An ABC transporter required for intercellular transfer of developmental signals in a heterocystous cyanobacterium

Patrick Videau[a,1], Orion S. Rivers[a], Kelly C. Higa[a,2] and Sean M. Callahan[a*]

[a]Department of Microbiology, University of Hawai'i at Mānoa, USA

Present address: [1]Oregon State University, College of Pharmacy, 103 Memorial Place, 418 Weniger Hall, Corvallis, OR 97331; [2]University of Colorado Anschutz Medical Campus, Department of Immunology & Microbiology, 12800 E. 19th Ave., RC1-North Tower, Aurora, CO 80045

[#]**For correspondence:** E-mail scallaha@hawaii.edu; Tel. (+1) 808 956 8015; Fax (+1) 808 956 5339

Supporting Information

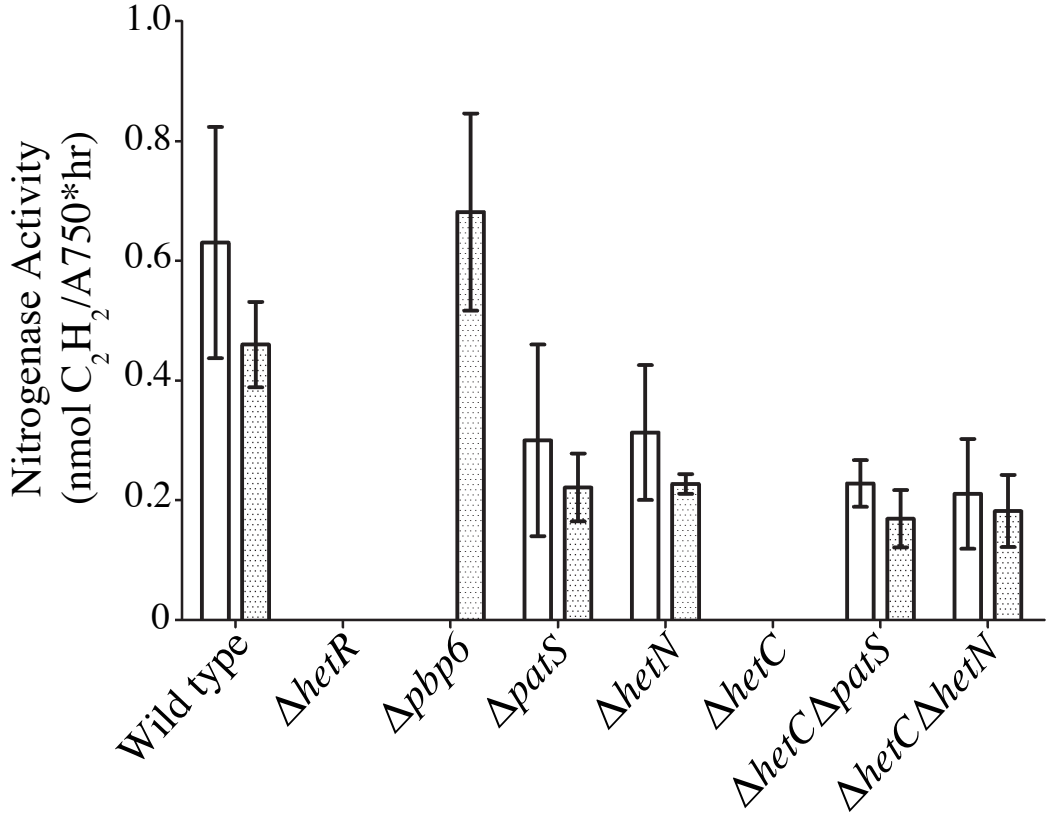

Figure S1. Acetylene reduction assay with *hetC*-mutant strains conducted in aerobic (clear bars) or anaerobic (dotted bars) conditions either 24 h (wild type, Δ*hetR*, Δ*patS*, and Δ*hetC* Δ*patS* strains) or 48 h (Δ*hetC*, Δ*hetN*, and Δ*hetC* Δ*hetN* strains) after the removal of combined nitrogen. Error bars represent one standard deviation.

