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Inactivation of three RG(S/T)GR pentapeptide-containing negative regulators of HetR results in lethal differentiation of *Anabaena* PCC 7120

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Abstract: The filamentous cyanobacterium *Anabaena* sp. PCC 7120 expresses during the differentiation of heterocysts a short peptide PatS and a protein HetN, both containing an RGSGR pentapeptide essential for activity. Both act on the master regulator HetR to guide heterocyst pattern formation by controlling the binding of HetR to DNA and its turnover. A third small protein, PatX, with an RG(S/T)GR motif is present in all HetR-containing cyanobacteria. In nitrogen-depleted medium, inactivation of *patX* does not produce a discernible change in phenotype, but its overexpression blocks heterocyst formation. Mutational analysis revealed that PatX is not required for normal intercellular signaling, but it nonetheless is required when PatS is absent to prevent rapid ectopic differentiation. Deprivation of all three negative regulators – PatS, PatX, and HetN – resulted in synchronous differentiation. However, in nitrogen-containing medium, such deprivation leads to extensive fragmentation, cell lysis, and aberrant differentiation, while either PatX or PatS as the sole HetR regulator can establish and maintain a semiregular heterocyst pattern. These results suggest that tight control over HetR by PatS and PatX is needed to sustain vegetative growth and regulated development. The mutational analysis has been interpreted in light of the opposing roles of negative regulators of HetR and the positive regulator HetL.

Keywords: cyanobacteria; heterocyst, regulation of differentiation

1. Introduction

In response to different environmental cues, a subpopulation of vegetative cells from nostocalean cyanobacteria can differentiate into different endpoints: terminally differentiated heterocysts for aerobic nitrogen fixation, suicidal necridia for filament fragmentation to produce motile hormogonia for dissemination, and dormant akinetes for survival in harsh environments [1-4]. The model strain *Anabaena*/*Nostoc* sp. PCC 7120 (hereafter *Anabaena* PCC 7120) does not produce akinetes and hormogonia in laboratory conditions, although it contains all genes known to be necessary for at least hormogonia production (Figure S5 in [5]). It has been widely used to study different aspects of heterocyst differentiation and nitrogen fixation, including the mechanisms governing what is arguably the simplest and most ancient example of a linear semi-regular biological pattern, that formed by heterocysts along filaments of vegetative cells. It is evident that despite their apparent simplicity, the regulatory networks underlying heterocyst pattern formation are highly complex and multilayered [3,6].

According to a widely accepted model for the initiation of heterocyst differentiation, the accumulation of 2-oxoglutarate (2-OG) in cyanobacteria under nitrogen deprivation [7] is perceived as a nitrogen starvation signal by the global transcriptional regulator NtcA [8]. Activated by its positive effector 2-OG [9,10], NtcA can act as either an activator or repressor of numerous genes [11].

2-OG-activated NtcA activates the transcription of nrrA [12], which in turn activates the transcription of *hetR*[13]. HetR is a master regulator of differentiation specific to filamentous cyanobacteria [14]. The expression of multiple heterocyst-specific genes depends on HetR, but only a few have been shown to be regulated directly, and repression of several promoters in vegetative cells has been reported (see [15] for recent review).

NtcA and HetR constitute a mutually dependent positive regulatory circuit [16]. Transcriptional regulation of *hetR* is complex -- it is transcribed from multiple promoters that are regulated both temporally and spatially [17]. There is more than transcriptional regulation, however, as replacement of normal transcriptional control of the chromosomal *hetR* with ectopic expression from a copper-regulated *petE* promoter resulted in a wild-type-like heterocyst patterning, which depended on posttranscriptional regulation of HetR-protein levels [18].

Proteins from three distinct genes, a small peptide PatS [19], a small protein PatX [14], and HetN protein [20,21], are known to suppress heterocyst differentiation when overexpressed. The only feature they all have in common is an RGSGR motif (a few PatX alleles including that in *Anabaena* PCC 7120 contain RGTGR instead), which was shown to be essential for suppression of heterocyst formation [19,22]. Both PatS and HetN have been shown to block the positive auto-regulation of *hetR* [23,24]. *patS* and *patX* expression is induced early (6-8 h after combined nitrogen deprivation) in regularly spaced cells that will become heterocysts [14,19] from DIF1 motif (TCCGGA)-containing, NtcA- and HetR-dependent promoters [25,26]. While *patS* expression is downregulated in mature heterocysts, expression of *hetN* first localizes to committed heterocysts [27] and continues also after heterocyst maturation [27,28].

Inactivation of *patS* leads initially to a phenotype of multiple contiguous heterocysts (Mch) during *de novo* heterocyst differentiation, but eventually, the normal pattern partially reappears [19,29]. In contrast, inactivation of *hetN* results in normal *de novo* pattern but Mch formation during successive rounds of differentiation [23]; thus PatS appears to be responsible for establishing the spatial heterocyst pattern, while HetN is responsible for its maintenance. Addition to the medium of synthetic penta- or hexapeptides (denoted PatS-5 for RGSGR and PatS-6 for ERGSGR) corresponding to the C-terminal (in PatS) or internal (in HetN) motifs inhibits differentiation but cannot restore a wild-type heterocyst pattern in a *patS* mutant [19,30]. Analysis of HetN deletion variants suggested that the RGSGR motif is required for both inhibitory and patterning activity, and thus PatS and HetN regulate different stages of heterocyst patterning utilizing the same amino acid motif for intercellular signaling [22,31].

