The heterocyst differentiation transcriptional regulator HetR of the filamentous cyanobacterium *Anabaena* forms tetramers and can be regulated by phosphorylation

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Running Title: *Anabaena* HetR tetramers and phosphorylation

Key words: *Anabaena*; Heterocyst differentiation; Post-translational modification

16 **Summary**

17 Many filamentous cyanobacteria respond to the external cue of nitrogen scarcity by the
18 differentiation of heterocysts, cells specialized in the fixation of atmospheric nitrogen in
19 oxic environments. Heterocysts follow a spatial pattern along the filament of two
20 heterocysts separated by ca. 10-15 vegetative cells performing oxygenic
21 photosynthesis. HetR is a transcriptional regulator that directs heterocyst
22 differentiation. In the model strain *Anabaena* sp. PCC 7120, the HetR protein was
23 observed in various oligomeric forms in vivo, including a tetramer that peaked with
24 maximal *hetR* expression during differentiation. Tetramers were not detected in a *hetR*
25 point mutant incapable of differentiation, but were conspicuous in an over-
26 differentiating strain lacking the PatS inhibitor. In differentiated filaments the HetR
27 tetramer was restricted to heterocysts, being undetectable in vegetative cells. HetR co-
28 purified with RNA polymerase from *Anabaena* mainly as a tetramer. In vitro, purified
29 recombinant HetR was distributed between monomers, dimers, trimers and tetramers,
30 and it was phosphorylated when incubated with (γ-^{32}P)ATP. Phosphorylation and PatS
31 hampered the accumulation of HetR tetramers and impaired HetR binding to DNA. In
32 summary, tetrameric HetR appears to represent a functionally relevant form of HetR,
33 whose abundance in the *Anabaena* filament could be negatively regulated by
34 phosphorylation and by PatS.

35

36 **Introduction**

37 Cyanobacteria are phototrophic prokaryotes that perform oxygenic photosynthesis and
38 are responsible for an important fraction of the primary production in the Earth. Many
39 filamentous cyanobacteria can fix atmospheric nitrogen under oxic conditions and, to
40 protect the nitrogen fixation machinery from oxygen, they produce cells specialized in
41 the fixation of N_2, called heterocysts, where nitrogenase is confined (Herrero *et al*.,
42 2013; Wolk *et al*., 1994). The differentiation of a heterocyst from a vegetative cell

responds to the cue of nitrogen deprivation and depends on two transcription factors: the global regulator NtcA, which is a CRP homolog (Herrero *et al*., 2004; Picossi *et al*., 2014), and HetR, which is considered a master regulator of differentiation (Buikema and Haselkorn, 1991a) and may also control other cellular processes in non-heterocystous cyanobacteria (El-Shehawy *et al*., 2003). Recently, the crystal structure of HetR from *Fischerella* sp. strain MV11 has been solved, showing the protein as a homodimer that comprises a central DNA-binding domain with two helix-turn-helix motifs (N-terminal regions), two flap domains extending in opposite directions, and a hood domain over the central core (C-terminal region) (Kim *et al*., 2011, 2013). HetR directly or indirectly regulates transcription from many gene promoters activated during heterocyst differentiation (Herrero *et al*., 2013; Flaherty *et al*., 2014; Mitschke *et al*., 2011), and in some of them direct interaction with the promoter has been shown in vivo (Flaherty *et al*., 2014) or in vitro (e.g., Camargo *et al*., 2012; Higa and Callahan, 2010; Huang *et al*., 2004). In the model heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena*), the expression of both genes *ntcA* and *hetR* is activated in a mutually dependent manner during heterocyst differentiation (Muro-Pastor *et al*., 2002). In addition, turnover or degradation of the HetR protein appears important for proper heterocyst differentiation and patterning (Risser and Callahan, 2007, 2009).

