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Mutagenesis of *hetR* Reveals Amino Acids Necessary for HetR Function in the Heterocystous Cyanobacterium *Anabaena* sp. Strain PCC 7120[▽]

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HetR is the master regulator of heterocyst differentiation in the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120. Genetic selection was used to identify 33 amino acid substitutions in HetR that reduced the proportion of cells undergoing heterocyst differentiation to less than 2%. Conservative substitutions in the wild-type HetR protein revealed three mutations that dramatically reduced the amount of heterocyst differentiation when the mutant allele was present in place of the wild-type allele on a replicating plasmid in a mutant lacking *hetR* on the chromosome. An H69Y substitution resulted in heterocyst formation among less than 0.1% of cells, and D17E and G36A substitutions resulted in a Het$^-$ phenotype, compared to heterocyst formation among approximately 25% of cells with the wild-type *hetR* under the same conditions. The D17E substitution prevented DNA binding activity exhibited by wild-type HetR in mobility shift assays, whereas G36A and H69Y substitutions had no affect on DNA binding. D17E, G36A, and H69Y substitutions also resulted in higher levels of the corresponding HetR protein than of the wild-type protein when each was expressed from an inducible promoter in a *hetR* deletion strain, suggesting an effect on HetR protein turnover. Surprisingly, C48A and S152A substitutions, which were previously reported to result in a Het$^-$ phenotype, were found to have no effect on heterocyst differentiation or patterning when the corresponding mutations were introduced into an otherwise wild-type genetic background in *Anabaena* sp. strain PCC 7120. The clustering of mutations that satisfied the positive selection near the amino terminus suggests an important role for this part of the protein in HetR function.

Anabaena sp. strain PCC 7120 is a filamentous cyanobacterium capable of fixing nitrogen in differentiated cells known as heterocysts, where the oxygen-labile nitrogenase complex is sequestered from atmospheric oxygen and processes that evolve O_2. When deprived of fixed nitrogen, heterocysts develop at regular intervals, approximately every 10th cell, along filaments (18). Along with spore formation by *Bacillus subtilis* and fruiting-body formation by *Myxococcus xanthus*, among others, heterocyst formation by *Anabaena* sp. has emerged as a paradigm system for the regulation of cellular differentiation and pattern formation in bacteria. HetR is the master regulator of heterocyst formation. The genetic network regulating differentiation consists of a series of interconnected positive and negative feedback loops intersecting at *hetR* (14, 19). Understanding the intricacies of HetR function is essential to understanding differentiation and patterning in heterocystous cyanobacteria.

The deletion of *hetR* results in an inability to form heterocysts, while overexpression promotes excessive differentiation even in media with a source of fixed nitrogen (3, 4). The induction of *hetR* transcription occurs early in the developmental process and is localized to clusters of cells around developing heterocysts, eventually resolving to those cells that will differentiate. *hetR* is positively autoregulated and may be posttranslationally modified upon nitrogen step-down (1, 20). HetR has been reported to be a novel, Ca^{2+}-dependent serine protease capable of degrading itself and CcbP, a calcium binding protein, and substitutions at residues S152 and S179 have been reported to abolish protease activity and heterocyst differentiation (9, 16, 21). S152 has been reported to be the active site. HetR binds to DNA of its own promoter region as well as to regions in the *patS* and *hepA* promoters. DNA binding by HetR as a homodimer has been reported to require a disulfide bond between monomers at C48, and a C48A substitution in HetR has been reported to prevent dimer formation and DNA binding in vitro and to result in a Het$^-$ phenotype in vivo (11). Two inhibitors of heterocyst formation, *patS* and *hetN*, have been shown to work at the level of HetR. An R223W substitution mutant protein results in increased differentiation when cells are deprived of combined nitrogen and is insensitive to *patS* and *hetN*, suggesting that both the *patS*- and *hetN*-dependent pathways converge at HetR (12). In vitro, the PatS pentapeptide (RGSGR) was shown to inhibit the DNA binding capability of HetR (11). Although much has been discerned about HetR, its exact function in promoting cellular differentiation as well as the nature of any posttranslational modifications has yet to be elucidated.

A genetic approach was used to isolate *hetR* mutant strains with altered levels of heterocyst differentiation in order to further characterize HetR. Here we report the results of two screens used to identify amino acid substitutions resulting in altered HetR function. Residues D17, G36, and H69 were found to be essential for normal heterocyst differentiation and levels of HetR protein turnover. Residue D17 was required for DNA binding activity. Contrary to previous reports, the S152

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and C48 residues of HetR were found not to be essential for heterocyst formation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The growth of *Escherichia coli* and *Anabaena* sp. strain PCC 7120 and its derivatives; concentrations of antibiotics; the induction of heterocyst formation in BG-11_0 medium, which lacks a combined nitrogen source; the regulation of P_{nir} expression; and conditions for photomicroscopy were as previously described with the exception that medium containing 10 mM NH_4 was supplemented with 30 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], pH 8.0, and *E. coli* cultures used for introducing the PCR mutagenesis library into PCC 7120 were diluted 1,000-fold to ensure that the selected exconjugant filaments arose from the conjugal transfer of a single plasmid (2, 8). To determine heterocyst percentages, 200 cells were counted and the number of heterocysts was recorded. All results are expressed as averages of results for three replicates, with error bars indicating 1 standard deviation, with the exception of data represented in Table 1, which are the percentages of heterocysts among 500 counted cells.

Plasmid construction. To create a library of plasmids containing mutations within the *hetR* coding frame, a 1,889-bp fragment containing the *hetR* coding region and 966 bp of upstream DNA was amplified via error-prone PCR (5) with 2 mM Mn^{2+}, 10 mM dTTP and dCTP, 2 mM dGTP and dATP, and 5% dimethyl sulfoxide (using primers hetR-F-BamHI [5′-ATCCCGGATCCCCTGCCAATG CAGAAGGTTAAAC-3′] and hetR-R-SacI [5′-CATTAGAGCTCCTTTTATT CACTCTGGGTGC-3′]) and cloned directly into pAM504 (17) as BamHI-SacI fragments.