Besides negative regulators, a positively acting factor, HetL, may be involved in HetR regulation. It is a member of a large family of poorly characterized pentapeptide repeat proteins abundant in cyanobacteria [35] and is composed of 40 pentapeptides (A(D/N)LXX). On a multicopy plasmid *hetL* restored the ability of the PatS overexpressing strain to differentiate heterocysts, while ectopic overexpression in wild-type induced Mch in nitrate-containing medium [33]. However, inactivation of *hetL* did not impair heterocyst development and diazotrophic growth [33]. A recent publication [34] sheds light on the interplay between HetL, HetR, PatS, and PatX, and provides evidence that HetR interacts with HetL at the same interface as PatS and PatX, but without inhibiting its DNA binding activity, suppressing inhibition of heterocyst differentiation. HetL competes with PatS and PatX for HetR binding, and thus it acts as a competitive activator of HetR, complicating its regulation.

We showed previously that the *hetR* gene arose in filamentous cyanobacteria, likely for the regulation of patterned differentiation of specialized cells, long before they learned how to secure nitrogenase in microoxic heterocysts, and it was invariably accompanied by *patX* and no other RG(S/T)GR-containing regulator protein [14]. It is not alone, however, in *Anabaena* PCC 7120, an unusual cyanobacterium with three RG(S/T)GR-containing negative regulators of HetR . It is therefore of interest to determine the role PatX plays along with PatS and HetN in regulating heterocyst differentiation. Here, we present the results of mutational analyses of the three RG(S/T)GR-containing proteins in Anabaena PCC 7120, alone and in combinations, under conditions that promote heterocyst differentiation, and show that unrestrained HetR activity is

lethal under different growth conditions. In nitrogen-depleted medium PatX has partially overlapping functions with PatS, but all three negative regulators show different lesions in intercellular signaling. We attribute these apparently contradictory results to interference of RG[S/T]GR-containing negative regulators with a competitive activator HetL due to a rivalry for HetR binding at the same interface. We also show that in nitrogen-replete medium PatX is required in the absence of PatS and PatS is required in the absence of PatX for pattern formation, irrespective of the presence or absence of HetN.

2. Materials and Methods

2.1. Strains, Growth Conditions and Microscopy

Anabaena sp. strain PCC 7120 was grown in nitrate-replete BG-11 medium or in BG-11_0 medium free of combined nitrogen [35] at 30°C in constant fluorescent light. For ammonium-containing medium, NH_4Cl (2.5 mM) and MOPS buffer (5 mM, pH 8.0) were added to BG-11_0. Single and double recombinants and strains carrying replicative plasmids were grown in the presence of appropriate antibiotics at the following final concentrations: neomycin, 25 μg/ml for solid medium and 15 μg/ml for liquid medium; erythromycin, 5 μg/ml; spectinomycin, 5 μg/ml plus streptomycin, 2.5 μg/ml.

To induce heterocyst formation and/or follow *gfp* reporter induction in liquid medium the filaments from fresh streaks on BG-11 plates were transferred with sterile toothpicks into a 96-well microtiter plate containing liquid BG-11_0 medium. Cells were routinely examined by bright-field microscopy with a Zeiss Axiostar plus microscope equipped with a Cannon EOS 1300D(W) digital camera. To visualize the heterocyst-specific envelope polysaccharide layer, a 0.5% Alcian blue solution in 50% ethanol was used for staining cyanobacterial filaments before microscopic examination [36].

2.2. Plasmid and strain construction.

The strains and plasmids used in this study are listed in Table 1. *patX* mutant strains RIAM1238 (DR929; *patX*::Ω; Sm^r/Sp^r) and RIAM1239 (DR931; Δ*patX*::Ω; Sm^r/Sp^r) were constructed as follows. A 2.9 kb SalI-XmnI fragment containing the 3'-end of *alr2333*, *asl2332* (*patX*), *alr2331, asl2329,* and the 3'-end of *alr2328* from anp03869 was ligated between the SalI and SmaI sites of pK18, producing pRIAM780, and an Ω cassette (a Sm^r/Sp^r determinant flanked by transcriptional terminators) was excised with SmaI from pAM684 and inserted into the internal ScaI site of *patX* in pRIAM780. A construct was selected with the orientation of *aadA* (Sm^r/Sp^r) parallel to *patX*, producing pRIAM796. Plasmid pRIAM 780 containing the 3'-end of *alr2333*, *asl2332* (*patX*), *alr2331*, *asl2329* and the 3'-end of *alr2328* derived from anp03869 was digested with ScaI and DraI, deleting most of *patX* and its 3'UTR, replacing the lost sequence with an Ω cassette excised with SmaI from pAM684. A construct was selected with the orientation of *aadA* (Sm^r/Sp^r) parallel to *patX*, producing pRIAM917. Plasmid pRIAM860 is a 4.65-kb derivative of anp03226 with the sequence between AfeI and SmaI sites deleted, leaving all of *all2333*, *asl2332* (*patX*), *alr2331*, *asl2329*, and the 3'-end of *alr2328*. The plasmid was cut with KpnI and partially with BspEI and ligated with pRIAM917 and pRIAM796 digested with KpnI+BspEI, producing pRIAM923 and pRIAM925, respectively. Finally, inserts from pRIAM925 and pRIAM923 were excised with SacI+PstI and placed between the same sites of suicide vector pRL271, producing pRIAM929 and pRIAM931, respectively. All steps of the constructions of pRIAM929 and pRIAM931 are depicted in Supplemental Figure S1.