To get insight into the mechanism of action of HetR, we have addressed the study of HetR protein complexes and of possible post-translational modification of HetR during heterocyst differentiation. We have found that HetR is present in different oligomeric forms in vivo, and that the largest one, likely a tetramer, is more abundant during heterocyst differentiation and in mature heterocysts than in vegetative cells. HetR tetramers are underrepresented in a HetR point mutant unable to differentiate heterocysts, but especially abundant in an over-differentiating mutant lacking the HetR inhibitor PatS. In vitro, HetR can be phosphorylated by ATP, and both phosphorylation and PatS impair HetR tetramer accumulation and HetR binding to DNA.

Results

Characterization of in vivo HetR complexes

In *Anabaena*, the expression of the *hetR* gene is up-regulated upon removal of combined nitrogen (Buikema and Haselkorn, 1991a). When cell extracts from *Anabaena* were used for immunoblotting with antibodies raised against the HetR protein expressed in *E. coli*, different specific protein bands were detected from filaments either grown on ammonium or grown on ammonium and incubated in the absence of combined nitrogen (Fig. 1A,B). The predominant band corresponded to a size of ca. 33 kDa (band 1), and another band corresponding to ca. 135 kDa (band 4) was also clearly observed. In addition, a faint band of ca. 100 kDa (band 3) was frequently observed. None of these bands was detected in the *hetR* mutant strain DR884a (Black *et al*., 1993) that does not express HetR, indicating that they contain HetR. Other HetR-specific bands corresponding to sizes between 63 and 75 kDa were inconsistently detected (band 2 in Fig. 1D). Finally, some bands with mobility corresponding to sizes between 35 and 48 kDa were inconsistently observed and, moreover, could be detected also in strain DR884a, indicating that they were not HetR-specific.

In a double mutant of the *patS* and *hetN* genes (strain CSL11) that produces more heterocysts than the wild type (Corrales-Guerrero *et al*., 2014), the HetR bands 1, 3 and 4 were clearly observed (Fig. 1C). Notably, in strain CSL11 the proportion of band 4 to band 1 was always higher than in the wild type, especially in the presence of ammonium where band 1 was hardly detected (see below). The theoretical molecular mass of the HetR monomer is 34.97 kDa (http://web.expasy.org/cgi-bin/compute_pi/pi_tool). Thus, band 1 could correspond to HetR in its monomeric state and band 4 to HetR tetramers (band 2 and 3 could correspond to HetR dimers and trimers, respectively). It should be pointed out that band 4 was observed only when

samples were not heated before loading into the gel, as done throughout this work. However, although HetR multimers appear to be at least partially stable through the SDS/PAGE, the predominant monomeric form could at least in part result from dissociation of HetR oligomers during the extraction and analysis procedures.

Concerning time-course of expression in the wild type, as observed in numerous gels (see e.g. Fig. 1A,B), band 4 was present in the ammonium-supplemented cultures and, upon incubation in the absence of combined nitrogen, it appeared to increase in abundance to reach its highest levels 4-6 h after nitrogen step-down, and to decrease thereafter exhibiting low levels after 24 h. Finally, when vegetative cells and heterocysts were separated from filaments grown on ammonium and incubated for 24 h in the absence of combined nitrogen, aside from band 1 that was always observed, band 4 was detected in the samples of heterocysts but not in those of vegetative cells (Fig. 1D). It should be pointed out that at 24 h the confinement of band 4 to heterocysts (ca. one in ten to fifteen cells of the differentiated filament) could explain its low level of detection at the whole filament level.

Association of HetR with RNA polymerase

In the course of experiments dealing with the purification by filtration through affinity chromatography columns of His-tagged RNAP from *Anabaena* (see Experimental procedures), we repeatedly observed that in the RNAP-enriched fractions (Fig. 2A) HetR could also be detected by immunoblotting (Fig. 2B). This association was detected using extracts from ammonium-grown filaments incubated or not in the absence of combined nitrogen. Moreover, the predominant HetR band detected would, by size, correspond to the tetramer. No HetR protein was readily retained in the affinity column when extracts from wild-type *Anabaena* were used as control (Fig. 2C,D). Thus, the retention of HetR required His-tagged RNAP. This observation is consistent

with the occurrence in vivo of the tetrameric form of HetR and suggests direct RNAP-HetR interactions preferentially involving tetrameric HetR.