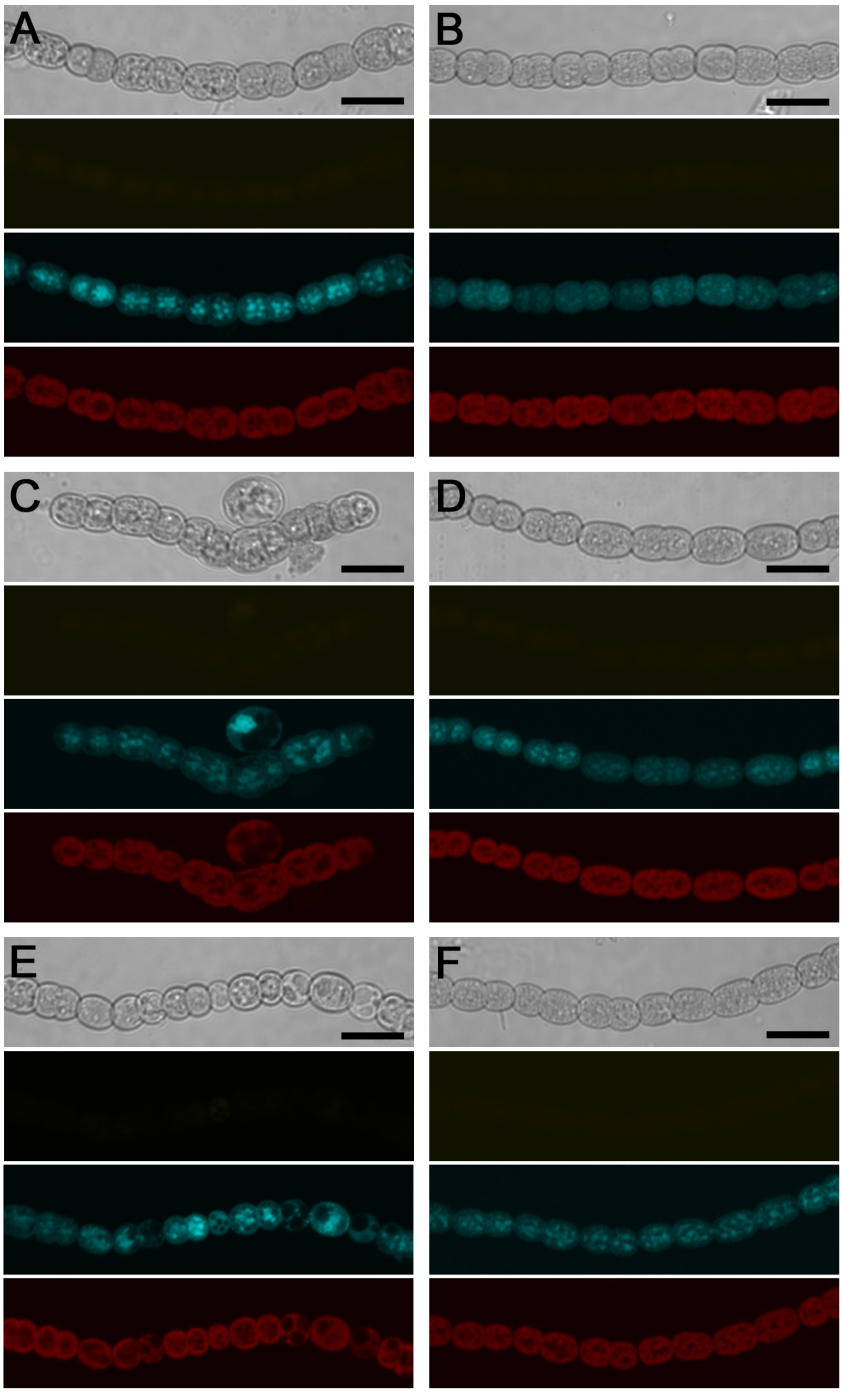

Figure S2. Source cells are not present at 0 h after the induction of differentiation. Plasmid pPJAV247 contains a PpatS-YFP transcriptional fusion divergently transcribed from P_{petE}-*hetR*(*H69Y*)-CFP and was introduced into the wild type (A), Δ*hetC* (B), Δ*patS* (C), Δ*hetC* Δ*patS* (D), Δ*hetN* (E), and Δ*hetC* Δ*hetN* (F) strains. Cultures were maintained in the presence of ammonia and were imaged 0 h after its removal. From top to bottom: bright field, yellow fluorescence from P_{patS}-YFP, blue fluorescence from P_{petE}-*hetR*(*H69Y*)-CFP, and red autofluorescence. Bar, 10 μm.