To delete *patS* from the *Anabaena* chromosome, an insert from pAM1035 was excised with BamH+SalI and moved to pK18, producing pRIAM1159. A C.CE3 Cm^r Em^r cassette was excised with EcoICRI from pRL1567 and inserted between the EcoRV and ScaI sites of RIAM1159, replacing a *patS*-bearing 0.38 kb chromosomal fragment with Cm^r Em^r genes parallel to excised *patS*, producing

TABLE 1. Strains and plasmids

Strain or plasmid	Derivation and/or relative characteristics	Source or reference
***Anabaena* strains**		
PCC 7120	Wild type	S. Callahan
7120PN	P_{petE}-*hetN*	[23]
RIAM1238	DR929; *patX*::Ω; Sm^r/Sp^r	This study
RIAM1239	DR931; Δ*patX*::Ω; Sm^r/Sp^r	This study
RIAM1241	PN DR929; P_{petE}-*hetN patX*::Ω; Sm^r/Sp^r	This study
RIAM1242	PN DR931; P_{petE}-*hetN* Δ*patX*::Ω; Sm^r/Sp^r	This study
RIAM1243	UHM114 DR931(pAM1714); Δ*patS* Δ*patX*::Ω(pAM1714); Sm^r/Sp^r Nm^r	This study
RIAM1245	PN DR929 DR1177; P_{petE}-*hetN patX*::Ω Δ*patS*::C.CE3; Sm^r/Sp^r Em^r	This study
RIAM1248	PN DR931 DR1177; P_{petE}-*hetN* Δ*patX*::Ω Δ*patS*::C.CE3; Sm^r/Sp^r Em^r	This study
RIAM1249	PN DR1177; P_{petE}-*hetN* Δ*patS*::C.CE3; Em^r	This study
RIAM1250	PN DR1177; P_{petE}-*hetN* Δ*patS*::C.CE3; Em^r	This study
UHM114	Δ*patS*	[39]
Plasmids		
anp03226	A *patX* (*asl2332*)-bearing bp 2805907 to 2813409 fragment of *Anabaena* sp. PCC 7120 chromosome in the BamHI site of pUC18; Ap^r	[43]
anp03869	A *patX* (*asl2332*)-bearing bp 2803179 to 2811405 fragment of *Anabaena* sp. PCC 7120 chromosome in the BamHI site of pUC18; Ap^r	[43]
pAM504	Shuttle vector for replication in *E. coli* and *Anabaena*; Km^r Nm^r	[46]
pAM684	A source of the Sp^r Sm^r Ω cassette; Ap^r Sp^r/Sm^r	[45]
pAM1951	pAM504 carrying P_{patS}-*gfp*; Km^r	[19]
pAM1956	Shuttle vector pAM504 with promoterless *gfp*; Km^r	[19]
pAM1035	*patS* on a 3.3 kb chromosomal fragment in pBluescript II KS(-); Ap^r	[19]
pK18	pBR322-derived cloning vector; Km^r	[44]
pRIAM780	A 2.9 kb SalI-XmnI fragment containing 3'-end of *alr233*, *asl2332* (*patX*), *alr2331* and 3'-end of *alr2330* from anp03869 ligated in SalI-SmaI sites of pK18; Km^r	This study
pRIAM796	Ω cassette inserted into internal ScaI site in *patX* ORF in pRIAM780; Km^r Sp^r/Sm^r	This study
pRIAM860	anp03226 derivative with an AfeI-SmaI fragment deleted; contains *patX* on remaining 4.65 kb insert; Ap^r	This study
pRIAM917	pRIAM780 with ScaI-DraI fragment containing most of *patS* ORF and 3' UTR replaced with Ω cassette; Km^r Sp^r/Sm^r	This study
pRIAM923	A 4.9 kb chromosomal region (bp 2805907 to 2810809) with ScaI-DraI fragment containing most of *patS* ORF and 3' UTR replaced with Ω cassette; reconstructed from pRIAM860 and pRIAM917; Km^r Sp^r/Sm^r	This study
pRIAM925	Same as pRIAM917, but the Ω cassette inserted into into internal ScaI site in *patX* ORF; reconstructed from pRIAM860 and pRIAM796; Km^r Sp^r/Sm^r	This study
pRIAM929	Insert from pRIAM925 moved into suicide vector pRL271; Cm^r Em^r Sp^r/Sm^r	This study
pRIAM931	Insert from pRIAM923 moved into suicide vector pRL271; Cm^r Em^r Sp^r/Sm^r	This study
pRIAM971	P_{patX}-*gfp* transcriptional reporter in pAM1956	[14]
pRIAM1159	Insert from pAM1035 moved in pK18 (probably as BamH-SalI fragment); Km^r	This study
pRIAM1175	C.CE3 Cm^r Em^r cassette excised with EcoICRI from pRL1567 and inserted into EcoRV-ScaI sites of RIAM1159, replacing *patS*-bearing 0.38 kb chromosomal fragment; Km^r Cm^r Em^r	This study
pRIAM1177	Insert from pRIAM1175 moved into SacI-PstI sites of pRL278; Km^r Cm^r Em^r	This study
pRL271	*sacB*-containing suicide vector; Cm^r Em^r	[40]
pRL278	*sacB*-containing suicide vector; Nm^r/Km^r	[40]
pRL1567	Source of C.CE3 Cm^r Em^r cassette; Ap^r Cm^r Em^r	[41]
pUC19	pBR322-derived cloning vector; Ap^r	[42]

pRIAM1175. The insert from this plasmid was moved as a SacI-PstI fragment between the same sites of suicide vector pRL278, producing pRIAM1177. All steps of the constructions of pRIAM1177 are depicted in Supplemental Figure S2.

The plasmids pRIAM929, pRIAM931, and pRIAM1177 were transformed into *E. coli* strain AM1359 [19] containing a broad host range plasmid pRL443 with conjugal functions and a pRL623 plasmid to provide both methylation and mobilization functions and then conjugated into *Anabaena* strains using standard protocols [37]. The next day conjugation plates were underlaid with appropriate antibiotics to select for single recombinants. Subsequent selection for double recombinants using the *sacB* gene present on the vectors was performed as described by [38]. After multiple rounds of successive cloning the genotypes of all constructed mutant strains were confirmed by PCR analysis (Supplemental Figures S3).

3. Results

3.1. Mutational analysis suggests that in nitrogen-depleted medium patX is impaired in cell-cell signaling and acts cell-autonomously.