In vitro phosphorylation of HetR

In search for possible post-translational modifications of HetR, we assayed whether it could be phosphorylated in vitro. For that, a form of HetR C-terminally fused to Strep-tag was expressed in *E. coli* and purified. Purified Strep-HetR was distributed between four protein bands, which by size could correspond to monomers (1), the predominant one, dimers (2), trimers (3) and tetramers (4), the least represented form (the size of the Strep-HetR monomer is approximately 37.9 kDa) (Fig. 3). In vitro phosphorylation assays were performed with (γ-^{32}P)ATP, and the reaction products were analyzed by immunoblotting and radioactivity detection. Upon incubation with (γ-^{32}P)ATP, radioactivity was detected in the three main Strep-HetR bands (Fig. 4A, upper panel). To control that the observed phosphorylation did not require the Strep-tag, purified His-HetR protein (Fig. 5A) was used in similar phosphorylation assays, again resulting in phosphorylated HetR forms, at least monomers and dimers (Fig. 5B, upper panel). It should be pointed out that the forms 3 and, especially, 4 were only faintly detected with His-HetR (see Fig. 5A and 5B lower panel; monomeric His-HetR is ca. 35.8 kDa). It is possible that the 6His addition to the C-terminus, which lies in the "hood" domain of HetR (Kim *et al*., 2011), interferes with HetR tetramer formation.

The effect of alkaline phosphatase (AP) on ^{32}P-labeled Strep-HetR protein was tested. After 1 h of incubation with (γ -^{32}P)ATP, which produced the three main Strep-HetR-labeled bands described above, treatment with AP led to removal of radioactivity from all of them (Fig 4B, upper panel). However, the three protein bands remained present in the preparations (Fig. 4B, lower panel). Note that, although not detected by immunoblotting (Fig. 4B, lower panel), some amount of form 3 should be present in the sample before the AP treatment, as detected by the incorporated radioactivity (Fig. 4B,

upper panel). Moreover, the AP treatment increased the abundance of protein in band 3 (Fig. 4B, lower panel). The faint radioactive bands showing up in the preparation after AP treatment (Fig. 4B, upper panel, open arrowheads) could correspond to labeled AP, dimer and monomer (70-80 kDa), to which the phosphate is transferred during catalysis. These results corroborate that Strep-HetR is phosphorylated by (γ-^{32}P)ATP. Moreover, the three main aggregation bands present in Strep-HetR preparations can be detected in a phosphorylated form.

Finally, the effect of the reducing agent DTT on in vitro phosphorylation of Strep-HetR was analyzed. As shown in Fig. 4C (upper panel), when present at a concentration of 1 mM, DTT inhibits Strep-HetR phosphorylation, although some phosphorylation could still be detected in the more abundant band 1.

Effect of phosphorylation on HetR oligomerization

Because in the assays shown in Fig. 4 (A and B) it appeared that phosphorylation impaired the accumulation of the larger oligomers of HetR, we addressed this point using gels with higher resolution. Strep-HetR preparations were incubated with different amounts of ATP under phosphorylation assay conditions and then resolved in large 8% gels and subjected to immunobloting with HetR antibodies (Fig. 6A). Quantification of the relative amounts of protein in the different HetR bands with regard to the control incubated in the absence of ATP showed that in this representative experiment increasing the ATP concentration led to a gradual decrease specifically in the HetR band 4 (Fig. 6B).