Plasmids pDR137, pDR138, pDR144, and pDR145 were used to express *hetR* carrying the mutation corresponding to S152A [*hetR*(*S152A*)], *hetR*, *hetR*(*C48A*), and *hetR*(*S179N*), respectively, from the native *hetR* promoter. A 966-bp fragment upstream of the *hetR* coding region was amplified via PCR (using primers PhetR-KpnI-F [5′-GGTACCCCTGCCAATGCAGAAGGTT AAAC-3′] and PhetR-NdeI-R [5′-CATATGACAAATAGTTGAATAGCAC GCTTATTAG-3′]) to give P_{hetR} and cloned into pGEM-T (Promega). P_{hetR} was subsequently cloned into pBluescript SK(+) (Stratagene) as an ApaI-PstI fragment to create pDR133. *hetR* and *hetR*(*S179N*) were amplified from genomic DNA of strain PCC 7120 and strain 216 (3), respectively (using primers hetRcf-NdeI [5′-CATATGAGTAACGACATCGATCT GATC-3′] and hetR6H-r [5′-TTAGTGATGGTGATGGTGATGATCT TCTTTTCTACCAAACACCATTTG-3′]). *hetR*(*S152A*) and *hetR*(*C48A*) were created using overlap extension PCR (10) using primers hetR152A-F (5′-GTTA GTTACTGCCTTTGAGTTTTTGGAGTTGATC-3′) and hetR152A-R (5′-CAAAA ACTCAAAGGCAGTAACTAACTGAGCGTCGGG-3′) and hetR48-F (5′-GCGGC TAAGGCCGCCATTTACATGACTTATCTAGAGC-3′) and hetR48-R (5′-GTAA ATGGCGGCCTTAGCCGCCGTTGCTG-3′), respectively, with hetRcf-NdeI and hetR6H-r. The subsequent fragments containing alleles of *hetR* were cloned into pGEM-T and then moved to pDR133 as an NdeI-PstI fragment using NdeI sites introduced by the primer. The subsequent P_{hetR} and *hetR* fragments were cloned into pAM504 as KpnI-SacI fragments.

Plasmid pDR147 was used to replace the wild-type allele of *hetR* in strain PCC 7120 with *hetR*(*S152A*). *hetR*(*S152A*) in pGEM-T was moved into pBluescript as a KspI-SacI fragment and subsequently cloned into pRL278 (1) as a BamHI-SacI fragment to create pDR147.

Plasmid pDR167 was used to replace the wild-type allele of *hetR* in strain PCC 7120 with *hetR*(*C48A*). *hetR*(*C48A*) fused to P_{hetR} [P_{hetR}-*hetR*(*C48A*)] was excised from pBluescript SK(+) and reintroduced into pBluescript SK(+) as a KspI fragment in order to create flanking SpeI sites. P_{hetR}-*hetR*(*C48A*) was then moved into pRL277 (1) as a SpeI fragment to create pDR167.

Plasmid pDR203 is a shuttle vector used to express *hetR*(*E254G*) from its native promoter. *hetR*(*E254G*) was created via overlap extension PCR (using internal primers hetR254E-G F [5′-CGAGCCTTAGAAGGACTCGATGTGCCACCAG AG-3′], hetR254E-G R [5′-GCACATCGAGTCCTTCTAAGGCTCGCATAGCG TTTG-3′], hetR-F-BamHI, and hetR-R-SacI) and cloned into pAM504 as a BamHI-SacI fragment using the restriction sites introduced on the primers to create pDR203.

To create a library containing mutations within pDR203, this plasmid was introduced into the hypermutator *E. coli* strain XL1-Red (Stratagene). Plasmid was isolated from the resultant colonies and introduced into a Δ*hetR* strain, UHM103 (2), via conjugation.

To create conservative substitutions within the *hetR* coding frame, overlap extension PCR was used with the overlapping inner primers described in Table 1 and the outer primers hetR-BamHI-F and hetR-SacI-R, and the PCR fragments containing mutant coding regions were cloned into pAM504 as BamHI-SacI fragments, with the exception of *hetR*(*G295A*), which was amplified with hetR-BamHI-F and hetRG295A-R (Table 1).

For the overexpression of recombinant HetR carrying the D17E substitution ($HetR_{D17E}$), $HetR_{G36A}$, and $HetR_{H69Y}$ from *E. coli*, the appropriate *hetR* allele was amplified via PCR using the primers hetRcf-NdeI and hetRH6-BamHI-R, cloned into the SmaI site of pBluescript SK(+) as a blunt-end fragment, and then moved into pET21a as an NdeI-BamHI fragment. For the overexpression of recombinant HetR, $HetR_{C48A}$, $HetR_{S152A}$, and $HetR_{S179N}$, the appropriate *hetR* allele was moved from pDR137, pDR138, pDR144, or pDR145 to pET21a (Novagen) as an NdeI-NotI fragment.

Plasmid pSMC188 was created to transcriptionally fuse *hetR* to the P_{nir} promoter. The EcoRI site of plasmid pAM504 was removed by cutting with EcoRI and blunt-ending with T4 polymerase followed by ligation. A fragment of DNA containing P_{nir} was generated by PCR as previously described (8) and cloned as a BamHI-SacI fragment into the resulting vector to create pSMC188. *hetR* mutant alleles were then amplified from appropriate template DNA (using the primers hetR-EcoRI-F [5′-CTATTGAATTCATGAGTAACGACATCGATCT GATC-3′] and hetRH6-BamHI-R [5′-TTACTGGATCCTTAGTGATGGTGA TGGTGATGATCTTCTTTTCTACCAAACACCATTTG-3′] for carboxy-terminal six-His-tagged constructs or hetR-EcoRI-F and hetR-BamHI-R [5′-TTA CTGGATCCTTAATCTTCTTTTCTACCAAACACCATTTG-3′]) and cloned into pSMC188 as EcoRI-BamHI fragments. All plasmid-borne constructs described herein were sequenced to verify integrity.

Strain construction. All strains and oligonucleotides not listed in the text are described in Table 1. The replacement of *hetR* with *hetR*(*C48A*) and *hetR*(*S152A*) was performed as previously described (2) with the following exceptions. Double recombinants were screened for the presence of mutant alleles of *hetR* via PCR using a pair of primers whose final one or two 3′-end nucleotides corresponded to those of either the wild-type or the mutant allele of *hetR* [hetR152wt-F (5′-GACGCTCAGTTAGTTACTT-3′) and *hetR*152A-F (5′-GACGCTCAGTTAGTTACTG-3′) for *hetR*(*S152A*) or hetR48wt-F (5′-AGCAACGGCGGCTAAGTG-3′) and hetR48A-F (5′-AGC AACGGCGGCTAAGGC-3′) for *hetR*(*C48A*)] along with hetR6H-r. The *hetR* region from isolates that tested positive for the mutant allele was amplified via PCR with two sets of primers, each containing at least one primer whose sequence was outside the region on the plasmid used to construct the corresponding strain (hetR-5′ F [5′-TTTAGATCTGTCGTTCTC AGCCACAGAGATTTGTCC-3′] and hetR6H-r or hetRcf-NdeI and hetR3′ R [5′-TTTCTGCAGATGTCTTGGCTCAGTCGCGGATGATGG-3′]), and the PCR products were sequenced to ensure the presence of only the intended mutation. The *hetR* region from these isolates was then amplified with primers corresponding to sequences inside the *hetR* coding frame (hetRD17E-F and hetRE254D-R [5′-GCACATCGAGATCTTCTAAGGCT CGCATAGCG-3′]) and sequenced to ensure that another copy of *hetR* was not present in the chromosome. Finally, Southern blot analysis was performed on chromosomal DNA from strains PCC 7120, R48, R152, and UHM103 (a Δ*hetR* strain), and the DNA was digested with SpeI or HindII and probed with the entire *hetR* coding region.