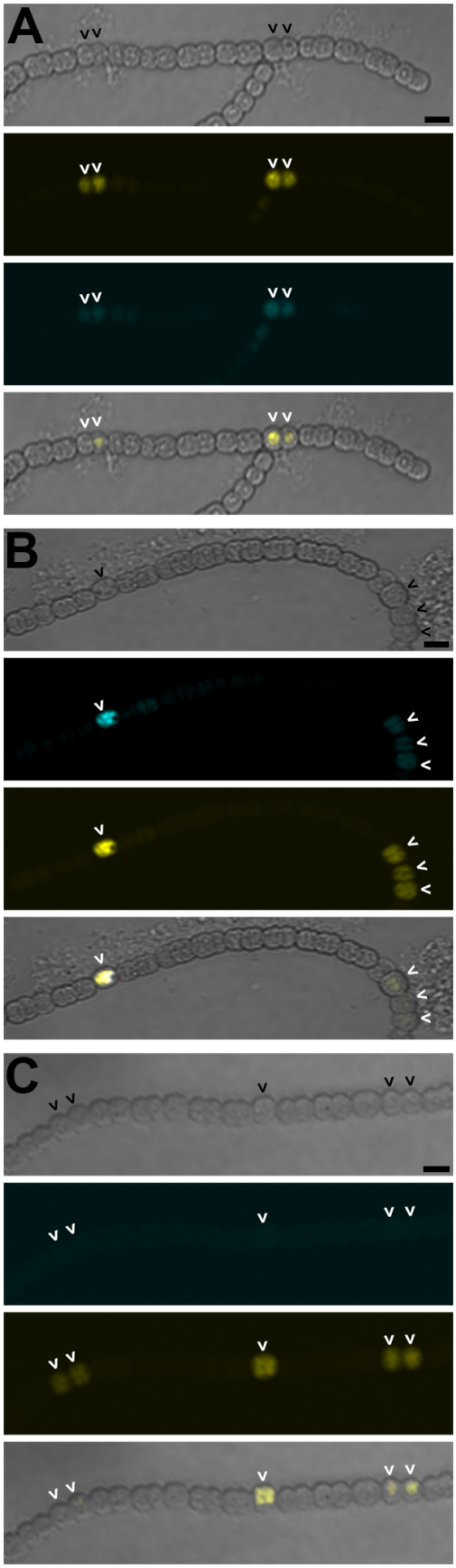
A
B
C

Figure S3. Short cell intervals between heterocysts do not display measureable signal ranges. The wild type (A), Δ*hetN* (B), Δ*hetN*Δ*hetC* (C) strains harboring pPJAV247, which encodes YFP expressed from the *patS* promoter to mark proheterocysts and HetR(H69Y)-CFP expressed from the copper-inducible *petE* promoter, were imaged 9 h after the removal of combined nitrogen. From top to bottom: bright field, yellow fluorescence from P_{patS}-YFP, blue fluorescence from HetR(H69Y)-CFP, and the composite image. Carets indicate proheterocysts. Bar, 10 μm.