Binding to DNA of phosphorylated HetR

HetR has been described to bind in vitro to DNA sequences in the promoter region of the *hetP* gene involved in heterocyst differentiation (Higa and Callahan, 2010). To study a possible effect of phosphorylation on HetR binding to DNA, mobility shift

assays were performed with purified Strep-HetR protein that had been incubated or not under phosphorylation conditions and a 29-bp double stranded DNA fragment including *hetP* promoter sequences to which HetR binding has previously been described (Higa and Callahan, 2010). A retarded band that would correspond to a DNA-Strep-HetR complex was observed and its proportion with respect to total DNA increased with increasing amounts of Strep-HetR in the assay (Fig. 7A). When similar amounts of Strep-HetR that had been incubated under phosphorylation conditions and afterwards enriched in phosphorylated form were used, the proportion of retarded band was 4-10 times lower than with Strep-HetR that had not been subjected to phosphorylation (Fig. 7A). Also, when DNA-HetR complexes had been formed with non-saturating amounts of untreated Strep-HetR, the increase in the amount of retarded band due to the addition of more protein was higher with untreated than with treated protein (not shown). These results suggest that phosphorylation has a negative effect on HetR binding to the used DNA fragment. A synthetic pentapeptide recreating the C-terminal sequence of the *patS* gene product (PatS-5) has been described to inhibit in vitro binding of HetR to a DNA fragment of the *hetR* gene promoter region (Huang *et al*., 2004) and to the *hetP* upstream fragment used here (Feldmann *et al*., 2011). Fig. 7B shows that PatS-5 inhibits binding of Strep-HetR, either in its phosphorylated or unphosphorylated form, to the *hetP* promoter DNA.

Effect of PatS-5 on HetR oligomerization

To test whether PatS-5 could influence HetR oligomerization, purified Strep-HetR was incubated with PatS-5 and analyzed by immunobloting. When increasing concentrations of PatS-5 were used, the proportion of HetR oligomers, and especially of the HetR tetramer, progressively decreased (Fig. 8A, B, D, E). Immunobloting with antibodies raised against PatS-5 showed that PatS-5 co-migrates exclusively with the HetR monomer (Fig. 8C). Thus, it appears that PatS-5 binds to the HetR monomer and inhibits HetR oligomerization.

206

207 *Characterization of HetR complexes and phosphorylation in strain 216*

208 *Anabaena* mutant strain 216 expresses a version of HetR with the change $S^{179}N$ and
209 fails to produce heterocysts (Buikema and Haselkorn, 1991b). We studied which HetR
210 forms could be detected in strain 216 in comparison to wild-type *Anabaena*. Fig. 9A
211 shows that in strain 216 the proportion of HetR band 4 (which would correspond to
212 tetramers) was lower than in the wild type, whereas bands corresponding to lower
213 sizes (including bands 2 and 3, which might correspond to HetR dimers and trimers,
214 respectively) appeared more represented. Moreover, in strain 216 the total amount of
215 HetR, including band 1 (monomeric HetR), appeared to increase after 24 h of N step-
216 down, becoming higher than in the wild type. An increase in the level of monomeric
217 HetR at 24 h after N step-down in strain 216 has previously been reported (Dong *et al*.,
218 2000).

219 Purified Strep-HetR $S^{179}N$ was used for in vitro phosphorylation assays as
220 shown above for the Strep-HetR protein. Figure 9B shows that upon incubation with (γ-
221 ^{32}P)ATP, Strep-HetR $S^{179}N$ can be phosphorylated with an inhibitory effect of DTT
222 similarly to Strep-HetR (upper panels). No binding to the 29-bp DNA fragment of the
223 *hetP* promoter region could be detected with purified Strep-HetR $S^{179}N$ either in its
224 phosphorylated (Fig. 7B, lane 6) or unphosphorylated (Fig. 7B, lane 5) form.

