Phenotypic assessment suggests multiple start codons for HetN, an inhibitor of heterocyst differentiation, in *Anabaena* sp. strain PCC 7120

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Supplemental Material

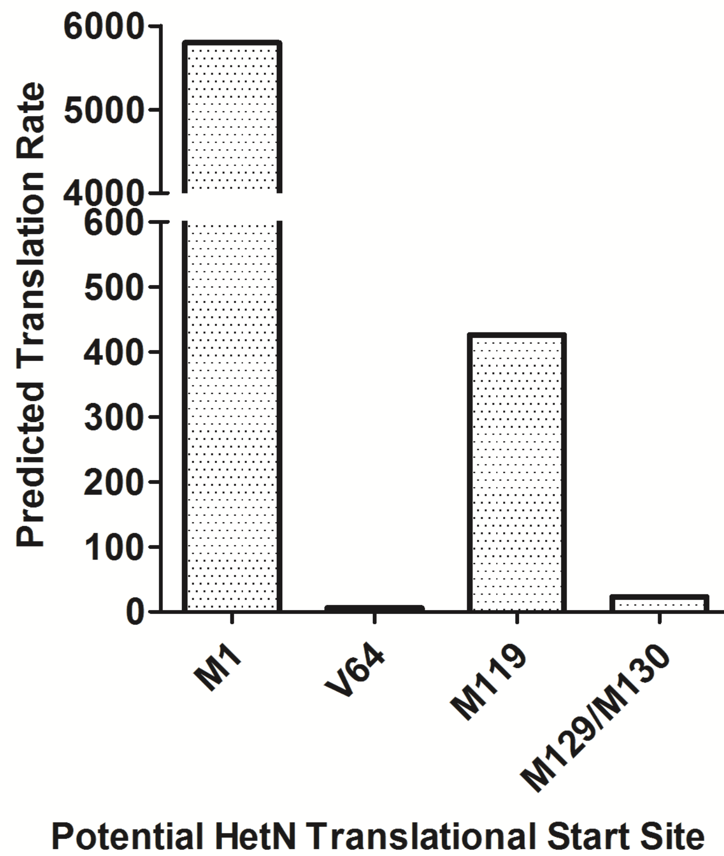

Figure S1. Bioinformatic prediction of the strength of ribosomal binding sites upstream of the potential translational start sites upstream of and within *hetN* using the RBS Calculator v2.0 program. Higher predicted translation rates indicate stronger predicted ribosomal binding sites.