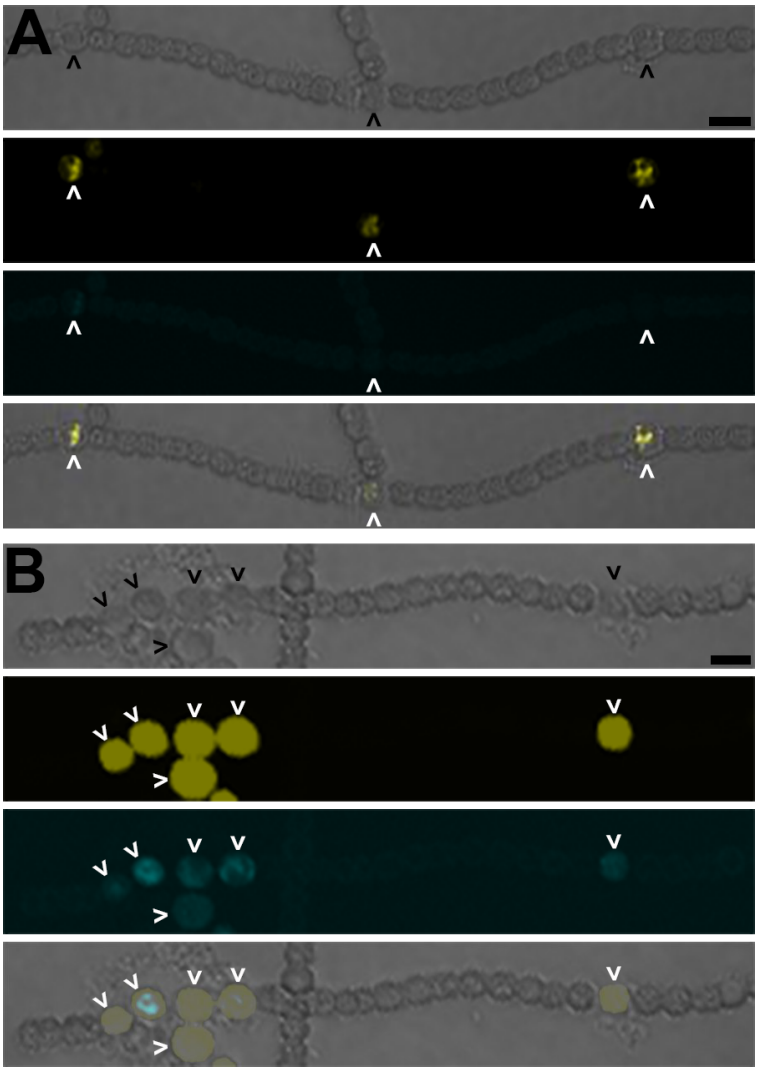

Figure S4. Short cell intervals between heterocysts do not display measurable signal ranges. The wild type (A) and Δ*hetN* (B) strains harboring pPJAV247, which encodes YFP expressed from the *patS* promoter to mark proheterocysts and HetR(H69Y)-CFP expressed from the copper-inducible *petE* promoter, were imaged 24 (A) or 48 h (B) after the removal of combined nitrogen. From top to bottom: bright field, yellow fluorescence from P_{patS}-YFP, blue fluorescence from HetR(H69Y)-CFP, and the composite image. Carets indicate heterocysts. Bar, 10 μm.

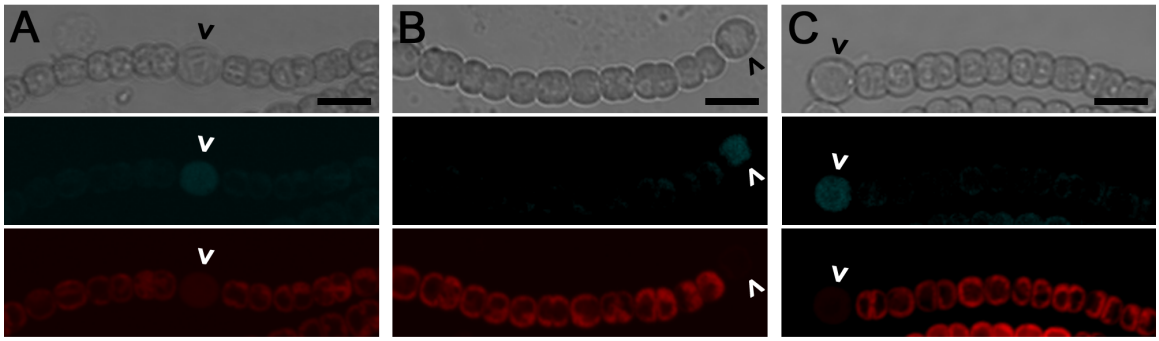

Figure S5. Transcription of *hetN* is unaltered in heterocysts produced by strains containing a *hetC* mutation. The plasmid pPJAV341, which contains a transcriptional fusion of the *hetN* promoter to the FMN-dependent fluorophore EcFbFP, was introduced into the wild type (A), Δ*hetC* (B), and Δ*patS* Δ*hetC* (C) strains. Cultures were imaged 48 h after the removal of combined nitrogen. From top to bottom: bright field, blue fluorescence from P_{hetN}-EcFbFP, and red autofluorescence. Carets indicate heterocysts. Bar, 10 μm.