Western blot analysis. One hundred-milliliter cultures of the appropriate strain were concentrated and resuspended in 1 ml of lysis buffer (10 mM Tris, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, pH 7.5, containing 1 mg/ml lysozyme) at 4°C and lysed via sonication. The lysate was cleared by centrifugation at 10,000 × *g* for 30 min, and the protein concentration was determined using the DC protein assay (Bio-Rad Laboratories). Lysate containing 50 μg of protein was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretic transfer onto polyvinylidene difluoride membrane. HetR was detected with Penta-His antibodies (QIAGEN) and chemiluminescent detection (Western Breeze; Invitrogen). Coomassie staining of the membrane was performed using Simply Blue safe stain (Invitrogen). Quantification of Western blot signals was done using Scion Image (Scion Corp.).

Overexpression and purification of recombinant HetR. Recombinant HetR from *E. coli* BL21(DE3) transformed with the appropriate pET21a derivative was obtained as previously described (20) with the exceptions that β-mercaptoethanol was used in place of dithiothreitol (DTT) and inclusion bodies were refolded in a solution containing 20 mM Tris base, 300 mM NaCl, 0.1 mM EGTA, and 20% glycerol, pH 8.0, at 4°C (TSEG), incubated several days at 4°C, and then purified using Ni-nitrilotriacetic acid affinity chromatography (QIAGEN). The column was washed three times with 1 column volume of TSEG containing 20 mM imidazole, and recombinant proteins were eluted in TSEG containing 250 mM imidazole.

DNA binding assays. Electrophoretic mobility shift assays were performed using the Light Shift chemiluminescent electrophoretic mobility shift assay kit

TABLE 1. Mutational analysis of *hetR*

HetR residue or strain	Amino acid substitution in $HetR_{E254G}$	Phenotype[a]	$HetR^+$ conservative substitution	Heterocyst frequency (%)		Sequence(s) of primer(s) used for overlap extension (5′–3′)[b]
				In BG-11	In BG-11_0	
D17	E	N	E	0	0	CAGTGCAATGGAACAGATCATGCTATATCTGGC/GCATGATCTGTTCCATTGCACTGGGGCCAAG
D17			N	0	0	CAGTGCAATGAACCAGATCATGCTATATCTGGC/GCATGATCTGGTTCATTGCACTGGGGCCAAG
I19	N	R	L	21.8	22.4	CAATGGATCAGCTAATGCTATATCTGGCTTTTAG/GATATAGCATTAGCTGATCCATTGCACTGGG
R29	G	R	K	8.8	11.2	CTTTTAGCGCCATGAAAACGAGCGGGCATAGGCATG/GCCCGCTCGTTTTCATGGCGCTAAAAGCCAG
H33	R	R	Y	19	34	GACGAGCGGGTATAGGCATGGAGCATTCTTAG/CTCCATGCCTATACCCGCTCGTCCTCATGG
G36	E	N	A	0	0	GCATAGGCATGCAGCATTCTTAGATGCAGCAGC/CTAAGAATGCTGCATGCCTATGCCCGCTCGTC
F38	C	N	W	11.8	20.8	GCATGGAGCATGGTTAGATGCAGCAGCAACG/CTGCATCTAACCATGCTCCATGCCTATGCC
L39	V	R	V	0	<1%	CATGGAGCATTCGTAGATGCAGCAGCAACGGCG/CTGCTGCATCTACGAATGCTCCATGCCTATGC
A41	V	R	G	3.2	3.8	CATTCTTAGATGGAGCAGCAACGGCGGCTAAGTG/CCGTTGCTGCTCCATCTAAGAATGCTCCATGC
A42	V	R	G	0.6	11.6	CTTAGATGCAGGAGCAACGGCGGCTAAGTGTGC/CCGCCGTTGCTCCTGCATCTAAGAATGCTCCATG
A43	V	N	G	5.4	4.8	CTTAGATGCAGCAGGAACGGCGGCTAAGTGTGCC/CTTAGCCGCCGTTCCTGCTGCATCTAAGAATGCTC
T44	M	R	S	17.4	39.8	GATGCAGCAGCATCGGCGGCTAAGTGTGCCATTTAC/CACTTAGCCGCCGATGCTGCTGCATCTAAGAATG
A45	V	N	G	6.2	12.8	CAGCAGCAACGGGAGCTAAGTGTGCCATTTACATG/CACACTTAGCTCCCGTTGCTGCTGCATCTAAG
A46	V	R	V	4.6	16	CAGCAACGGCGGGAAAGTGTGCCATTTACATGAC/GTAAATGGCACACTTTCCCGCCGTTGCTGCTGCATC
L61	P	N	V	9	25.8	CAAGGACAAAACGTCCGGATGACCGGACATTTGC/CGGTCATCCGGACGTTTTGTCCTTGCTCTAGATAAG
M63	T	R	L	8.2	22.2	CAAAACCTCCGGCTGACCGGACATTTGCACCACC/CAAATGTCCGGTCAGCCGGAGGTTTTGTCCTTGCTC
L67	V	R	V	0	6.2	GACCGGACATGTGCACCACCTAGAGCCAAAACG/CTAGGTGGTGCACATGTCCGGTCATCCGGAG
H69	R	N	Y	0	<0.1%	GACATTTGCACTATCTAGAGCCAAAACGGGTC/GTTTTGGCTCTAGATAGTGCAAATGTCCGGTCATCC
P72	L	R	S	5.2	13.4	CCACCTAGAGAGCAAACGGGTCAAAATCATTGTAG/GATTTTGACCCGTTTGCTCTCTAGGTGGTGCAAATGTC
V79	E	R	L	0.6	21.4	GTCAAAATCATTTTAGAGGAAGTCAGACAAGCCCTG/CTGACTTCCTCTAAAATGATTTTGACCCGTTTTG
V82	A	R	L	12.2	24.4	CATTGTAGAGGAATTAAGACAAGCCCTGATGGAGG/GGGCTTGTCTTAATTCCTCTACAATGATTTTGAC
A85	T	R	G	9.2	24.8	GAAGTCAGACAAGGACTGATGGAGGGCAAACTGC/CCTCCATCAGTCCTTGTCTGACTTCCTCTACAATG
K90	E	R	R	11.2	26	GATGGAGGGCAGACTGCTCAAGACCTTAGGTTC/GTCTTGAGCAGTCTGCCCTCCATCAGGGCTTGTC
W110	R	N	Y	15.4	38	GTTTCCCTATGTCTATATGGAACAGTACCCTTGG/GTACTGTTCCATATAGACATAGGGAAACTGAATC
I159	N	R	L	7	14.6	GTTTTTGGAGTTGTTAGAATTTCTCCATAAGCGATC/GGAGAAATTCTAACAACTCCAAAAACTCAAAGG
L178	F	R	V	8	23.8	CATCGCATGGAGGTGAGCGAAGCCCTGGCAGAGC/GGGCTTCGCTCACCTCCATGCGATGTTCTGG
A181	V	N	G	1.6	16.2	GTTAAGCGAAGGACTGGCAGAGCATATCAAGC/GCTCTGCCAGTCCTTCGCTTAACTCCATGCGATG
R189	L	R	K	0.2	12.6	CATATCAAGCGTAAGCTGCTTTACTCTGGCACGGTG/GAGTAAAGCAGCTTACGCTTGATATGCTCTGCCAG
L190	P	N	V	19.4	44.6	CAAGCGTCGTGTGCTTTACTCTGGCACGGTGAC/CAGAGTAAAGCACACGACGCTTGATATGCTCTG
V232	T	N	G	6.6	16.6	GGAAGATACCGGACGGTATTTCCGCATGATGAAAG/GGAAATACCGTCCGGTATCTTCCACCATAATATAAG
W241	R	N	Y	22.2	45.4	GATGAAAGACTATGCAGAAAAGCGGCCAAACG/GCTTTTCTGCATAGTCTTTCATCATGCGGAAATAC
V257	A	R	L	23	50	GAAGAACTCGATTTACCACCAGAGCGCTGGGATG/GCTCTGGTGGTAAATCGAGTTCTTCTAAGGCTC
A265	D	N	G	11.2	25.6	GCTCTGGTGGTAAATCGAGTTCTTCTAAGGCTC/GTTCCTGCATTCCTTCATCCCAGCGCTCTGGTG
G295	D	R	A	25.2	35.2	ATATAGAGCTCTTAATCTTCTTTTCTGGCAAACACCATTTGTAAAATCATG
Wild type				14.4	26.2	
S179			N	0	0	