PatX shares the ability of PatS and HetN to suppress heterocyst differentiation when over-expressed [14]. The phenotypes of *patS* and *hetN* single and double mutants were previously described in detail. The *patS* deletion single mutant strain UHM114 [47] and *patS* replacement mutant [19,29] produce both single heterocysts and Mch in BG-11$_0$ medium. A conditional P$_{petE}$-*hetN* mutant transferred into copper-free BG-11$_0$ initially displays the wild-type pattern, but later Mch starts to appear [23], while a Δ*hetN* strain CSL7 forms Mch without a lag [31]. Unlike the *patS* and *hetN* mutants, inactivation of *patX* did not cause any visibly aberrant phenotype in nitrogen-depleted medium. The mutant strains RIAM1238 (*patX*::Ω) and RIAM1239 (Δ*patX*::Ω) mutant strains behaved similarly to each other. Both the time course of differentiation (not shown) and heterocyst pattern were essentially the same as in the wild-type strain (Figure 1a, b). Since the seemingly normal phenotype of *patX* mutants growing in nitrogen-depleted medium could be caused by a functional redundancy or impaired function of PatX, we attempted to construct and examine phenotypes of strains with different combinations of *patX*, *patS*, and *hetN* mutations.

It was previously reported that inactivation of *hetN* results in an unstable Mch phenotype tending to change to a Het$^-$ phenotype upon extended subculturing [20]. To avoid the instability, we exploited an approach used previously by [23], who constructed strain 7120PN in which the coding region of *hetN* was fused to the *petE* promoter controlled by the level of copper. At the concentration of copper in BG-11 medium (0.3 μM) the ectopic expression of *hetN* from this promoter is sufficient to completely suppress heterocyst differentiation in *Anabaena* PCC 7120, while in copper-free medium residual *hetN* expression is low enough to virtually eliminate the HetN-mediated suppression of HetR activity [23,39]. The phenotype of a Δ*patS* derivative of 7120PN was described earlier [39].

In order to assess whether the functional role of PatX is masked by PatS and HetN, we made use of the original conditional mutant strain 7120PN (P$_{petE}$-*hetN*) [23] with *patX* knocked out: RIAM1241 (P$_{petE}$-*hetN* Δ*patX*::Ω) and RIAM1242 (P$_{petE}$-*hetN* Δ*patX*::Ω). In addition, we looked at the phenotypes of two strains similar to the previously reported 7120PN Δ*patS* mutant UHM100 [39]: RIAM1249 and RIAM1250 (independent isolates of P$_{petE}$-*hetN* Δ*patS*::C.CE3). Both Δ*patS* mutants showed identical phenotypes, similar to that of UHM100 but different from 7120PN Δ*patX*.

In N$^-$ Cu$^-$ medium RIAM1241 and RIAM1242 behaved exactly like the parental strain, the *patX*$^+$ *hetN* conditional mutant 7120PN: one day after induction single semiregular heterocyst formed (Supplemental Figure S4a and b), but during consecutive rounds of differentiation Mch started to appear due to the formation of new heterocysts adjacent to existing single or multiple heterocysts (Figure 1c). Subsequent to this excessive differentiation, growth continued at a slower rate.

Transfer of RIAM1250 filaments in liquid N$^-$ Cu$^-$ medium resulted in a deteriorated heterocyst pattern with production of Mch pattern at 24 h (Supplemental Figure S4c) and progressive ectopic