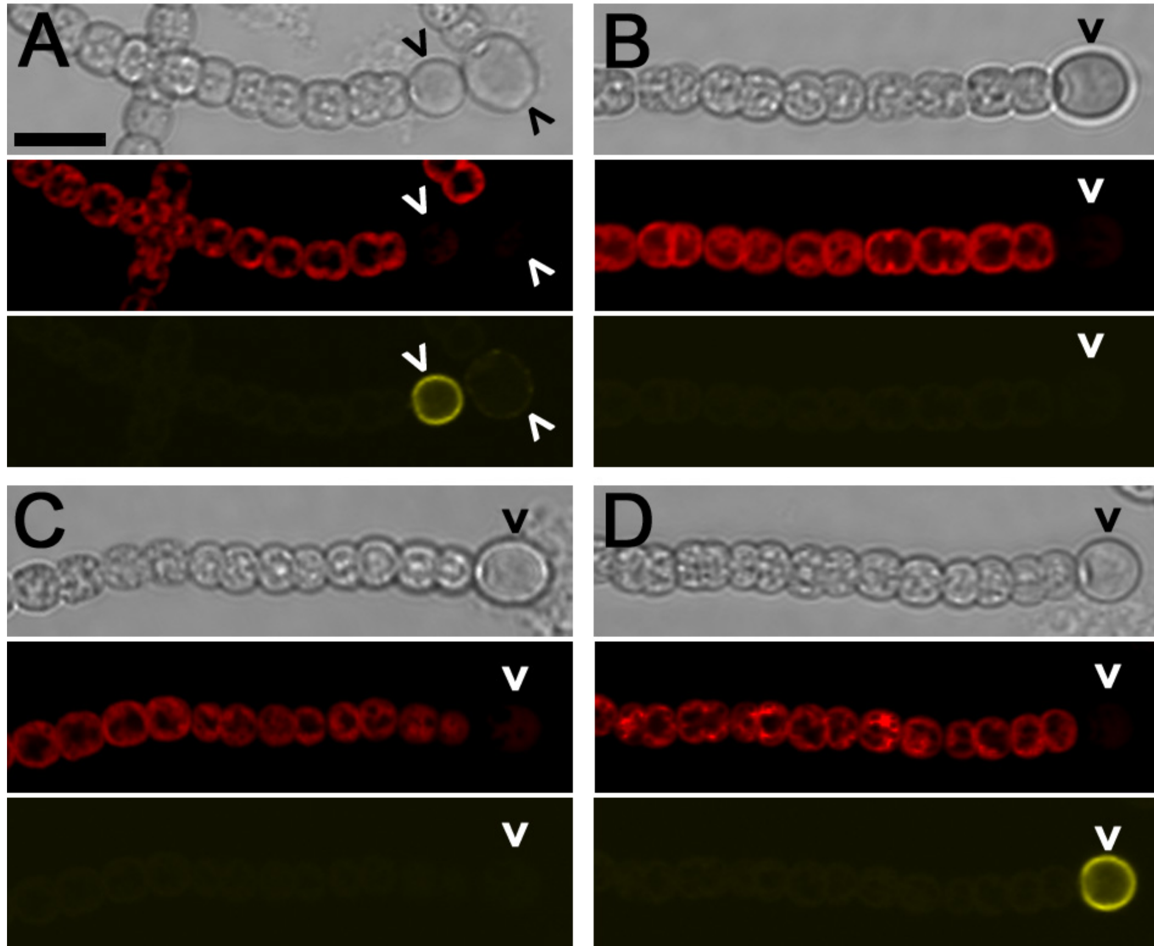

Figure S2. The M1 residue of *hetN* is required for heterocyst-specific translation and localization of a YFP fusion protein expressed from the native promoter. Strain UHM163, which contains *hetR*(*R250K*) at the native locus and forms heterocysts even when *hetN* is overexpressed, 24 h after the removal of combined nitrogen with the following plasmids: pAD127 containing P_{hetN}-*hetN*-YFP (A); pAD126 containing P_{hetN}-*hetN*(*M1L*, *M119L, M129L, M130L*)-YFP (B); pAD124 containing P_{hetN}-*hetN*(*M1L, M129L, M130L*)-YFP (C); or pAD125 containing P_{hetN}-*hetN*(*M119L, M129L, M130L*)-YFP (D). From top to bottom: bright-field, red autofluorescence, yellow fluorescence from HetN-YFP alleles. Carets indicate heterocysts. Bar in panel A, 10 µm.