[a] The phenotype of strain UHM103 carrying a version of $HetR_{E254G}$ with the indicated substitution. N, no heterocysts; R, less than 2% of cells differentiated into heterocysts.
[b] Slashes indicate the end of the sequence of the first primer in a pair and the beginning of the sequence of the second primer.

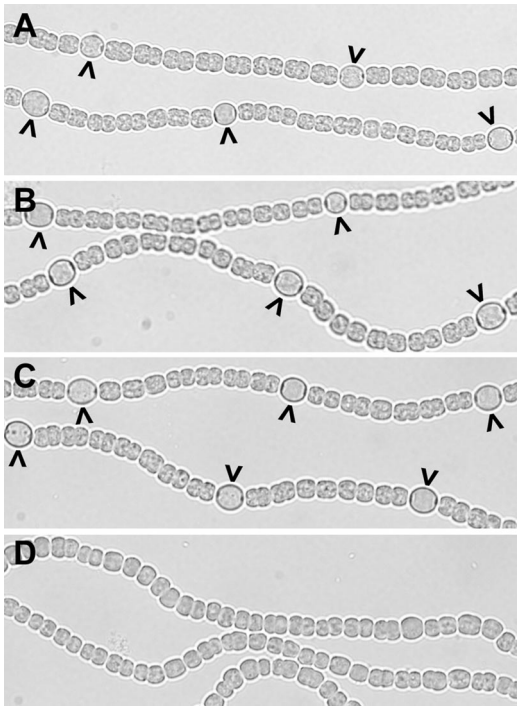

FIG. 1. Phenotypes of strains carrying amino acid substitutions in HetR 48 h postinduction. Images correspond to *Anabaena* sp. strains PCC 7120 (A), R48 (B), R152 (C), and 216 (D). Carets indicate heterocysts.

(Pierce) and NuPAGE 6% DNA retardation gels (Invitrogen). The F_{hetR}-1 region of the *hetR* promoter was amplified via PCR as previously described with the primers FhetR-1-F (biotin-5′-CAGATAAGTTCCGGATAATAGGG-3′) and FhetR-1-R (5-5TATAACTAGCAACAGTGTTGTTA-3′) (11). Ten-microliter binding reaction mixtures containing 20 fmol F_{hetR}-1 or Epstein-Barr virus biotinylated DNA, 15% glycerol, 0.5 μg poly(dI-dC), 1× binding buffer (Pierce), and recombinant HetR or Epstein-Barr virus nuclear extract as indicated in Fig. 5 were incubated for 20 min at room temperature prior to electrophoresis.

RESULTS

S152 and C48 of HetR are not required for heterocyst differentiation. To search for alleles of *hetR* resulting in a Het$^-$ phenotype, error-prone PCR was used to generate a library of the *hetR* promoter and coding regions containing random point mutations. This library was cloned into a shuttle vector and introduced into a *hetR* deletion strain, UHM103 (2). Exconjugants were selected and cultured in BG-11, then transferred into BG-11_0. Colonies unable to grow without combined nitrogen turned brown and were rescued to BG-11. One hundred five Het$^-$ isolates were identified. As controls, plasmids containing wild-type *hetR* (pDR138), *hetR*(*C48A*) (pDR144), *hetR*(*S152A*) (pDR137), and *hetR*(*S179N*) (pDR145) were also introduced into the Δ*hetR* strain UHM103. *hetR*(*C48A*), *hetR*(*S152A*), and *hetR*(*S179N*) have previously been reported to be unable to cause differentiation when introduced on a plasmid or into the chromosome of a *hetR* mutant strain (3, 9, 11). Surprisingly, two of the alleles used as negative controls for complementation did complement the mutant. *hetR*(*S152A*) and *hetR*(*C48A*) on a plasmid complemented UHM103 in BG-11_0, resulting in heterocyst frequencies comparable to that conferred by wild-type *hetR*. Only *hetR*(*S179N*) was found not to complement the Δ*hetR* strain. To determine if complementation of UHM103 by *hetR*(*C48A*) and *hetR*(*S152A*) was a result of extra copies of the genes on a plasmid and represented a limitation of our screen, *hetR*(*C48A*) and *hetR*(*S152A*) unmarked substitutions were created in the chromosome of the wild-type strain, PCC 7120. The resultant strains, R48 and R152, had double and single base pair substitutions in the chromosome, respectively, that replaced the cysteine or serine with an alanine residue in HetR. Each strain had a phenotype similar to that of the wild-type strain, PCC 7120 (Fig. 1A to C), with heterocyst frequencies of 8.6% (R48), 8.6% (R152), and 9.5% (PCC 7120). Strain 216 (3), which carries *hetR*(*S179N*) in the chromosome, was incapable of heterocyst formation (Fig. 1D). The reason for the discrepancy between our results and those of previous studies (9, 11) is unclear, but it appears that neither cysteine-48 nor serine-152 is essential for HetR function.