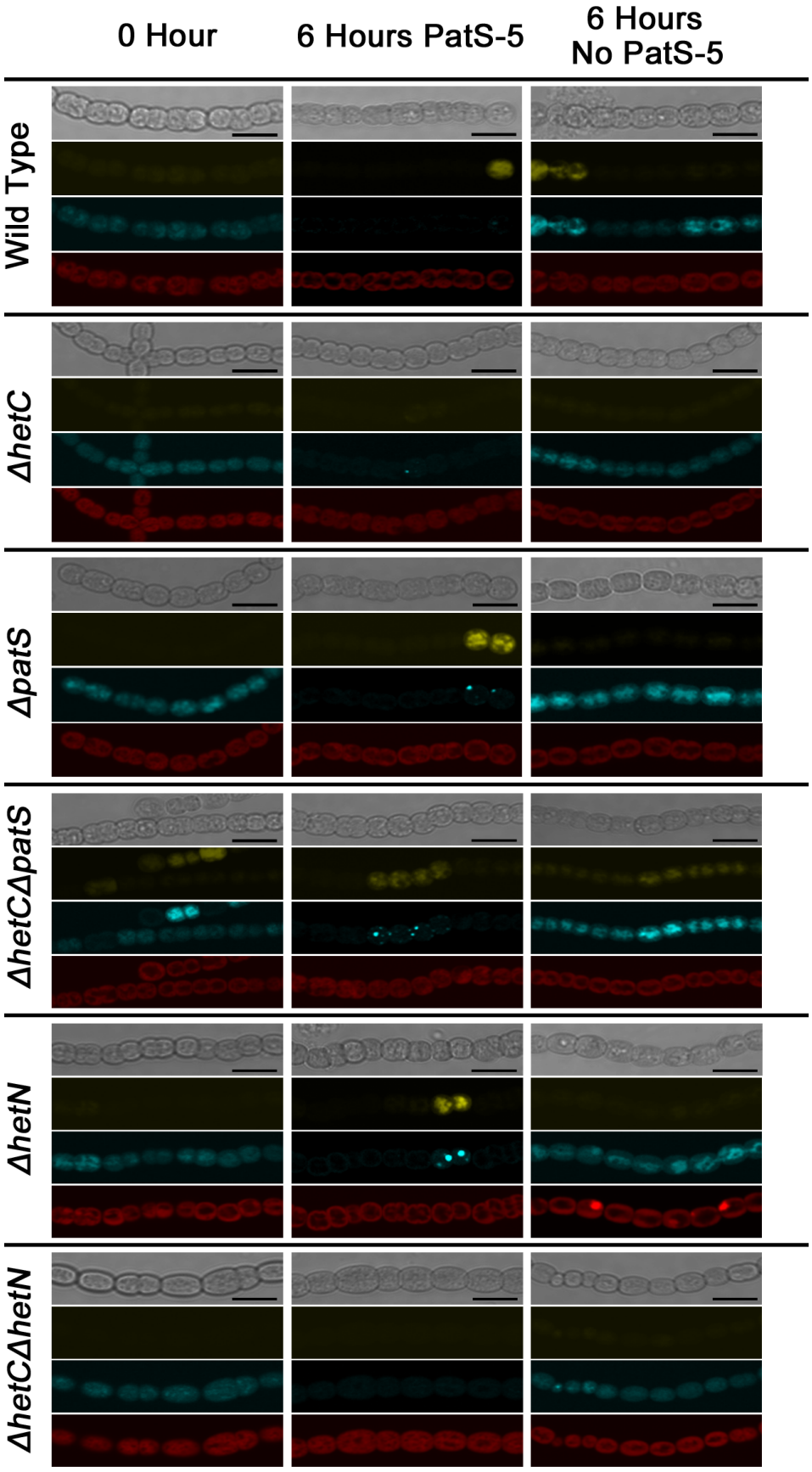
0 Hour
6 Hours PatS-5
6 Hours
No PatS-5
Wild Type
ΔhetC
ΔpatS
ΔhetCΔpatS
ΔhetN
ΔhetCΔhetN

Figure S6. Degradation of HetR-CFP in response to RGSGR peptide. Plasmid pPJAV247 contains a PpatS-YFP transcriptional fusion divergently transcribed from P_{petE}-*hetR*(*H69Y*)-CFP and was introduced into the indicated strains. Cultures were maintained in the presence of nitrate and imaged before and 6 hours after the addition of 10 μM PatS-5 pentapeptide (RGSGR) or a control to which nothing was added. From top to bottom: bright field, yellow fluorescence from P_{patS}-YFP, blue fluorescence from P_{petE}-*hetR*(*H69Y*)-CFP, and red autofluorescence. Bar, 10 μm.

Table S1. Patterns of heterocysts produced by strains of *Anabaena*.