225

226 **Discussion**

227 Early events in heterocyst differentiation in *Anabaena* require induction of the *hetR*
228 gene. HetR is dispensable for growth in the presence of combined nitrogen (Buikema
229 and Haselkorn, 1991b), although it has been detected in filaments grown with nitrate,
230 forming monomers and dimers (Huang *et al*., 2004). In the presence of combined
231 nitrogen, given the positive auto-regulation of *hetR* expression, some HetR might be
232 needed for a quick response to changes in the availability of nitrogen (Black *et al*.,

1993). Additionally, HetR could have a role as a transcriptional regulator also in the presence of combined nitrogen and, in fact, repressive effects of HetR in the presence of ammonium have already been noted (e.g., Flaherty *et al*., 2014; Camargo *et al*., 2012; López-Igual *et al*., 2010).

In this work, we have detected different HetR forms in *Anabaena*, both in filaments grown in the presence of ammonium and in those incubated in the absence of combined nitrogen. Besides the monomer, which can be favored by the analytical procedure, the predominant form detected was the tetrameric one. To the best of our knowledge, this is the first report of a HetR tetramer visualized by PAGE. In our hands, a band that would correspond to a HetR dimer is clearly observed only in samples from vegetative cells 24 h after N step-down (Fig. 1D) and in the 216 point mutant (Fig. 9A), none of which show HetR tetramers. Thus, HetR tetramers may form from HetR dimers (see structural data by Kim *et al*., 2013). Moreover, we have observed tetrameric HetR only when heating the samples before loading into the gels was avoided, indicating that they are not heat-stable. This fact could account for the differences in aggregation forms found by us with regard to previous work (e.g., Huang *et al*., 2004; Risser and Callahan, 2007). It would also be possible that the form of highest MW corresponded to HetR in complex with a different protein. However, the fact that purified HetR, both from *Anabaena* in association with RNA polymerase (Fig. 2) or after cloning in *E. coli* (Figs. 3 and 5A), is also detected forming a complex of a size consistent with a tetramer supports the occurrence of HetR tetramers in vivo.

The HetR tetramer is present in the ammonium-grown filaments and its amount responds to N step-down. In differentiated filaments, the tetramer is present in heterocysts but scarcely represented in vegetative cells (Fig. 1). Thus, regarding the level of HetR tetramers, the ammonium-grown cells differ from the vegetative cells of diazotrophic cultures. Upon N step-down, the spatiotemporal distribution of HetR tetramers resembles that of induction of the *hetR* gene, which in *Anabaena* takes place shortly after combined nitrogen deprivation, preferentially in differentiating cells (Black

261 *et al*., 1993; Rajagopalan and Callahan, 2010; Corrales-Guerrero *et al*., 2015).
262 Moreover, in strain 216, which is unable to differentiate heterocysts, the formation of
263 HetR tetramers is impaired (Fig. 9). Thus, in the cells differentiating into heterocysts,
264 activation of the expression of *hetR* might be accompanied by substantial formation of
265 HetR tetramers, suggesting that the tetrameric one could be a form of HetR relevant for
266 HetR activity during heterocyst differentiation. Strain CSL11 lacks the heterocyst
267 differentiation inhibitor PatS (as well as HetN) (Corrales-Guerrero *et al*., 2014) that
268 interacts with HetR (see Feldmann *et al*., 2011) and produces more heterocysts than
269 the wild type. In the wild type grown in the presence of ammonium, PatS is present in
270 the only cell type of the filament, where it influences the HetR activity (Corrales-
271 Guerrero *et al*., 2015). In contrast, after N step-down PatS is restricted to the non-
272 differentiating cells (Corrales-Guerrero *et al*., 2013). The higher propensity of HetR to
273 form tetramers in CSL11 than in the wild type, especially in the presence of ammonium
274 (Fig. 1), and the negative effect of PatS-5 on HetR oligomerization in vitro (Fig. 8)
275 support that PatS inhibits HetR tetramer formation in vivo and is consistent with HetR
276 activity being associated to HetR tetramers.