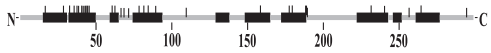

FIG. 2. Schematic diagram of HetR depicting mutation sites and secondary structure predictions. Mutations (thin, vertical black lines), α-helices (thick, horizontal black lines), and random coils and extended strands (gray lines) are represented.

Isolation of *hetR*(*E254G*) and positive selection for Het$^-$ alleles of *hetR*. Because the mutation rate generated by the error-prone PCR method was higher than expected (approximately 5/kb), alleles containing single mutations were subcloned or recreated using overlap extension PCR to identify those responsible for the Het$^-$ phenotype. One recreated allele of hetR with a glutamate-to-glycine substitution at residue 254 could not be introduced into the Δ*hetR* strain presumably because cells that receive the allele differentiate into heterocysts, which do not divide and so are not recovered. This mutation was initially isolated with another mutation resulting in an A41V substitution that was found to reduce heterocyst formation and was likely responsible for our ability to originally isolate the E254G mutation. A more detailed analysis of the *hetR*(*E254G*) mutant will be published elsewhere.

Because *hetR*(*E254G*) resulted in essentially lethal differentiation when introduced on a plasmid even in BG-11, it could be used in positive selection for mutations in *hetR* resulting in reduced HetR function. The plasmid containing P_{hetR}-*hetR*(*E254G*) (pDR203) was introduced into XL1-RED, a hypermutator strain of *E. coli*, in order to introduce random mutations into the *hetR* promoter and coding frame. Plasmid was isolated from this strain and introduced into the Δ*hetR* strain UHM103. Neo^r exconjugants that arose on BG-11 medium represented isolates that had reduced or no heterocyst formation due to a mutation within pDR203 or a second site mutation within the genome. To confirm that the mutation responsible for the observed phenotype was carried on pDR203, plasmid was isolated from these strains and reintroduced into UHM103. Colonies of all mutants turned brown when transferred to BG-11_0 and showed no discernible growth when streaked onto solid BG-11_0. Sequencing revealed 33 unique amino acid substitutions that conferred a Het$^-$ or reduced heterocyst phenotype compared to pDR203 (Table 1). Of the 33 substitutions, 19 reduced the percentage of hetero-

cysts to below 2% of cells (Table 1) and 14 resulted in no heterocyst formation (Table 1). Over 30% of the mutations occurred in one predicted α-helix near the N terminus of HetR, located at residues H33 to I50, which comprises only 6% of the protein (7) (Fig. 2). All of the sequenced mutant alleles of *hetR* differed from the wild-type allele by the substitution, deletion, or insertion of a single base pair.

Conservative substitution of amino acids identified by the *hetR*(*E254G*) screen. Although 33 unique amino acid substitutions were identified that alter the function of $HetR_{E254G}$, it was unclear whether these were due to the replacement of a residue required for a specific function of HetR, such as DNA binding, or were simply the result of disrupted protein structure induced by a nonconservative substitution. To determine which residues were truly essential to HetR function, conservative substitutions were created in an otherwise wild-type copy of *hetR* at each of the 33 residues identified in the previous screen and introduced on a plasmid into the Δ*hetR* strain UHM103 (Table 1). While most of the conservative substitutions resulted in a lower frequency of heterocysts compared to wild-type *hetR*, two substitutions, D17E and G36A, resulted in alleles of *hetR* completely incapable of promoting heterocyst differentiation, and an H69Y substitution resulted in the differentiation of less than 0.1% of cells. Because glutamate has been reported to mimic the phosphorylated state of aspartate residues in some proteins (13), a D17N substitution was also tested to see if it would increase the rate of differentiation but was found to be incapable of promoting differentiation as well.

Dominant-recessive testing of Het$^-$ alleles of *hetR*. Extra copies of *hetR*(*S179N*) on a plasmid have been reported to suppress heterocyst formation in a wild-type background (11). To determine if *hetR*(*D17E*), *hetR*(*G36A*), or *hetR*(*H69Y*) has a similar effect, plasmids containing the *hetR* promoter and the respective alleles were introduced into PCC 7120 along with 17 nonsense and frameshift mutant alleles isolated from the XL1-Red library to test for dominance. PCC 7120 containing *hetR*(*H69Y*) on a plasmid had levels of differentiation similar to those in the negative control, PCC 7120 containing a plasmid bearing a fusion of P_{hetR} to the gene encoding the green fluorescent protein (P_{hetR}-*gfp*), under all conditions, and so *hetR*(*H69Y*) appears to have no effect on the level of differentiation (data not shown). Twenty-four hours postinduction with BG-11_0, PCC 7120 containing *hetR*(*S179N*), *hetR*(*G36A*), and *hetR*(*883+T*) [*hetR*(*E254G*) with a thymine insertion at nucleotide +883 with respect to the start codon, resulting in a G295W substitution, followed by the introduction of a stop codon] had reduced heterocyst frequency (<0.5%) compared to PCC 7120 containing a plasmid bearing P_{hetR}-*gfp* (pSMC127) (8.3%) (6) (Fig. 3A). By 120 h postinduction, the strain with *hetR*(*S179N*) had reached a heterocyst frequency (8.67%) similar to that in the P_{hetR}-*gfp* control (8.83%) while strains containing *hetR*(*G36A*) and *hetR*(*883+T*) continued to show reduced heterocyst frequencies (3.83% and 2%, respectively). Surprisingly, compared to PCC 7120 containing P_{hetR}-*gfp* (Fig. 3A and B), PCC 7120 containing *het*(*RD17E*) (Fig. 3A and C) exhibited excessive differentiation and perturbed heterocyst patterning, including both multiple contiguous heterocysts and irregularly long vegetative cell intervals in some parts of filaments. Excessive heterocyst differentiation caused by extra copies of *hetR*(*D17E*) in PCC 7120 was not as severe as the effects of extra copies of wild-type *hetR* (Fig. 3A).