Strain (Genotype)	Hours N-	Heterocyst Percentage	Mean Vegetative Cell Interval	Heterocyst Occurrence	Diazotrophic Growth
Wild type	24	9.13 ± 0.64	10.9 ± 0.26	95 ± 1.53; 5 ± 1.53	YES
	48	7.31 ± 0.61	13 ± 0.3	94.11 ± 0.96; 5.44 ± 1.17 0.44 ± 0.51	
	72	9.06 ± 0.42	13.2 ± 0.34	94.11 ± 1.35; 5.89 ± 1.35	
	96	8.73 ± 1.01	14.3 ± 0.37	93.22 ± 1.02; 6 ± 0.88; 0.78 ± 0.19	
	120	8.93 ± 0.31	14.2 ± 0.36	92.78 ± 1.07; 5.67 ± 1.2; 1.56 ± 0.51	
UHM115 (Δ*hetN*::Ω Sp^r/Sm^r)	24	8.8 ± 0.53	11 ± 0.35	86.78 ± 1.64; 12.89 ± 1.64; 0.33 ± 0.33	YES
	48	13.5 ± 1.29	6.2 ± 0.38	44.67 ± 1.2; 29.78 ± 0.77; 25.67 ± 0.58	
	72	17.13 ± 1.47	5.7 ± 0.39	34.89 ± 1.17; 28.11 ± 1.58; 37 ± 1.67	
	96	17.7 ± 0.7	6.1 ± 0.44	39.33 ± 0.88; 26.22 ± 0.84; 34.67 ± 01.15	
	120	17.8 ± 1.4	6.7 ± 0.48	40 ± 2.73; 20.11 ± 0.84; 39.89 ± 2.5	
UHM334 (Δ*patS*::Ω Sp^r/Sm^r)	24	24.9 ± 2.7	3 ± 0.2	58.22 ± 0.84; 40.78 ± 0.77; 1 ± 1.2	YES
	48	20.13 ± 1.14	3.5 ± 0.21	61.78 ± 2.52; 35.33 ± 2.73; 2.67 ± 1.2	
	72	17.47 ± 1.94	4.3 ± 0.25	63.33 ± 0.67; 29.44 ± 1.84; 7.33 ± 2.08	
	96	15.7 ± 2.1	6.3 ± 0.34	67.44 ± 2.67; 25 ± 0.88; 7.56 ± 1.9	
	120	16.6 ± 1.31	6.3 ± 0.32	69.78 ± 0.96; 24.56 ± 0.84; 5.67 ± 1.2	
UHM232 (Δ*hetC*)	24	0	-	-	NO
	48	2.67 ± 1.2	-	98.78 ± 0.69; 1.22 ± 0.69	
	72	3.78 ± 1.49	21 ± 0.4	94.78 ± 1.5; 5.11 ± 1.39	
	96	4.7 ± 1.25	19.7 ± 0.42	93.44 ± 1.17;	

				6.56 ± 1.17	
	120	4.4 ± 1.67	18.5 ± 0.45	91.44 ± 1.68; 8.56 ± 1.68	
UHM224 (Δ*hetC* Δ*patS*::Ω Sp^r/Sm^r)	24	16.27 ± 1.7	3.8 ± 0.24	70.44 ± 0.84; 27.78 ± 0.69; 1.78 ± 0.51	YES
	48	17.8 ± 1.31	6 ± 0.33	72.33 ± 1.76; 23.89 ± 1.68; 3.78 ± 1.02	
	72	15.2 ± 1.78	11 ± 0.51	73.89 ± 1.68; 22.44 ± 0.84; 3.56 ± 1.26	
	96	14.87 ± 1.22	12 ± 0.54	73.11 ± 3.17; 22.78 ± 2.67; 4.11 ± 0.51	
	120	13.67 ± 1.33	11.9 ± 0.56	77 ± 1; 19 ± 1.53; 4.33 ± 0.88	
UHM225 (Δ*hetC* Δ*hetN*::Ω Sp^r/Sm^r)	24	0.33 ± 0.31	-	96.89 ± 0.84; 3.11 ± 0.84	YES
	48	6.93 ± 0.99	10.6 ± 0.43	72.11 ± 1.26; 24.33 ± 1.33; 3.56 ± 0.51	
	72	9.6 ± 0.87	10.8 ± 0.51	57.78 ± 3.24; 29.89 ± 2.04; 12.33 ± 1.2	
	96	10.33 ± 1.21	10.2 ± 0.53	51.78 ± 0.69; 29.67 ± 1.2; 18.33 ± 1.86	
	120	8.27 ± 0.88	11.6 ± 0.53	55.11 ± 1.17; 24.56 ± 1.35; 20.33 ± 1.2	