277 In the crystal structure of HetR complexed with palindromic DNA targets, HetR
278 interacts with DNA as a dimer when using a short DNA fragment. However, declared
279 surprising by the authors (Kim *et al*., 2013), the structure of the HetR complex with a
280 29-bp DNA duplex shows a tetramer of the protein involving two DNA duplexes, with a
281 more extended protein-DNA interface than in the DNA-HetR dimer complex (Kim *et al*.,
282 2013). The tetramer is formed mainly by interaction of the flap domains of HetR (Kim *et*
283 *al*., 2013) and, indeed, the S^{179} mutated in HetR of strain 216 resides in the flap domain
284 of HetR (Kim *et al*., 2011), consistent with our observation of deficiency of HetR
285 tetramers in this strain (Fig. 9A). Moreover, in contrast to the reported lack of effect of
286 the $S^{179}N$ mutation on in vitro binding of HetR to a DNA fragment of the *hetR* gene
287 promoter (Huang *et al*., 2004), we have found that interaction with the *hetP* promoter
288 DNA is impaired (Fig. 7B). Our data indicates that HetR tetramers are not a result of

289 the crystallization procedure only, but they are present in vivo and can have a
290 functional relevance. Furthermore, we have observed specific interactions of HetR with
291 *Anabaena* RNAP, which appears to preferentially involve the tetrameric form of HetR
292 (Fig. 2). Indeed, our observations would be consistent with the in vivo occurrence of
293 HetR as monomers only in the soluble, un-complexed form (or when bound to PatS,
294 see Fig. 8C), whereas being present mainly as tetramers in the RNAP-HetR complex.

295 In contrast to the regulation of *hetR* gene expression, which has been the
296 subject of multiple reports, little is known about possible mechanisms of HetR post-
297 translational modifications, although the turnover of HetR has been considered to play
298 an important role in the regulation of heterocyst differentiation (Risser and Callahan,
299 2007, 2009). Previously it has been described that purified recombinant HetR has
300 autoproteolytic activity sensitive to serine-type protease inhibitors, and that this activity
301 is absent from a recombinant version of the HetR protein present in strain 216 (Zhou *et*
302 *al*., 1998) and in a HetR $S^{152}A$ version (Dong *et al*., 2000). In these reports, it was
303 claimed that HetR is a serine-type protease and that this activity is required for
304 heterocyst differentiation. However, an *Anabaena* derivative expressing the mutant
305 $S^{152}A$ HetR was later reported to be similar to the WT with regard to heterocyst
306 differentiation (Risser and Callahan, 2007). We have observed only limited degradation
307 of our Strep-HetR versions from both strain PCC 7120 or 216 (Figs. 3 and 6).
308 Considering the widespread distribution of protein phosphorylation events in biological
309 regulation, we have tested whether HetR could be phosphorylated in vitro.

310 We have shown that our purified Strep-HetR protein exhibits four forms that by
311 size would correspond to monomers, dimers, trimers and tetramers (Fig. 3) (to the best
312 of our knowledge recombinant HetR tetramers in solution have not been previously
313 reported), and that HetR can be phosphorylated by ATP (Fig. 4). Because we detected
314 several phosphorylated HetR oligomers, we cannot discern whether the different HetR
315 aggregation forms could be phosphorylated, or whether phosphorylation takes place
316 preferentially in one of them, which then interconverts into the other forms. In any case,

317 it appears that phosphorylation has a negative effect on the accumulation of the larger
318 HetR oligomers (Fig. 6). Importantly, we have shown that a different form of
319 recombinant HetR, a 6His-tagged protein, can also be phosphorylated exhibiting at
320 least phosphorylated monomers and dimers (Fig. 5B). These results make it unlikely
321 that the phosphorylated site of recombinant HetR resides in the tag.