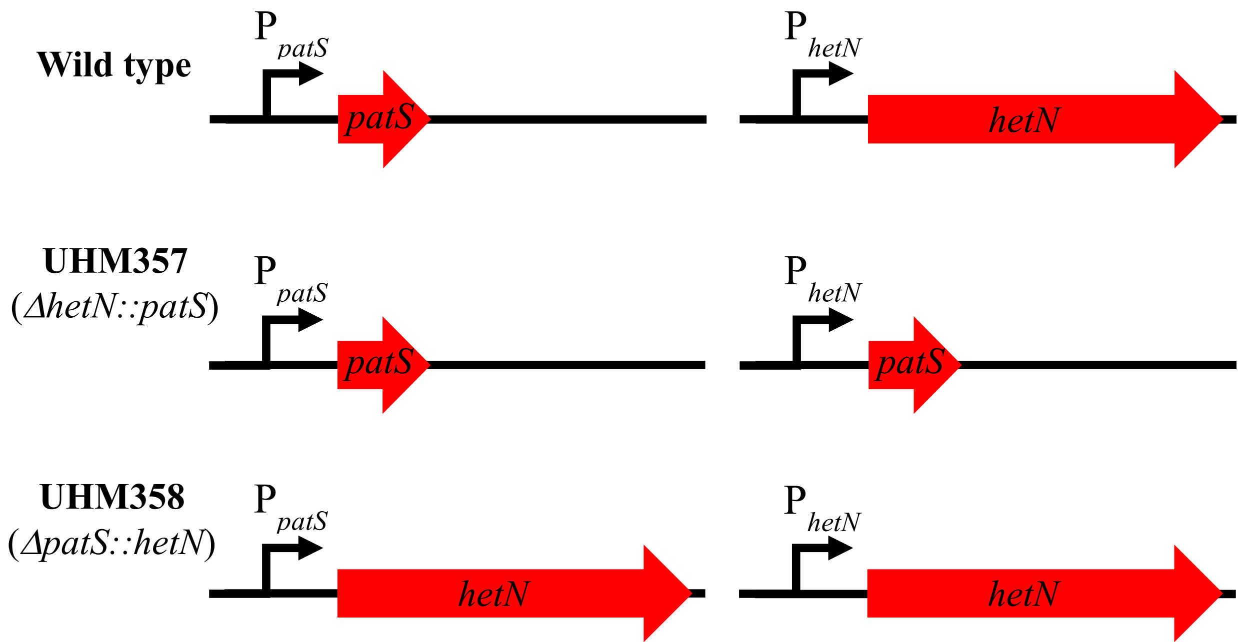

Figure S3. Schematic depicting the mutations introduced into the UHM357 and UHM358 strains. The wild type has a copy of *patS* controlled by P_{patS} and *hetN* controlled by P_{hetN}, both at the native loci. UHM357 has had the coding region of *hetN* replaced with the *patS* coding region, and UHM358 has had the coding region of *patS* replaced with the *hetN* coding region, both at the native loci.

Table S1. Oligonucleotide primers used in this study.

