription factors and sRNAs are frequent regulatory elements in bacterial gene regulation and have also been described or discussed for other sRNAs, e.g., Spot42 or FnrS (38, 50). A delay in GS inactivation might be of importance in habitats showing strong fluctuations in nitrogen availability.

Through the regulation of IF7, a GS inactivating factor, NsiR4 influences NH_4^+ assimilation. The mechanism described in the present study represents a regulatory node upstream of amino acid synthesis. With these features, NsiR4 is, to our knowledge, the first analyzed sRNA for the regulation of macronutrient assimilation in bacteria.

Materials and Methods

Strains and Growth Conditions. *Synechocystis* 6803 strain GT-Kazusa was grown in Cu^{2+} -free BG11 (34) buffered with 20 mM N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), pH 8.0, at 30 °C at 50–80 $\mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of white light. To induce nitrogen deficiency, cells were spin down by centrifugation and resuspended in NO_3^- -free BG11. *Anabaena* 7120 WT and the *ntcA* mutant strain CSE2 (51) were grown in CO_2 -enriched air, 1% (vol/vol), in BG11 without NO_3^- and supplemented with 6 mM NH_4Cl , 12 mM TES-NaOH (pH 7.5), and 10 mM NaHCO_3 . The generation of NsiR4 mutants and further growth conditions are described in SI Appendix in detail.

Genome and Promoter Analysis and Computational Prediction of NsiR4 Targets.

The 70-nt sequence of *Synechocystis* NsiR4 (positions 1289326–1289257, complementary strand) was used to search the JGI database. To verify *nsiR4* promoter activity in vivo in *Synechocystis*, the nucleotides –130 to +49 (TSS at +1) were fused to *luxAB* reporter genes. The selection of the reporter strains and bioluminescence measurements were performed as previously described (52). Target prediction was performed using CopraRNA (38, 42) with the NsiR4 homologs of *Synechocystis* 6803, *Microcystis aeruginosa* NIES-843, *Cyanothece* sp. ATCC 51142, *Cyanothece* sp. PCC 7424, *Cyanothece* sp. PCC 7822, *Synechococcus* sp. PCC 7002, *Pleurocapsa* sp. PCC 7327, and *Stanieria cyanosphaera* PCC 7437 and default settings.

RNA Extraction, Microarrays, and Northern Blots. The collection of *Synechocystis* cells, the RNA extraction, DNase treatment, labeling, and hybridization to microarrays was performed as previously described (53, 54). The microarray data are accessible at GEO database under accession number GSE73840. For Northern hybridization, *Synechocystis* 6803 RNA was separated on denaturing agarose gels, transferred to Hybond-N⁺ membranes (Amersham), and hybridized as previously described (55).

Protein Extraction and Immunoblots. For analysis of inactivating factor (IF) accumulation, 2 μM CuSO_4 was added to induce the *petE* promoter 8 h before addition of 10 mM NH_4Cl and 20 mM TES-NaOH (pH 7.5). Extracts were prepared using glass beads as previously described (56). Proteins were fractionated by 15% (mass/vol) SDS/PAGE and immunoblotted with anti-IF7 (1:2,000), anti-IF17 (1:2,000), or anti-TrxA (1:3,000). Anti-IF7, anti-IF17, and anti-TrxA antisera were obtained from M. I. Muro-Pastor and F. J. Florencio and used as previously described (57, 58).

Reporter Assays for the in Vivo Verification of Targets. We used the reporter system described before (59) with sGFP plasmid pXG-10-SF (41). The primers used for cloning and the resulting plasmids are given in SI Appendix, Tables S2 and S3. Further details of the generation of tested constructs can be found in SI Appendix.

Quantification of Amino Acids. Cells were grown in 100 mL BG11 containing 17.6 mM NO_3^- . After reaching an OD_{750} of ~0.8, 2 μM CuSO_4 , and after further 24 h 10 mM NH_4Cl were added. Samples of 2 mL were taken before and in a narrow time series after adding ammonia. Samples were shortly centrifuged and cell pellets frozen in liquid nitrogen. Free amino acids were extracted from frozen cyanobacterial cells with 80% ethanol at 65 °C for 3 h. After centrifugation, the extraction was repeated for the remaining cell pellet. Afterward, both supernatants were combined, dried in a vacuum

centrifuge, and redissolved in sample buffer. Individual amino acids were quantified via HPLC (60).

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