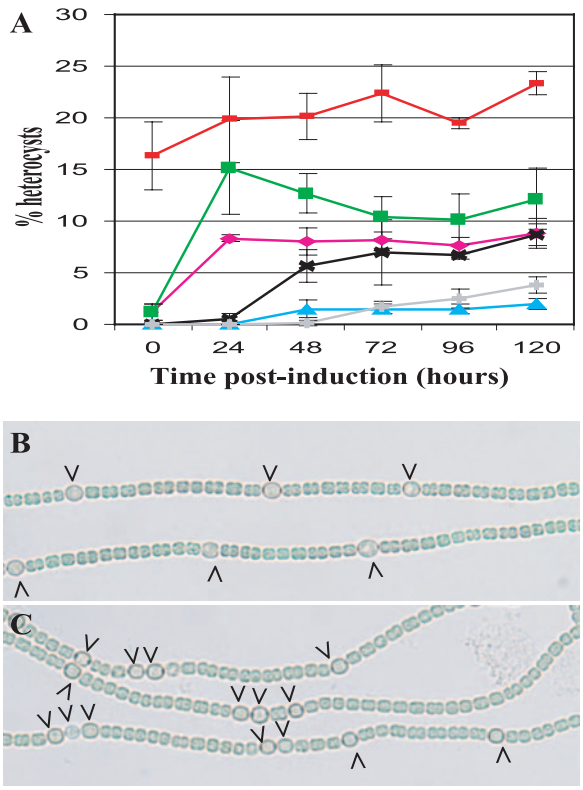

FIG. 3. Heterocyst frequency versus time postinduction and the phenotype of PCC 7120 with extra copies of *hetR*(*D17E*). (A) Heterocyst frequency over time for PCC 7120 carrying extra copies of *hetR* alleles on a plasmid. P_{hetR}-*gfp*, diamonds; *hetR*, rectangles; *hetR*(*S179N*), x's; *hetR*(*833+T*), plus signs; *hetR*(*G36A*), triangles; *hetR*(*D17E*), squares. (B) PCC 7120 carrying P_{hetR}-*gfp* 48 h postinduction. Carets indicate heterocysts. (C) PCC 7120 carrying extra copies of *hetR*(*D17E*) on a plasmid 48 h postinduction.

HetR turnover is affected by D17E, G36A, and H69Y substitutions. HetR has been reported to be a serine protease capable of autodegradation, and a strain with an S179N substitution, which prevents protease activity and differentiation, has been shown to have higher levels of HetR protein than the wild-type strain (9, 21). To determine if *hetR*(*D17E*), *hetR*(*G36A*), or *hetR*(*H69Y*) also results in higher HetR protein levels, these alleles were ectopically overexpressed in PCC 7120 from the P_{nir} promoter to bypass any effects the mutations may have on transcription, and the level of HetR was assessed by Western blot analysis. Plasmids containing *hetR*, *hetR*(*S179N*), *hetR*(*S152A*), *hetR*(*C48A*), *hetR*(*D17E*), *hetR*(*G36A*), and *hetR*(*H69Y*) under the control of P_{nir} were introduced into the Δ*hetR* strain, UHM103. When transcription from P_{nir} was induced by the removal of ammonium and the addition of nitrate, the level of HetR in UHM103 containing *hetR*(*D17E*), *hetR*(*G36A*), *hetR*(*H69Y*), and *hetR*(*S179N*) was substantially higher than that in the same strain containing wild-type *hetR*, *hetR*(*C48A*), or *hetR*(*S152A*) (Fig. 4A and B). This suggests that $HetR_{D17E}$, $HetR_{G36A}$, and $HetR_{H69Y}$ have reduced turnover rates like $HetR_{S179N}$. The fact that UHM103 containing *hetR*(*S152A*) had levels of HetR protein similar to those of UHM103 containing

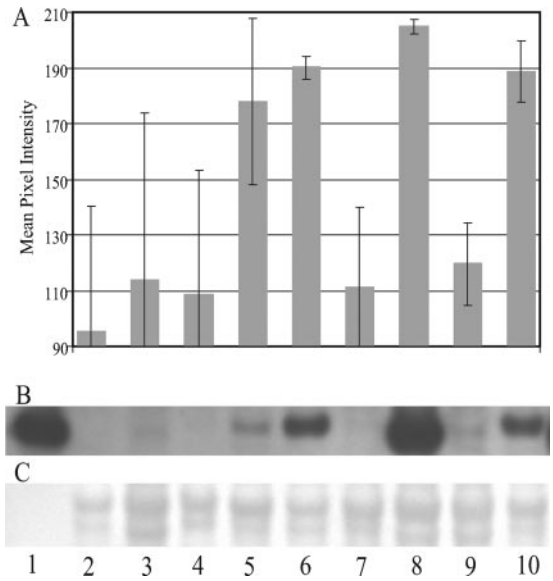

FIG. 4. Characterization of HetR levels corresponding to different alleles. (A) Mean pixel densities in Western blot analysis of various *hetR* alleles in UHM103. (B) Representative results from Western blot analysis of HetR from different alleles in UHM103. (C) Coomassie-stained section of the membrane showing loading with equal amounts of protein. Lanes 1, 2, and 4 are controls containing recombinant HetR from *E. coli* with the addition of six histidine residues to the C terminus, protein from strain UHM103 carrying pSMC188, and wild-type HetR from UHM103 without the six-His tag, respectively. Remaining lanes contain variations of HetR from UHM103 with a six-His tag at the carboxy terminus: HetR (lane 3), $HetR_{D17E}$ (lane 5), $HetR_{G36A}$ (lane 6), $HetR_{C48A}$ (lane 7), $HetR_{H69Y}$ (lane 8), $HetR_{S152A}$ (lane 9), and $HetR_{S179N}$ (lane 10).