Oligonucleotide primers	Sequence*
3'patSBglIIR	AGATCTGGGAGTAAATTGTAAATCATAGAAC
5'patSSacF	GAGCTCCGCATCTTTTATTCAAGCTAACTAGC
dhetNup-S-OEX-F	GTGAATTTCTGTGATGAGCGCGGTAGTGGTAGATAGCTCCGCAGT TGCTTAGGGAATGAG
dhetNup-S-OEX-R	CTCATCACAGAAATTCACTAACATAATTGCCTTCATTGTAACCTG CTAGTCTCCAAATTC
down-hetN-R	GGATCCGCCCATTAATATAAGTCTC
dpatS-N-OEX-F	TTTAAGATTATGACAACTCTTACAGGTAAGACAGTAC
dpatS-N-OEX-R	ACACTCGTTTCATGAGCGATGAGACTCAACAGCTACATAG
dpatSdn-N-OEX-F	CGCTCATGAAACGAGTGTAAAATTCTGCCTCGACTATCG
dpatSup-N-OEX-R	AGTTGTCATAATCTTAAAATCGGTGAATTACTTTTCAACAG
hetIR-SacI	GAGCTCTGGAACCAGGGCAAGTTAAATTT
hetMF-BamHI	GGATCCTAGAACGCTGGTCTGATGAACAA
HetN E->A Fwd	GCATGATGGCACGCGGTAGTGGTCGGATTG
HetN E->A Rev	CAATCCGACCACTACCGCGTGCCATCATGC
HetN E->L Fwd	GCATGATGCTACGCGGTAGTGGTCGGATTG
HetN E->L Rev	CAATCCGACCACTACCGCGTAGCATCATGC
HetN E->Q Fwd	GCATGATGCAACGCGGTAGTGGTCGGATTG
HetN E->Q Rev	CAATCCGACCACTACCGCGTTGCATCATGC
hetN Mod-M1 F	ACTAGCAGGTTACACTCACAACTCTTACAGG
hetN Mod-M1 R	CCTGTAAGAGTTGTGAGTGTAACCTGCTAGT
hetN SacI-R	CGCCGAGCTCGCTGCTATTAACCTTGCAAAGTTC
hetN-BamHI-R	ATATAGGATCCTCATGAGCGATGAGACTCAAC
HetN-EcoRI-F-petE	ATATAGAATTCATGACAACTCTTACAGGTAAG
HetN-GTG-to-GTT-QC-F	GCGATCGCTATTCCTTTTGATGTTAGGAACACATCACAATTATCG
HetN-GTG-to-GTT-QC-R	CGATAATTGTGATGTGTTCCTAACATCAAAAGGAATAGCGATCGC
hetN-Tln-BglII-R	AGATCTTGAGCGATGAGACTCAACAGCTACATAGC
HetN(M1L)-EcoRI-F	ATATAGAATTCCTCACAACTCTTACAGGTAAG
HetN-Mod-M2-QC-F	CACTAATCTATTGGCTGCTCTCGAATTAACACGTTTGTTAC
HetN-Mod-M2-QC-R	GTAACAAACGTGTTAATTCGAGAGCAGCCAATAGATTAGTG
MMERGSGR-Sub Met-F2	CTACCCAGCCTCCTCGAACGCGGTA
MMERGSGR-Sub Met-R2	ACCGCGTTCGAGGAGGCTGGGTAGT
patSfor	GATATCTAATCGATGCCACATCTAAG
patSrev	CACATTAATCTCACTAACTTCTACATC
PhetN-Bam-F	ATATAGGATCCAGGAGAAGACGCGATGAATC
PhetN-MunI-F	ATATACAATTGAGGAGAAGACGCGATGAATC
PpetE-MunI-R	TATATCAATTGGCTGAGGTACTGAGTACACAGC
up-hetN-F	GAGCTCGGCAAGCAGAGTTAATC

* Oligonucleotides are shown in the 5’ to 3’ direction.

Table S2. Phenotypic and statistical analysis of *Anabaena* strains harboring plasmids expressing various *hetN* alleles fused to YFP.

Plasmid Construct:	% Heterocysts 24H N_2	t-test comparison to the Empty Vector	YFP detected in Wt from P*petE*	YFP detected in *hetR(R250K)* from P*petE*	YFP detected in *hetR(R250K)* from P*hetN*
Empty Vector	7.6 ± 0.4	(--)	No	No	No
hetN	0	0.0001	Yes	Yes	Yes
hetN(M1L)	0	0.0001	No	No	No
hetN(M119L)	0	0.0001	Yes	Yes	Yes
hetN(M129L/M130L)	0	0.0001	Yes	Yes	Yes
hetN(M1L, M119L)	6.13 ± 0.11	0.003	No	No	No
hetN(M1L, M129L/M130L)	0.06 ± 0.11	0.0001	No	No	No
hetN(M119L, M129L/M130L)	0	0.0001	Yes	Yes	Yes
hetN(M1L, M119L, M129L/M130L)	6.86 ± 0.9	0.26	No	No	No

Table S3. Statistical analysis of the heterocyst percentages produced from *Anabaena* strains expressing various *hetN* alleles from the native locus compared to the wild type and Δ*hetN* strain.