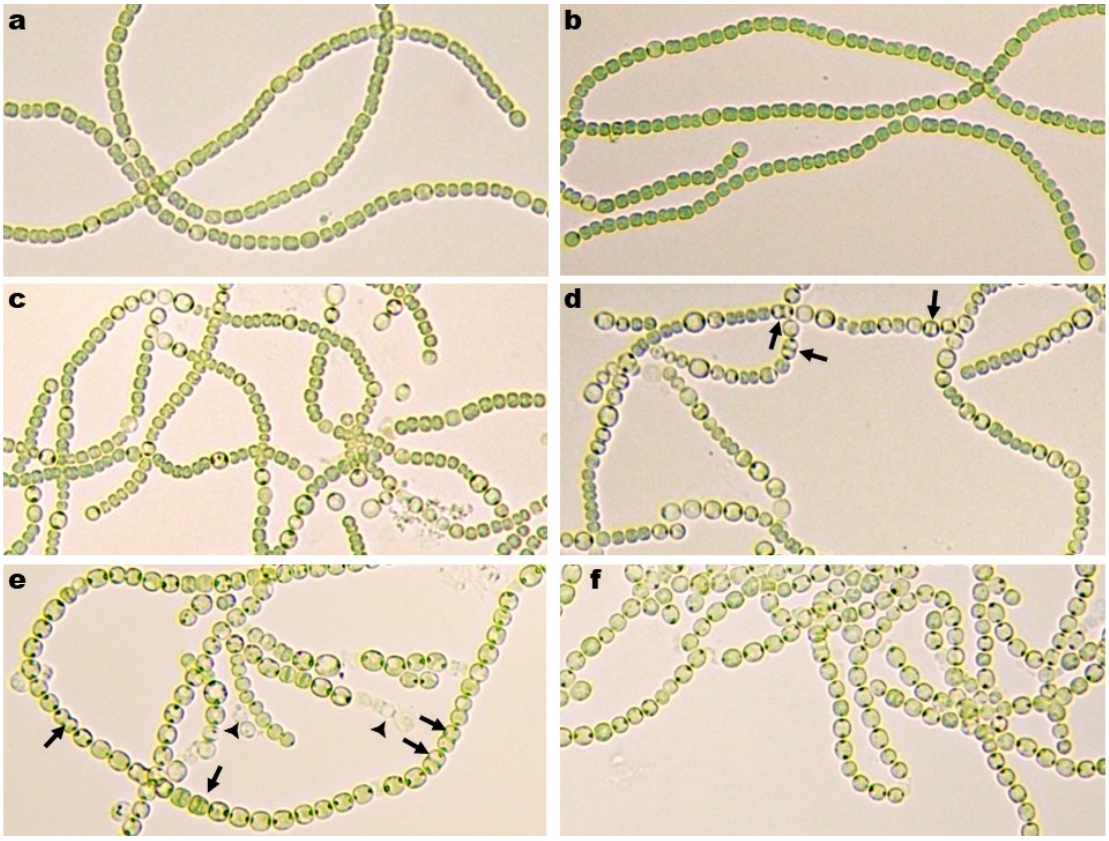

Figure 1. Phenotypes of the wild-type and mutant strains after transfer from a BG-11 plate to liquid BG-11$_0$ copper-free medium.Wild-type *Anabaena* 7120 (a) and Δ*patX*::Ω single mutant RIAM1239 (b), 1 day of incubation; P$_{petE}$-*hetN* Δ*patX* conditional double mutant RIAM1242, 4 days of incubation (c); P$_{petE}$-*hetN* Δ*patS* conditional double mutant RIAM1250, 2 days of incubation (d); Δ*patS* Δ*patX*::Ω(pAM1714) conditional double mutant RIAM1243 after 3 days of incubation (e) and *hetN patX patS* conditional triple mutant RIAM1248 after 2 days of incubation (f). Possibly dividing heterocysts in d and e are indicated by arrows, necridia in e and f by arrowheads.

differentiation upon further incubation (Figure 1d), so that nearly complete differentiation occurred after 4-5 days. There was no growth and cell sediment bleached. This behavior was essentially the same as described earlier for for a strain UHM100, also lacking *patS* and conditional in *hetN* expression [39]. The significance of the possibly dividing heterocysts (Figure 1d,e) is discussed later. In the case of our double and triple mutants, the relative abundance of aberrant heterocysts varied in particular mutants and was most prominent in RIAM1243 (ca. 20%), perhaps due to the presence of a functional copy of *hetN*, which could influence the activity of HetR at late stages and somehow compromise the block of division.

Our initial attempts to construct a Δ*patS* Δ*patX*::Ω double mutant failed: while single recombinants were easily obtained, positive selection for double recombinants [38] on sucrose plates produced only very rare colonies. All retained the Em^R marker of the pRL271 suicide vector, indicating the absence of gene replacement by double recombination. However, after conjugation of a P$_{petE}$-*patS*-containing plasmid pAM1714 marked by Nm resistance [19] into the Δ*patS* SR*patX*::Ω/pRL271 single recombinants, we got many Em^S Nm^R colonies on sucrose plates, indicating double recombination. A completely segregated conditional double mutant, RIAM1243, bleached upon transfer to copper-free medium and apparently stopped growing, even in nitrate-containing medium. Massive synchronous heterocyst differentiation was visible after 1-2 days in nitrogen-depleted medium (Figure 1e; Supplemental Figure S4e), single necridia were rare in some filaments but formed multiple contiguous necridia in others, and filaments of differentiated

cells contained abundant aberrant (pro)heterocysts with mid-cell envelope constrictions and/or division planes.

A triple mutant RIAM1248, defective in *patS*, in *patX*, and conditionally in *hetN* was constructed, with viability maintained by *hetN* expression from a copper-regulated promoter. As with the *patS patX* double mutant (see above), transfer of the triple mutant into medium depleted of both nitrogen and copper resulted in synchronous ectopic differentiation at 24 h (Supplemental Figure S4e). After 48 h, filaments showed little fragmentation and consisted almost exclusively of proheterocysts and heterocysts (Figure 1f). Aberrant (possibly dividing) heterocysts were comparatively rare in the triple mutant.

3.2. In nitrogen-replete medium, either patS or patX alone can promote semiregular pattern formation

On BG-11 plates RIAM1239 (defective in *patX*) did not form heterocysts; however, unlike the wild type, the mutant strain upon transfer from solid to liquid nitrate- or ammonium-containing BG-11 medium produced semiregular heterocysts in many filaments, although at a lower frequency than when transferred from solid BG-11 to liquid N^- medium (Figure 2a, b). Differentiation of heterocysts in nitrogen-replete medium resembles formation of constitutive heterocysts by *patS* and *hetN patS* mutants [19,29,39,47].

Upon transfer to N^+ Cu^- medium, the *hetN* Δ*patX* conditional mutant RIAM1242 formed semiregular predominantly single heterocysts (Figure 2c), and the *hetN* Δ*patS* conditional mutant RIAM1250 in N^+ Cu^- medium behaved similarly to RIAM1242 – it also formed semiregular single