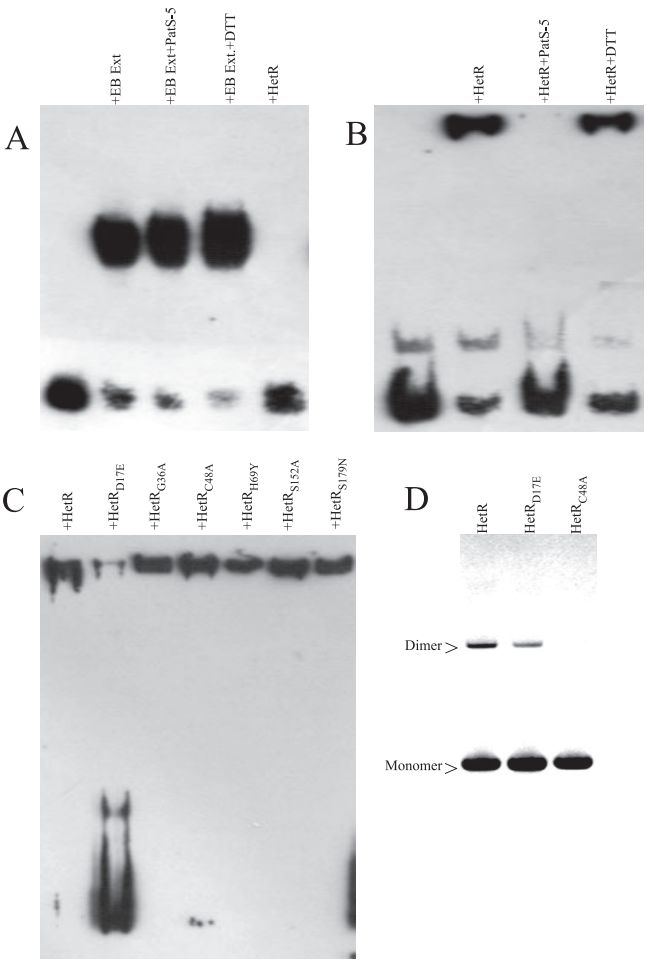

FIG. 5. Results from mobility shift assays of recombinant HetR and SDS-PAGE analysis. Biotinylated Epstein-Barr virus DNA (EB Ext, Epstein-Barr virus nuclear extract) (A) or F_{hetR}-1 (B) was incubated with additional factors as indicated prior to electrophoresis, demonstrating the specificity of HetR DNA binding and PatS-5 inhibition (HetR, PatS-5, and DTT were present at concentrations of 75 μM, 375 μM, and 50 mM, respectively). (C) F_{hetR}-1 was incubated with the indicated variants of recombinant HetR at a concentration of 300 μM. (D) Result from nonreducing SDS-PAGE analysis of recombinant HetR, $HetR_{D17E}$, and $HetR_{C48A}$. Positions of the monomer and dimer forms of HetR are indicated.

wild-type HetR suggests that the S152A substitution does not affect protein turnover. When *hetR* and *hetR*(*S179N*) were ectopically overexpressed from the P_{petE} promoter in BG-11_0, similar results were obtained, indicating that the presence of nitrate did not affect the assay (data not shown). *hetR*, *hetR*(*C48A*), and *hetR*(*S152A*) were capable of inducing heterocyst formation when overexpressed, while *hetR*(*D17E*), *hetR*(*G36A*), *hetR*(*H69Y*), and *hetR*(*S179N*) were not.

D17 but not C48 is necessary for DNA binding activity of HetR. It was previously reported that HetR binds to DNA from its own promoter region as well as that of the *hepA* and *patS* promoter regions and that under reducing conditions DNA binding and dimer formation by HetR are prevented (11). A C48A substitution and PatS-5 have also been reported to prevent DNA binding (11). To determine if any of the mutant alleles for HetR isolated in this study were deficient in DNA binding activity, recombinant HetR was overexpressed, purified from *E. coli*, and tested for DNA binding activity. Control mobility shift assays showed that wild-type HetR was capable of binding specifically to the previously defined F_{hetR}-1 region of DNA and was inhibited by a fivefold excess of PatS-5, consistent with previous findings (11), and that neither PatS-5 nor DTT affected the DNA binding in a control reaction containing Epstein-Barr virus DNA and Epstein-Barr virus nuclear extract (Fig. 5A and B). $HetR_{D17E}$ had decreased binding activity, shifting only a small portion of DNA at a protein concentration sufficient to completely shift all the biotinylated F_{hetR}-1 DNA for HetR, $HetR_{G36A}$, $HetR_{H69Y}$, $HetR_{S179N}$, and surprisingly, $HetR_{C48A}$ (Fig. 5C). At lower protein concentrations, no shift was detected only for $HetR_{D17E}$ (data not shown). Contrary to previous findings (11), 50 mM DTT did not abolish DNA binding activity. Dimer formation could be detected for wild-type HetR, $HetR_{D17E}$, $HetR_{G36A}$, and $HetR_{H69Y}$ but not for $HetR_{C48A}$ (Fig. 5D and data not shown).

DISCUSSION

Prior to this study, three amino acid residues had been reported to be important for HetR function. An S179N substitution mutant was initially isolated by Buikema and Haselkorn to implicate *hetR* in heterocyst differentiation and show that the substitution prevents differentiation (3). Subsequently, it was proposed that HetR is a protease that can degrade itself,

that the S179N substitution prevents proteolysis in vitro, and that protein levels in a strain carrying the mutation are higher than those in the wild type (20, 21). Our results are consistent with these previous findings.

The active-site serine of HetR was later reported to be S152, and a *hetR* mutant strain, 884a, carrying the mutant allele on a plasmid was reported to be incapable of heterocyst differentiation (9). Strain 884a has a chromosomal copy of *hetR* with DNA corresponding to amino acid residues 35 to 168 of HetR replaced by DNA containing *luxAB* reporter genes, *oriT* and *oriV* from plasmid pMB1, and a gene encoding resistance to neomycin (1, 9). A strain with *hetR*(*S152A*) introduced into the chromosome of strain 884a by single recombination with a plasmid bearing the mutant allele was also reported to be incapable of differentiation (9). As described previously, the strain, designated S152A, should contain two copies of the *hetR* promoter, one promoting transcription of the mutant allele, followed by the pET3a vector and an Sm^r-Sp^r cassette, and the other promoting a fragment of *hetR* from residues 1 to 35, followed by *luxA* and *luxB*, the pMB1 *oriV* and *oriT*, a neomycin resistance cassette, and residues 168 to 299 of *hetR*. $HetR_{S152A}$ protein was also reported to lack autodegradation in vitro, and levels of the mutant protein were higher in strain S152A than were levels of the wild-type protein in the wild-type strain. Our results are inconsistent with most of these previous findings. The introduction of a *hetR* mutant producing an alanine residue at position 152 [*hetR*(*S152A*)] on a plasmid promoted heterocyst differentiation like the wild-type allele in strain UHM103, which has the *hetR* coding and promoter regions cleanly deleted from the chromosome. In addition, the replacement of a single nucleotide in the chromosome of the wild-type strain to give an S152A substitution had no discernible effect on heterocyst differentiation or patterning. In addition, HetR protein levels in a strain expressing *hetR*(*S152A*) from an inducible promoter in a Δ*hetR* background were similar to those in a strain expressing the wild-type allele under the same conditions. The simplest explanation for the discrepancy in the two sets of results is the difference in the strains that were used to express the mutant alleles and the methods for creating the chromosomal mutations.

HetR has DNA binding activity, and residue C48 has been reported to be necessary for this activity (11). It was also reported that incubation with a reducing agent prevents dimer formation by wild-type HetR in vitro and that the recreation of *hetR*(*C48A*) in the chromosome of the *hetR* mutant strain 884a did not restore heterocyst differentiation. Here we have confirmed that a reducing agent and a C48A substitution prevent the formation of a covalent linkage between two HetR monomers in vitro, suggesting the formation of a disulfide bond by the wild-type protein. However, in our hands, $HetR_{C48A}$ exhibited DNA binding activity, *hetR*(*C48A*) on a plasmid complemented a Δ*hetR* mutant, and the replacement of 2 bp in the chromosome of PCC 7120 to generate a C48A substitution did not affect heterocyst differentiation or patterning. Although differences in mutant construction between the work presented here and the previous work, which relied on strain 884a as well, could explain the different phenotypes of the mutant *Anabaena* strains, the difference in DNA binding results is enigmatic. Regardless of the cause of the discrepancy between our findings and previous results, it is clear that C48 and S152 are not necessary for HetR function in vivo.