322 DTT has a negative effect on Strep-HetR phosphorylation (Figs. 4C and 9B),
323 although at the concentration used (1 mM) it does not prevent the formation of at least
324 dimers and trimers. Moreover, DTT facilitates the observation of Strep-HetR $S^{179}N$
325 oligomers (Fig. 9B). HetR has a single Cys residue (C^{48}) (Buikema and Haselkorn,
326 1991a). Regarding the possibility of inter-subunit formation of disulfide bonds involving
327 C^{48} and its relevance for differentiation, contrasting results have been published
328 (Huang *et al*., 2004; Risser and Callahan, 2007). Recently, by means of DEER-EPR
329 and size exclusion chromatography, the C^{48} residue was found not to be required for
330 HetR dimer formation, which could take place in the presence of 10 mM DTT, although
331 because a HetR $C^{48}A$ dimer was not observed in SDS-PAGE, it was considered that
332 the dimer could be stabilized by an interchain disulfide bond (Feldmann *et al*., 2011).
333 Our results are consistent with recent structural data indicating that a disulfide bond is
334 not possible between the two chains in the HetR dimer (Kim *et al*., 2011). In this
335 scenario, the negative effect of DTT on HetR phosphorylation that we have observed
336 suggests a different effect of C^{48} reduction on the HetR conformation. Finally, we have
337 observed that phosphorylated HetR binds a DNA fragment including the best HetR
338 target identified to date (Higa and Callahan, 2010) with lower affinity than
339 unphosphorylated HetR (Fig. 7). The extent to which phosphorylation modulates HetR
340 interaction with regulated promoters in vivo, and whether or not HetR binding to target
341 promoters is the only step affected during regulation of gene expression by HetR,
342 should be further investigated.

343 In summary, our results support the notion that the HetR tetramer is relevant for
344 the in vivo activity of the protein at gene activation during heterocyst differentiation.

345 Also, the negative regulator PatS appears to influence the formation of HetR tetramers.
346 We have shown for the first time that HetR is susceptible of phosphorylation (in vitro)
347 and that phosphorylation interferes with HetR tetramer accumulation and impairs HetR
348 interaction with DNA. It is tempting to speculate that also in vivo phosphorylation could
349 be a mechanism of negative regulation of the HetR activity. This relationship would
350 resemble that described for the response regulator HnoC in the *Shewanella oneidensis*
351 nitric oxide signaling network. In its unphosphorylated form HnoC exists as a tetramer
352 that associates tightly to DNA, whereas phosphorylation causes tetramer dissociation
353 and detachment of subunits from promoter DNA (Plate and Marletta, 2013). Finally, in
354 our in vitro system, HetR is phosphorylated when incubated with ATP in the absence of
355 any additional factors added. This might imply autophosphorylation, although the
356 presence in the preparations of traces of *E. coli* kinases cannot be strictly ruled out.
357 However, in vivo phosphorylation of HetR might be catalyzed by a trans-kinase. The
358 expression of the *pknE* gene (encoding a Ser/Thr kinase) is concentrated in cells
359 differentiating into heterocysts at medium-to-late times after N step-down (12 h), likely
360 after the peak of HetR-promoted gene activation, and *pknE* over-expression blocks
361 differentiation downstream from HetR (Saha and Golden, 2011). Thus, as previously
362 proposed (Saha and Golden, 2011), one effect of HetR regulation by phosphorylation
363 could be limiting the activity of HetR in the mature heterocysts (see a model in Fig. 10).
364

365 **Experimental procedures**

366 *Strains and growth conditions*

367 *Anabaena* sp. strain PCC 7120 and derivative strains DR884a (an insertional mutant of
368 the *hetR* gene [Black *et al*., 1993]), 216 (which expresses a HetR version with the
369 change $S^{179}N$ [Buikema and Haselkorn, 1991b]) and an *Anabaena* derivative strain that
370 expresses an RNA polymerase (RNAP) β' subunit C-terminally fused to poly-His (see
371 Valladares *et al*., 2008) were grown photoautotrophically in $BG11_0$-based medium as

described (Camargo *et al*., 2012; Rippka *et al*., 1979), supplemented with 5 μg ml^{-1} neomycin sulfate for DR884a, and 2 μg ml^{-1} each streptomycin sulfate and spectinomycin dihydrochloride pentahydrate for the latter strain.