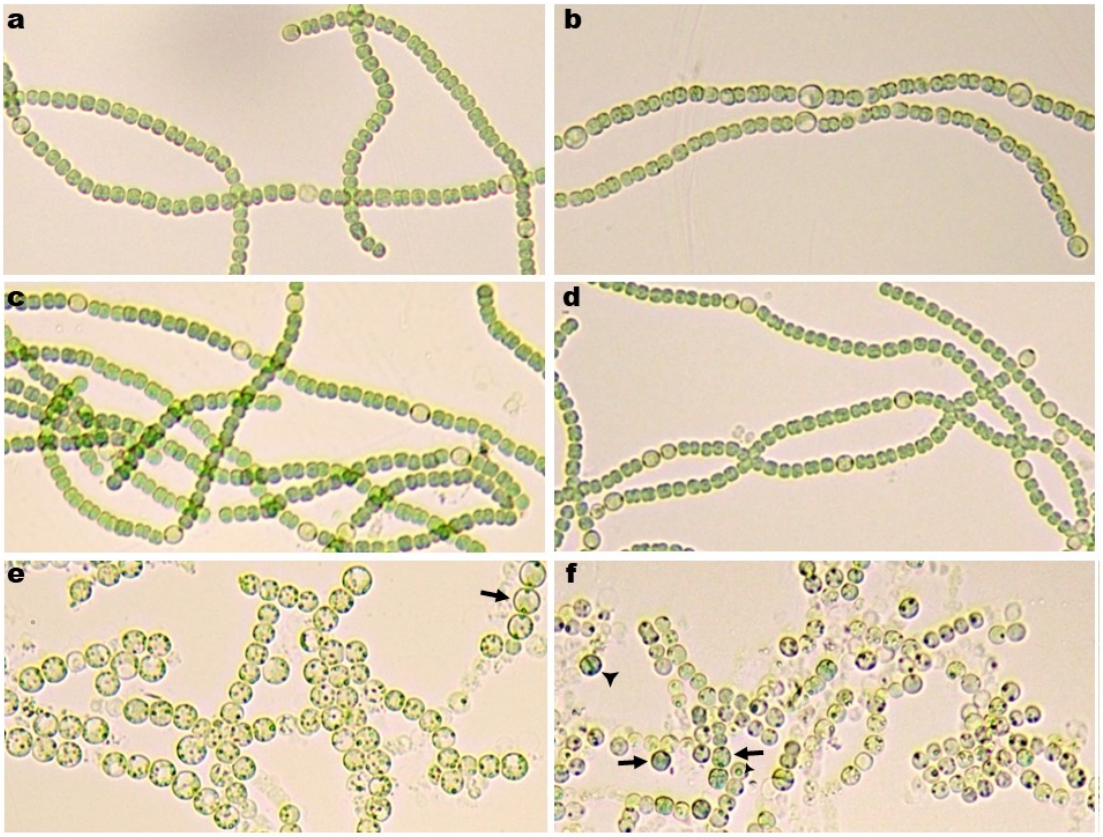

Figure 2. Phenotypes of mutants after transfer from a BG-11 plate into liquid copper-free nitrate-containing (a, c-f) or ammonium-containing (b) BG-11 medium. Δ*patX*::Ω single mutant RIAM1239, 1 day of incubation (a, b); P_{petE}-*hetN* Δ*patX* conditional double mutant RIAM1242 (c) and P_{petE}-*hetN* Δ*patS* conditional double mutant RIAM1250 (d) after 2 days of incubation; Δ*patS* Δ*patX*::Ω(pAM1714) conditional double mutant RIAM1243 after 3 days of incubation (e) and *hetN patX patS* conditional triple mutant RIAM1248 after 4 days of incubation (f). Cells resembling heterocysts in e and f are indicated by arrows and aberrant round cells with division planes in f by arrowheads. Multiple necridia (cell ghosts) are visible in e and f.

and some double heterocysts, but no Mch (Figure 2d). However, 5-6 days after the transfer, RIAM1250 started to fragment, first at vegetative cell-heterocyst junctions. Further incubation resulted in progressive fragmentation to very short filaments and single vegetative cells (not shown). Evidently, both PatS and PatX are necessary to restrain HetR sufficiently to prevent differentiation in nitrogen-replete liquid medium, while only one of them is necessary to produce a semiregular pattern of heterocysts.

A double mutant defective in both *patS* and *patX* exhibited a more extreme phenotype under nitrogen-replete conditions. The Δ*patS* Δ*patX*::Ω(pAM1714) conditional double mutant RIAM1243 grew as filaments of green vegetative cells on BG-11 but upon transfer into N^+ Cu^- liquid medium fragmented into chains of bleached enlarged round cells with small cyanophycin-like granules in the cytoplasm as well as single and multiple contiguous necridia (Figure 2e). Most of the enlarged round cells morphologically did not resemble heterocysts, but a few of them had a thicker envelope and polar nodules similar to those located in the neck regions of heterocysts (Figure 2e, indicated by arrows). Apparently in nitrogen-replete medium the differentiation process is induced by transfer into liquid BG-11 but is disorganized and in most cases ceases soon after the initial and early stages. These stages include disintegration of thylakoids and loss of pigmentation and deposition of the additional outer polysaccharide layer as indicated by Alcian blue staining (Supplemental Figure S4e).

The RIAM1248 triple mutant behaved differently from the RIAM1243 double mutant. After transfer to copper-free nitrogen-replete medium, the induced differentiation process was also disorganized but proceeded more slowly, and during the first two days many cells retained their shape and size but gradually lost pigmentation, and necridia-like cells appeared. After 4 days, the filaments bleached and fragmented due to massive necridia formation, and some enlarged pale-green round cells with thickened envelopes and division planes were visible but without any sign of envelope constriction. Also seen were cells with colorless transparent cytoplasm with large granules (Figure 2f). Both RIAM1243 and RIAM1248 produced multiple necridia looking like cell ghosts, which increased in number during prolonged incubation (Figures 2e and f).

3.4. Instability of conditional mutants overexpressing patS or hetN.

In the course of our experiments with conditional mutants we observed very high mutation rates of strains overexpressing either *patS* or *hetN*. These strains were constructed to conditionally block differentiation and thus avoid lethality or eliminate a detrimental burden of extra heterocyst production during cultivation. Although this approach permitted us to obtain and document the phenotypes of strains with multiple mutations, working with such mutants was tricky and required constant microscopic control and frequent cloning to prevent otherwise rapid changes in mutant phenotypes.