At the indicated times following nitrogen stepdown, 500 cells were counted in triplicate and total heterocysts are presented as the mean ± the standard deviation. The presence of single (top line), double (second line), or multiple contiguous heterocysts (third line) was determined for 300 heterocyst occurrences in triplicate and are presented as the average percent ± the standard deviation. The number of vegetative cell between heterocysts was counted for 300 intervals and is presented as the mean ± the standard deviation of the mean. The ability to grow diazotrophically was assessed visually following two weeks of growth on solid BG-11 medium lacking a combined nitrogen source.

Table S2. Statistical analysis of differences in signal ranges 9 h following the removal of combined nitrogen. Pairwise comparisons of signal ranges recorded from the indicated strains were analyzed with a t-test. P-values are indicated for each pairwise comparison. Values below 0.05 were considered indicative of a significant difference between signal ranges.

	WT	Δ*patS*	Δ*hetN*	Δ*hetC*	Δ*hetC*Δ*patS*	Δ*hetC*Δ*hetN*
WT		< 0.0001	0.16	0.59	< 0.0001	0.2
Δ*patS*			< 0.0001	< 0.0001	1	0.0006
Δ*hetN*				0.55	< 0.0001	0.65
Δ*hetC*					< 0.0001	0.26
Δ*hetC*Δ*patS*						0.0046
Δ*hetC*Δ*hetN*						

Table S3. Statistical analysis of differences in signal ranges 24 or 48 h following the removal of combined nitrogen. Pairwise comparisons of signal ranges recorded from the indicated strains were analyzed with a t-test. P-values are indicated for each pairwise comparison. Values below 0.05 were considered indicative of a significant difference between signal ranges.

	WT	Δ*patS*	Δ*hetN*	Δ*hetC*	Δ*hetC*Δ*patS*	Δ*hetC*Δ*hetN*
WT		N/A	0.92	< 0.0001	< 0.0001	< 0.0001
Δ*patS*			N/A	N/A	N/A	N/A
Δ*hetN*				< 0.0001	< 0.0001	< 0.0001
Δ*hetC*					0.46	1
Δ*hetC*Δ*patS*						0.57
Δ*hetC*Δ*hetN*						

Table S4. Strains, plasmids, and oligonucleotide primers used in this study.

Strain or plasmid	**Characteristic(s)***	**Source or reference**
***Anabaena* sp. strains**		
PCC 7120	Wild type	Pasteur Culture Collection
UHM103	Δ*hetR*	(1)
UHM115	Δ*hetN*::Ω Sp^r/Sm^r	(1)
UHM224	Δ*hetC* Δ*patS*::Ω Sp^r/Sm^r	This study
UHM225	Δ*hetC* Δ*hetN*::Ω Sp^r/Sm^r	This study
UHM232	Δ*hetC*	This study
UHM334	Δ*patS*::Ω Sp^r/Sm^r	This study
Plasmids		
pBlueScript SK+	Cloning vector, Ap^r	Stratagene
pAM504	Shuttle vector for replication in *E. coli* and *Anabaena*; Km^r Nm^r	(2)
pUC57-PS12-cfp	Plasmid used as template for CFP	(3)
pUC57-PS12-yfp	Plasmid used as template for YFP	(3)
pRL277	Suicide vector; Sp^r/Sm^r	(4)
pAM1951	pAM504 with P_{patS}-*gfp*	(5)
pSMC126	pAM504 with P_{hetN}-*gfp*	(6)
pSMC164	Suicide plasmid used to replace *patS* with Sp^r/Sm^r Ω interposon	(7)
pSMC182	Suicide plasmid used to replace *hetN* with Sp^r/Sm^r Ω interposon	(1)
pDR306	pAM504 with P_{petE}-*hetR*(*H69Y*)-*gfp*	(8)
pPJAV123	pAM504 with the NdeI site removed	(9)
pPJAV341	pAM504 with P_{hetN}-EcFbFP	(9)
pPJAV243	pAM504 with P_{petE}-CFP	This study
pPJAV247	pAM504 with P_{petE}-*hetR*(*H69Y*)-CFP transcribed divergently from P_{patS}-YFP	This study
pKH206	pRL277 used to delete *hetC*	This study
Oligonucleotide[a]	**Sequence**	
CCFP2-PpetE-OEX-F	GATCCTCCCGCGGATCGGCGTCAGCTATGAGCGGGGGCGAGGAGCTGTTCGCTGGC	
CCFP2-PpetE-OEX-R	CGATCCGCGGGAGGATCCCCCGGGCATATGGTTCTCCTAACCTGTAGTTTTATTTTTC	
CCFP2-SacI-R	ATATAGAGCTCTTAGCGGTACAGCTCGTCCATGCCGTG	
del-hetC-dn-F	AAACAGACCCGGGTCTAGTCAGTTGTCAG	
del-hetC-dn-R	TATATGAGCTCTACTAGGTAATGAGG	
del-hetC-up-F	TATATAGATCTCAACCAAGGGAAAATG	
del-hetC-up-R	TGACTAGACCCGGGTCTGTTTGGTGTGTAAAC	
del-hetC-dn-out	TTTTCATGAATGTCACCCG	
del-hetC-up-out	AACCAGTGTTAACAATTTTCGG	
HetR-F-NdeI-express	ATCGATCGCATATGAGTAACGACATCGATCTGATC	
hetR-tln-BamHI-R	GGATCCATCTTCTTTTCTACCAAACACCATTTG	
down-hetN-R	GGATCCGCCCATTAATATAAGTCTC	
up-hetN-F	GAGCTCGGCAAGCAGAGTTAATC	
patSfor	GATATCTAATCGATGCCACATCTAAG	
patSrev	CACATTAATCTCACTAACTTCTACATC	
PpatS-MunI-F	ATATACAATTGTGAATTTGTTTTGGGAACACTTAAG	
PpatS-OEX-R	CGCCGCTGCTCATGTATATCTCCTTCTTAAATCTAGCGCTCATC	
PpetE-XhoI-F	TATATCTCGAGGCTGAGGTACTGAGTACACAGC	