	Wild type	Δ*hetN*
ANOVA P-value	$1.92 * 10^{-15}$	$1.41 * 10^{-15}$
Strain:	Pairwise t-test (P-value)	Pairwise t-test (P-value)
hetN(M1L)	0.14	<0.0001
hetN(M119L)	<0.0001	0.0007
hetN(M129L/M130L)	0.24	<0.0001
hetN(M1L, M119L)	0.0008	0.18
hetN(M1L, M129L/M130L)	0.07	<0.0001
hetN(M119L, M129L/M130L)	0.0004	0.47
hetN(M1L, M119L, M129L/M130L)	<0.0001	0.23
hetN(M1L, V64V(GTG to GTT), M129L/M130L)	0.05	<0.0001
hetN(E131A)	0.0004	0.01
hetN(E131L)	0.0001	0.03
hetN(E131Q)	0.01	<0.0001

MATERIALS AND METHODS

Plasmids pAD120, pAD121, pAD122, pAD123, pAD124, pAD125, pAD126, and pAD127 are mobilizable shuttle vectors based on pAM504 (1) containing P_{hetN}-*hetN*(*M1L*)-YFP, P_{hetN}-*hetN*(*M119L*)-YFP, P_{hetN}-*hetN*(*M129L*/*M130L*)-YFP, P_{hetN}-*hetN*(*M1L*, *M119L*)-YFP, P_{hetN}-*hetN*(*M1L*, *M129L*/*M130L*)-YFP, P_{hetN}-*hetN*(*M1L*, *M119L*, *M129L*/*M130L*)-YFP, and P_{hetN}-*hetN*-YFP, respectively. P_{hetN}-*hetN*(*M1L*), P_{hetN}-*hetN*(*M119L*), P_{hetN}-*hetN*(*M129L*/*M130L*), P_{hetN}-*hetN*(*M1L*, *M119L*), P_{hetN}-*hetN*(*M1L*, *M129L*/*M130L*), P_{hetN}-*hetN*(*M1L*, *M119L*, *M129L*/*M130L*), or P_{hetN}-*hetN* were amplified by PCR with the primers PhetN-MunI-F and hetN-Tln-BglII-R from the plasmids pOR101, pOR102, pOR103, pOR104, pOR105, pOR106, pOR107, and pDR382 (2), respectively, digested with *Mun*I-*Bgl*II, and individually cloned into the *Eco*RI-*Bam*HI sites of pPJAV153 (3) to create pAD120, pAD121, pAD122, pAD123, pAD124, pAD125, pAD126, and pAD127.

Plasmids pAD128, pAD129, pAD130, pAD131, pAD132, pAD133, pAD134, and pAD135 are mobilizable shuttle vectors based on pAM504 containing P_{petE}-*hetN*(*M1L*)-YFP, P_{petE}-*hetN*(*M119L*)-YFP, P_{petE}-*hetN*(*M129L*/*M130L*)-YFP, P_{petE}-*hetN*(*M1L*, *M119L*)-YFP, P_{petE}-*hetN*(*M1L*, *M129L*/*M130L*)-YFP, P_{petE}-*hetN*(*M1L*, *M119L*, *M129L*/*M130L*)-YFP, and P_{petE}-*hetN*-YFP, respectively. P_{petE}-*hetN*(*M1L*), P_{petE}-*hetN*(*M119L*), P_{petE}-*hetN*(*M129L*/*M130L*), P_{petE}-*hetN*(*M1L*, *M119L*), P_{petE}-*hetN*(*M1L*, *M129L*/*M130L*), P_{petE}-*hetN*(*M1L*, *M119L*, *M129L*/*M130L*), and P_{petE}-*hetN* were amplified by PCR with the primers PpetE-MunI-F and hetN-Tln-BglII-R from the plasmids pOR108, pOR109, pOR110, pOR111, pOR112, pOR113, pOR114 and pDR320 (4), respectively, digested with *Mun*I-*Bgl*II, and individually cloned into the *Eco*RI-*Bam*HI sites of pPJAV153 to create pAD128, pAD129, pAD130, pAD131, pAD132, pAD133, pAD134, and pAD135.