Several passages on BG-11 plates with or without appropriate antibiotics of fully segregated conditional mutants resulted in a rapid accumulation of secondary mutations. For example, Figure 3 shows a double mutant strain RIAM1243 before and after several subcultures on selective BG-11+Sp, Sm, Nm plates and subsequent transfer and incubation in N^- Cu^- medium for 2 weeks. While a freshly isolated RIAM1243 clone after prolonged incubation exhibited only rare short stretches of vegetative cells originating from rare single cells that remained undifferentiated (Figure 3a), long filaments of vegetative cells (Het^- phenotype), sometimes with terminal single or multiple heterocysts (PatA-like or PatA Mch phenotypes), accumulated in suspensions of fragmented chains of heterocysts (Figure 3b). Similar instability was exhibited by the conditional P_{petE}-*hetN* Δ*patS* mutant RIAM1250 (Figure S4f) and the triple mutant RIAM1248 (not shown).

There was no growth in both cases, and both cultures contained a light yellowish-grey sediment. We used in experiments only freshly isolated clones with all filaments showing similar phenotypic characteristics.

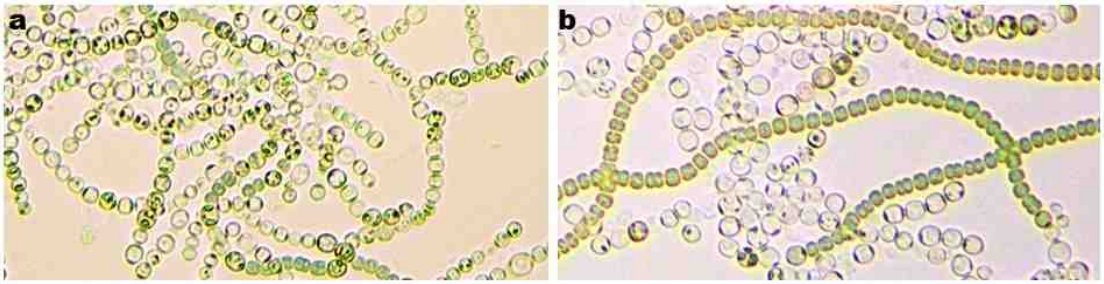

Figure 3. Phenotypes of Δ*patS* Δ*patX*::Ω(pAM1714) conditional double mutant RIAM1243 after transfer from a BG-11 plate into liquid copper-free nitrate-free BG-11$_0$ medium and incubation for 14 days. A freshly isolated RIAM1243 clone (a) and the same mutant after several subcultures on BG-11+Sp, Sm, Nm plate (b).

4. Discussion

PatX is an ancient regulator of HetR and the only one that always accompanies HetR, but its function remains obscure. Prior to this work, all that was known was that ectopic overexpression of PatX blocked heterocyst differentiation in *Anabaena* 7120 [14], and so did PatX orthologs from *Mastigocladus laminosis* [48] (called 'alternative PatS') and from *Arthrospira platensis* NIES 39 [49] (called 'PatS'), a filamentous strain that does not form heterocysts. To clarify the role of PatX in heterocyst regulation we disrupted the patX gene, but the resulting mutant strain grown in the absence of a nitrogen source showed no heterocyst-related phenotype. This could be a consequence of functional redundancy or a functionality unrelated to heterocyst formation. Loss of PatX also produced no obvious phenotypic change in combination with a conditional *hetN* mutation. Under conditions in which *hetN* was not expressed, two mutant strains, one carrying conditional *hetN* and the other identical but with *patX* knocked out, displayed a similar delayed Mch phenotype (Figure 2c and Supplemental Figure S4a). Both strains were phenotypically different from the conditional *hetN patS* double mutant UHM100 described previously [39,47] and from an analogous double mutant RIAM1250 constructed in this work, which showed gradual asynchronous differentiation so that in a few days almost all cells became heterocysts (Figure 1d). These results demonstrate that the functionality of *patX* differs from that of *patS*. PatS is required for *de novo* pattern formation but is not sufficient to prevent later formation of contiguous heterocysts (Mch). PatX cannot replace PatS in directing *de* novo pattern under nitrogen-depleted conditions, nor can it replace HetN in maintaining pattern.

A residual functionality of *patX* becomes obvious on comparison of a Δ*patS* mutant with a Δ*patS* Δ*patX* double mutant RIAM1243, both retaining a wild-type *hetN* (Figure 1, reference 19, and results not shown). The double mutant is inviable unless *patS* is conditionally suppressed, by placing the gene under the control of the copper-regulated *petE* promoter. Upon removal of copper from the medium, this double mutant behaved essentially as the conditional *hetN patX patS* triple mutant RIAM1248 – both started nearly synchronous differentiation of all vegetative cells into heterocysts when incubated in N^- Cu^- medium (Figures 1e and 1f). The intact chromosomal copy of *hetN* in RIAM1243 could not prevent or slow down differentiation process. Thus PatX (along with PatS) is required to prevent a rapid synchronous differentiation of all vegetative cells.

These results indicate that upon combined nitrogen deprivation all three negative regulators are instrumental in promoting regular pattern formation and/or maintenance, but in different ways. The roles of PatS and HetN in pattern initiation and maintenance, respectively, are readily discernible, the role of PatX is masked by the presence of PatS. Our results are in line with those of [18], who observed the formation of concentration gradients of HetR in proximity to heterocysts, dependent on either *patS* or *hetN*. When both genes were deleted, no HetR gradients formed, indicating that either *patS* or *hetN* are required for establishing HetR concentration gradients after combined nitrogen removal. Their results demonstrate that *patX* (unknown at the time) is unable to induce HetR gradient formation in the absence of *patS* and *hetN* and thus does not participate in cell-cell signaling in the same way as *patS* and *hetN*.