Turbo-OEX-F	GAAGGAGATATACATGAGCAGCGGCGCCCTGCTGTTCCACGGC
YFP-MunI-R	ATATACAATTGTCAGCTGGTGTCTCCGGAAC

*Ap, ampicillin; Km, kanamycin; Nm, neomycin; Sp, spectinomycin; Sm, streptomycin.
[a]Oligonucleotides are shown in the 5'-to-3' direction.

References cited

1. **Borthakur, P. B., C. C. Orozco, S. S. Young-Robbins, R. Haselkorn, and S. M. Callahan.** 2005. Inactivation of *patS* and *hetN* causes lethal levels of heterocyst differentiation in the filamentous cyanobacterium *Anabaena* sp. PCC 7120. Mol. Microbiol. **57:**111-123.
2. **Wei, T.-F., R. Ramasubramanian, and J. W. Golden.** 1994. *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. J. Bacteriol. **176:**4473-4482.
3. **Norris, M. H., Y. Kang, B. Wilcox, and T. T. Hoang.** 2010. Stable site-specific fluorescent tagging constructs optimized for Burkholderia species. Appl. Env. Microbiol. **76:**7635-7640.
4. **Black, T. A., Y. Cai, and C. P. Wolk.** 1993. Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. Mol. Microbiol. **9:**77-84.
5. **Yoon, H.-S., and J. W. Golden.** 1998. Heterocyst pattern formation controlled by a diffusible peptide. Science **282:**935-938.
6. **Callahan, S. M., and W. J. Buikema.** 2001. The role of HetN in maintenance of the heterocyst pattern in *Anabaena* sp. PCC 7120. Mol. Microbiol. **40:**941-950.
7. **Orozco, C. C., D. D. Risser, and S. M. Callahan.** 2006. Epistasis analysis of four genes from *Anabaena* sp. strain PCC 7120 suggests a connection between PatA and PatS in heterocyst pattern formation. J. Bacteriol. **188:**1808-1816.
8. **Risser, D. D., and S. M. Callahan.** 2009. Genetic and cytological evidence that heterocyst patterning is regulated by inhibitor gradients that promote activator decay. Proc. Natl. Acad. Sci. USA **106:**19884-19888.
9. **Videau, P., R. T. Oshiro, L. M. Cozy, and S. M. Callahan.** 2014. Transcriptional dynamics of developmental genes assessed with an FMN-dependent fluorophore in mature heterocysts of *Anabaena* sp. strain PCC 7120. Microbiol. **160:**1874-1881.