Plasmid pAHB174 is a suicide vector based on pRL277 (5) to introduce *hetN*(*M1L*, *M129L*/*M130L*) with a point mutation to change the codon for V64 from a GTG to a GTT valine at the native locus. The plasmid pOR105 was amplified by PCR with the primers HetN-GTG-to-GTT-QC-F and HetN-GTG-to-GTT-QC-R and QuickChange was done to create pAHB174.

Plasmid pOR101 is a suicide vector based on pRL277 to introduce *hetN*(*M1L*) at the native locus. Regions up- and downstream of the HetN start codon were amplified from pDR382 with the primer pairs PhetN-Bam-F and hetN Mod-M1 R and hetN Mod-M1 F and HetN-SacI-R, respectively. The products harboring the mutation were fused by overlap extension PCR (6), digested with *Spe*I-*Sac*I, and cloned into the same sites of pDR382 replacing the native allele to create pOR101.

Plasmid pOR102 is a suicide vector based on pRL277 to introduce *hetN*(*M119L*) at the native locus. The plasmid pDR382 was amplified by PCR with the primers HetN-Mod-M2-QC-F and HetN-Mod-M2-QC-R and QuickChange was done to create pOR102.

Plasmid pOR103 is a suicide vector based on pRL277 to introduce *hetN*(*M129L*/*M130L*) at the native locus. Regions up- and downstream of *hetN*(*M129*/*M130*) were amplified from pDR382 with the primer pairs PhetN-Bam-F and MMERGSGR-Sub Met-R2 and MMERGSGR-Sub Met-F2 and HetN-SacI-R, respectively. The products harboring the mutation were fused by overlap extension PCR, digested with *Spe*I-*Sac*I, and cloned into the same sites of pDR382 replacing the native allele to create pOR103.

Plasmid pOR104 is a suicide vector based on pRL277 to introduce *hetN*(*M1L*, *M119L*) at the native locus. The plasmid pOR101 was amplified by PCR with the primers HetN-Mod-M2-QC-F and HetN-Mod-M2-QC-R and QuickChange was done to create pOR104.

Plasmids pOR105, pOR106, and pOR107 are suicide vectors based on pRL277 to introduce *hetN*(*M1L*, *M129L*/*M130L*), *hetN*(*M119L*, *M129L*/*M130L*), and *hetN*(*M1L*, *M119L*, *M129L*/*M130L*), respectively, at the native locus. Regions up- and downstream were amplified from pOR101, pOR102, and pOR104, respectively, with the primer pairs PhetN-Bam-F and MMERGSGR-Sub Met-R2 and MMERGSGR-Sub Met-F2 and HetN-SacI-R, respectively. The products harboring the mutation were fused by overlap extension PCR, digested with *Spe*I-*Sac*I, and cloned into the same sites of pDR382 replacing the native allele to create pOR105, pOR106, and pOR107.

Plasmids pOR108, pOR111, pOR112, and pOR114 are mobilizable shuttle vectors based on pAM504 containing P_{petE}-*hetN*(*M1L*), P_{petE}-*hetN*(*M1L*, *M119L*), P_{petE}-*hetN*(*M1L*, *M129L*/*M130L*), and P_{petE}-*hetN*(*M1L*, *M119L*, *M129L*/*M130L*), respectively. The *hetN*(*M1L*), *hetN*(*M1L*, *M119L*), *hetN*(*M1L*, *M129L*/*M130L*), and *hetN*(*M1L*, *M119L*, *M129L*/*M130L*) alleles were amplified by PCR from pOR101, pOR104, pOR105, and pOR107, respectively, with the primers HetN(M1L)-EcoRI-F and hetN-BamHI-R. The products were digested with *Eco*RI-*Bam*HI and cloned into the same sites in pPJAV213 to create pOR108, pOR111, pOR112, and pOR114.