A role for PatX in regulating heterocyst differentiation is more easily seen when Anabaena 7120 is grown in nitrogen-replete medium> Unlike the wild-type strain, the *patX* mutant RIAM1239 produced morphologically distinct semiregular heterocysts when transferred to liquid nitrate or ammonia-containing media (Figures 2a and b), indicating that its product was needed to prevent unnecessary differentiation. Conditional double mutants RIAM1242 (*hetN patX*) and RIAM1250 (*hetN patS*) also formed semiregular heterocysts in copper-free medium (Figures 2c and d), indicating that in nitrogen-replete conditions either PatS or PatX were sufficient for rapid pattern formation. At the same time both conditional double mutant RIAM1243 (*ΔpatS ΔpatX*::Ω(pAM1714)) and conditional triple mutant RIAM1248 (*hetN patX patS*) after removal of copper started ectopic aberrant differentiation. With both mutants, all cells differentiated into morphologically distinct heterocysts in N- Cu- medium, but in N+ Cu- medium, differentiation into heterocyst-like cells was rare, and aberrant differentiation led to the appearance of enlarged spherical cells that partially retained pigmentation and of necridia, resulting in cell lysis (Figure 2e and f). Apparently, unrestrained HetR in nitrogen-replete medium promotes differentiation that is completely dysregulated. The low level in N+ medium of NtcA and/or its activator 2-oxoglutarate [50] could exacerbate the situation.

We interpret the differences between heterocyst pattern characteristics of the *patX* mutant (RIAM1239), *hetN patX*, mutant (RIAM1242), and *hetN patS* mutant (RIAM1250) in N+ Cu- and N- Cu- medium in the following way. HetR expression and activity in nitrate and ammonia-containing media are suppressed because of the low concentration or activity of NtcA, combined with a basal level of PatX and PatS negative regulators sufficient to outcompete what little HetL positive regulator is present for binding to HetR [34]. Inactivation of *patX* or *patS* results in a partial activation of HetR, under the control of the remaining negative regulator. , and HetN may also have some effect since in vegetative cells grown with combined nitrogen a low level of HetN has been detected [24]. HetL seems to play no role in nitrogen-replete conditions, and either PatS or PatX suffice to establish and maintain semiregular pattern. Inactivation of both patX and patS is lethal under all conditions. These results apparently exclude any role of external and internal sources of combined nitrogen and of differences in the C/N balance of cells in heterocyst pattering mechanism. This would imply either the existence of a predetermined cryptic pattern or a very efficient patterning mechanism based on interactions between HetR and a single RG[S/T]GR-containing negative regulator, PatS or PatX. Upon combined nitrogen step down the situation becomes more complicated because HetL steps in and competes with negative regulators for HetR binding [34]. The competition between negative and positive regulators had been precisely tuned in terms of timing and effectiveness of expression, stability, diffusibility and relative affinities for HetR to insure formation of a semiregular pattern. In this situation inactivation of any negative regulator (except PatX) or combination thereof lead to more or less severe deterioration of pattern right up to disastrous complete differentiation.

There is a strong selective pressure for ongoing rapid accumulation of suppressor mutations allowing escape from lethal terminal differentiation in *patX patS* and *hetN patS* double mutants. Similar instability has been seen with strains overexpressing HetR [51] and strains with mutated *hetN* [21]. In our experiments, we tried to avoid this problem by using conditional mutants and maintaining them in a medium that blocked heterocyst development completely and thus presumably eliminated a negative selection pressure on mutants overproducing heterocysts [39,52]. Nevertheless, a high mutation rate was still observed resulting in rapid accumulation of Het$^-$ and PatA-like mutants producing only terminal heterocysts. HetR may well participate in protein interactions and/or influence expression of genes unrelated to heterocyst formation but important for vegetative growth, and its overexpression or inhibition of expression and activity could impose selective pressure for the appearance of suppressor mutations.

The observation that some heterocysts in multiply mutant strains appear to be undergoing cell division (Figures 1e, 2f) has been previously reported by others for different wild-type strains [53-55] and supports the proposed connection between cell division and regulation of heterocyst differentiation [1]. The decision to initiate differentiation is taken by a cell early in the cycle during

the first 8 hours from the division [56], and this prior division is apparently critical for proceeding through all further steps [57]. An arrest of division during differentiation is probably imposed at the commitment stage [58]. These authors observed the presence of heterocyst-like cells with active division plane at their midcell upon *hetR* ectopic overexpression in the Δ*hetZ*$_{354–762}$ mutant. Constriction of the heterocyst envelope at midcell gives an impression of cells undergoing active division. However, the mechanical constraints of the multilayered heterocyst envelope [59] make this possibility unlikely. A more attractive explanation is that a prerequisite for prior cell division can be overcome by the absence of negative regulator (PatS and PatX), and cells at different stages in the cell cycle are forced to leave off the division process in the middle and start differentiation.

In the presence of combined nitrogen, either PatX or PatS as the sole HetR regulator can establish and maintain a wild-type semiregular heterocyst pattern (Figures 2c and 2d), and in terms of current models of heterocyst pattern formation they are fully proficient in cell-cell signaling, a prerequisite for pattern formation. This basic mechanism rests on interactions between two players, HetR and PatX/PatS derivatives, perhaps as modeled by a Turing-like reaction-diffusion mechanism [3]., it works irrespective of nitrogen status and is hidden by additional layers of regulation, but can be revealed by mutational analyses. These additional layers may respond to the availability of combined nitrogen or other environmental cues and a set of downstream regulators (which can vary in different strains and complicate regulation) transmitting the pattering signals and converting them into morphogenetic and metabolic changes. We conjecture that this basic mechanism was initially designed by filamentous cyanobacteria for a purpose of dissemination by nonrandom fragmentation of trichomes through necridia formation, and later modified and adopted for patterned heterocyst differentiation.

Supplementary Materials: Figure S1: Construction of pRIAM929 and pRIAM931 (Δ*patX*), Figure S2: Construction of pRIAM1177 (Δ*patS*), Figure S3: Segregation of mutations, Figures S4: Differentiation of mutant strains

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