In this work, genetic selection was used to identify 33 amino acid substitutions in HetR that affect heterocyst differentiation. Conservative substitutions at each site revealed that D17, G36, and H69 have important roles in HetR activity. Recombinant protein with a D17E substitution had substantially less DNA binding activity. Conservative substitutions at all three positions gave protein levels higher than those of wild-type HetR, suggesting that all three positions are involved in protein turnover in PCC 7120, but we believe that the effect at D17 may be indirect. We have found that the absence of *patA* leads to elevated levels of HetR protein in vivo (D. D. Risser and S. M. Callahan, unpublished data) and that *hetR* is required for transcriptional upregulation of *patA* (S. S. Young and S. M. Callahan, unpublished data). Because $HetR_{D17E}$ lacks DNA binding activity, the elevated level of HetR in vivo may be due to decreased levels of PatA instead of a direct effect of the D17E substitution on HetR turnover. We are currently investigating this possibility.

Both $HetR_{D17E}$ and $HetR_{G36A}$ were found to form dimers in vitro. Huang and coworkers proposed that the suppression of heterocyst formation by extra copies of *hetR*(*S179N*) in a wild-type background resulted from dimer poisoning, rendering functional molecules of HetR inert by coupling them to an excess of nonfunctional $HetR_{S179N}$ (11). This model could also be applied to the suppression of differentiation when extra copies of *hetR*(*G36A*) were present in a wild-type background but does not explain the excessive differentiation resulting from extra copies of *hetR*(*D17E*). It is possible that a heterologous dimer between HetR and $HetR_{D17E}$ may be functional while a $HetR_{D17E}$ homodimer is not. Thus, extra copies of *hetR*(*D17E*) in a wild-type background would increase the number of functional HetR dimers. An alternative hypothesis is that $HetR_{D17E}$, which lacks DNA binding activity, may be functional in one or more other functions and that the addition of *hetR*(*D17E*) on a plasmid increased the activity of this function to promote differentiation.

Elevated levels of $HetR_{D17E}$, $HetR_{G36A}$, and $HetR_{H69Y}$ relative to that of the wild-type HetR extend the correlation between proper protein turnover and heterocyst differentiation, which was first demonstrated with $HetR_{S179N}$ (20). Unfortunately, we were unable to demonstrate protease activity resulting in autodegradation in vitro for any of the recombinant HetR proteins, including the wild-type version (data not shown) and, therefore, could not extend the correlation to HetR-protease activity. The turnover of HetR could be required to produce the active form of HetR from an apoprotein in developing cells. In this case, the degradation of HetR would be required for differentiation. Conversely, the protease activity of HetR could both limit the number of cells that differentiate by regulating the amount of HetR and also be required for the degradation of another protein, such as CcbP or an unidentified activator of differentiation, to promote differentiation, thus coupling the observance of low HetR protein levels and the ability to promote differentiation. Alternatively, both activities may be necessary for differentiation. From our results, it is not possible to tell if $HetR_{G36A}$, $HetR_{H69Y}$, and $HetR_{S179N}$ have lost protease activity or have lost the ability to be degraded. Separation of protease activity from HetR pro-

tein turnover or separation of turnover or protease activity from differentiation by mutation would help resolve questions regarding the regulation of differentiation by HetR.

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REFERENCES

1. **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.
2. **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.
3. **Buikema, W. J., and R. Haselkorn.** 1991. Characterization of a gene controlling heterocyst development in the cyanobacterium *Anabaena* 7120. Genes Dev. **5:**321–330.
4. **Buikema, W. J., and R. Haselkorn.** 2001. Expression of the *Anabaena hetR* gene from a copper-regulated promoter leads to heterocyst differentiation under repressing conditions. Proc. Natl. Acad. Sci. USA **98:**2729–2734.
5. **Cadwell, R. C., and G. F. Joyce.** 1992. Randomization of genes by PCR mutagenesis. PCR Methods Appl. **2:**28–33.
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. **Combet, C., C. Blanchet, C. Geourjon, and G. Deleage.** 2000. NPS@: network protein sequence analysis. Trends Biochem. Sci. **25:**147–150.
8. **Desplancq, D., C. Bernard, A. Sibler, B. Kieffer, M. Laurent, N. Potier, A. Van Dorsselaer, and E. Weiss.** 2005. Combining inducible protein overexpression with NMR-grade triple isotope labeling in the cyanobacterium *Anabaena* sp. PCC 7120. BioTechniques **39:**405–411.
9. **Dong, Y., X. Huang, X.-Y. Wu, and J. Zhao.** 2000. Identification of the active site of HetR protease and its requirement for heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **182:**1575–1579.
10. **Higuchi, R., B. Krummel, and R. K. Saiki.** 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.
11. **Huang, X., Y. Dong, and J. Zhao.** 2004. HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS. Proc. Natl. Acad. Sci. USA **101:**4848–4853.
12. **Khudyakov, I. Y., and J. W. Golden.** 2004. Different functions of HetR, a master regulator of heterocyst differentiation in *Anabaena* sp. PCC 7120, can be separated by mutation. Proc. Natl. Acad. Sci. USA **101:**16040–16045.
13. **Klose, K. E., D. S. Weiss, and S. Kutsu.** 1993. Glutamate at the site of phosphorylation of nitrogen-regulatory protein NTRC mimics aspartyl-phosphate and activates the protein. J. Mol. Biol. **232:**67–78.
14. **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.
15. Reference deleted.
16. **Shi, Y., W. Zhao, Y. Zi, and J. Zhao.** 2006. Regulation of intracellular free calcium concentration during heterocyst differentiation by HetR and NrcA in *Anabaena* sp. PCC 7120. Proc. Natl. Acad. Sci. USA **103:**11334–11339.
17. **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.
18. **Wolk, C. P., A. Ernst, and J. Elhai.** 1994. Heterocyst metabolism and development, p. 769–823. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria, vol. 1. Kluwer Academic Publishers, Dordrecht, The Netherlands.
19. **Zhang, C.-C., S. Laurent, S. Sakr, L. Peng, and S. Bedu.** 2005. Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. Mol. Microbiol. **59:**367–375.
20. **Zhou, R., Z. Cao, and J. Zhao.** 1998. Characterization of HetR protein turnover in *Anabaena* sp. PCC 7120. Arch. Microbiol. **169:**417–423.
21. **Zhou, R., X. Wei, N. Jiang, H. Li, Y. Dong, K.-L. Hsi, and J. Zhao.** 1998. Evidence that HetR is an unusual serine-type protease. Proc. Natl. Acad. Sci. USA **95:**4959–4963.