Plasmids pOR109, pOR110, and pOR113 are mobilizable shuttle vectors based on pAM504 containing P_{petE}-*hetN*(*M119L*), P_{petE}-*hetN*(*M129L*/*M130L*), and P_{petE}-*hetN*(*M119L*, *M129L*/*M130L*), respectively. The *hetN*(*M119L*), *hetN*(*M129L*/*M130L*), and *hetN*(*M119L*, *M129L*/*M130L*) alleles were amplified by PCR from pOR102, pOR103, and pOR106, respectively, with the primers HetN-EcoRI-F-petE and hetN-BamHI-R. The products were digested with *Eco*RI-*Bam*HI and cloned into the same sites in pPJAV213 to create pOR109, pOR110, and pOR113.

Plasmids pOR115 and pOR116 are suicide vectors based on pRL277 to introduce *hetN*(*E131A*) and HetN(E131L), respectively, at the native locus. Regions up- and downstream of *hetN*(*E131*) were amplified by PCR from pDR382 with the primers PhetN-Bam-F and HetN E->A Rev and HetN E->A Fwd and hetN SacI-R for E131A or PhetN-Bam-F and HetN E->L Rev and HetN E->L Fwd and hetN SacI-R for E131L. The products harboring the mutation were fused by overlap extension PCR, digested with *Spe*I-*Sac*I, and individually cloned into the same sites of pDR382 replacing the native allele to create pOR115 and pOR116.

Plasmid pPJAV348 is a suicide vector based on pRL277 to replace the coding region of *hetN* with *patS* at the native *hetN* locus. Regions directly up- and downstream of the *hetN* coding region were amplified by PCR from *Anabaena* chromosomal DNA with the primer pairs hetMF-BamHI and dhetNup-S-OEX-R and dhetNup-S-OEX-F and hetIR-SacI, respectively. The products were fused together by overlap extension PCR, such that the region of overlap on the primers introduced the *patS* coding region, and cloned into the *Nru*I site of pRL277 to create pPJAV348.

Plasmid pPJAV349 is a suicide vector based on pRL277 to replace the coding region of *patS* with *hetN* at the native *patS* locus. Regions directly up- and downstream of the *patS* coding region were amplified from *Anabaena* chromosomal DNA with the primer pairs 5'patSSacF and dpatSup-N-OEX-R and dpatSdn-N-OEX-F and 3'patSBglIIR, respectively, and the coding region of *hetN* was amplified with the primers dpatS-N-OEX-F and dpatS-N-OEX-R. The *hetN* coding region and regions up- and downstream of *patS* were fused together by overlap extension PCR and the product was cloned into the *Nru*I site in pRL277 to create pPJAV349.

Plasmid pPJAV369 is a suicide vector based on pRL277 to introduce *hetN*(*E131Q*) at the native locus. Regions up- and downstream of *hetN*(*E131*) were amplified by PCR from pDR382

with the primers PhetN-Bam-F and HetN E->Q Rev and HetN E->Q Fwd and hetN SacI-R, respectively. The products harboring the mutation were fused by overlap extension PCR, digested with *Spe*I-*Sac*I, and cloned into the same sites of pDR382 replacing the native allele to create pPJAV369.

REFERENCES

1. Wei T-F, Ramasubramanian R, Golden JW. 1994. *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. J Bacteriol 176:4473-4482.
2. Higa KC, Rajagopalan R, Risser DD, Rivers OS, Tom SK, Videau P, Callahan SM. 2012. The RGSGR amino acid motif of the intercellular signaling protein, HetN, is required for patterning of heterocysts in *Anabaena* sp. strain PCC 7120. Mol Microbiol 83:682-693.
3. Rivers OS, Videau P, Callahan SM. 2014. Mutation of *sepJ* reduces the intercellular signal range of a *hetN*-dependent paracrine signal, but not of a *patS*-dependent signal, in the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120. Mol Microbiol 94:1260-1271.
4. Risser DD, Callahan SM. 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.
5. Cai Y, Wolk CP. 1990. Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. J Bacteriol 172:3138-3145.
6. Higuchi R, Krummel B, Saiki RK. 1988. A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res 16:7351-7367.