Preparation of cell-free extracts and western blot analysis

For heterocyst and vegetative cell isolation, filaments were incubated 15 min at room temperature with 1 mg of lysozyme per ml of buffer A (50 mM Tris-HCl [pH 8.0], 200 mM NaCl, 10% glycerol), then centrifuged at 1,935 x *g* (4 ºC, 10 min), and the resulting supernatant was used as vegetative-cell extract. The pellet was resuspended in buffer A, and the resulting suspension passed through a French pressure cell at 3,000 psi three times and enriched in heterocysts after successive steps of low-speed centrifugation (200 x *g*, 10 min, 4 ºC) and washing with buffer A. Whole filament suspensions were supplemented with a protease inhibitor mixture tablet (*c*Omplete Tablets, Mini EDTA-free; Roche) and heterocyst suspensions also with phosphatase inhibitors (1 mM Tris-HCl [pH 8], 10 mM NaF, 10 mM NaN_3, 10 mM *p*-nitrophenylphosphate) and passed through a French pressure cell at 20,000 psi three times. Cell debris was removed by centrifugation at 16,100 x *g* (4 ºC, 10 min). Cell-free extracts from *Anabaena* expressing RNAP β'-His were prepared in buffer B (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 20% glycerol) supplemented with protease inhibitor (PMSF) by two passes through a French pressure cell at 20,000 psi followed by 60 min centrifugation at 141,750 x *g*. The precipitated material was discarded.

For immunoblotting, samples were mixed with 0.1 volume of 5x sample buffer, loaded and run in a Laemmli SDS-PAGE system of the indicated acrylamide percentages, and transferred to PVDF membrane filters. (Importantly, samples were not heated before loading into the gel.) Western-blot analysis was performed by standard procedures with a polyclonal antibody generated in rabbits against purified 6His-tagged HetR protein (Camargo *et al*., 2012) or with antibodies against PatS-5 (Corrales-Guerrero *et al*.,

2013). Antigen-antibody complexes were visualized with the WesternBright detection kit (Advansta).

Production and purification of Strep-tagged HetR (Strep-HetR)

Plasmid pCSAV225 consists of a 897-bp DNA fragment from the *Anabaena hetR* genomic region, amplified by PCR with primers alr2339-*Nco*I (CTATTTGTACCATGGGTAACGACATC, positions -11/+15 relative to the translation start of *hetR*) introducing an *Nco*I site (underlined) at the *hetR* start codon, and alr2339-18 (AGCGTCGAGtttttcgaactgcgggtggctccaATCTTCTTTTCTACCAAAC, positions +897/+879) introducing the Strep-tag sequence (lower case) at the C-terminus of the protein, and plasmid pCSAM105 (Camargo *et al*., 2012) as template, and cloned into NcoI- and BamHI-digested expression vector pET28b. In plasmid pCSAV225 the *Anabaena* DNA fragment begins in the start codon of the *hetR* gene, which is in frame with the site of initiation of translation located after the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter in pET28b. The nucleotide sequence of the *hetR* gene cloned in pCSAV225 was verified by sequencing. The same procedure, except that the template for PCR amplification was genomic DNA from strain 216, was followed to generate plasmid pCSAV223 for expression of Strep-tagged HetR ($S^{179}N$). Strep-HetR or Strep-HetR ($S^{179}N$) were purified from cultures of *E.coli* BL21 (DE3) (pREP4, pCSAV225) or (pREP4, pCSAV223), after induction with IPTG, by chromatography through a 1-ml Gravity flow Strep-Tactin^R Superflow^R high capacity column (Iba, Germany) charged with Strep-Tactin resin.

Electrophoretic Mobility Shift Assays (EMSA)

The 29-bp DNA fragment used was prepared by mixing 10 pmol of R-bind 29mer top and R-bind 29mer btm oligonucleotides (Higa and Callahan, 2010) in annealing buffer containing 10 mM Tris-ClH (pH 7.5), 50 mM NaCl, and 1 mM EDTA, then heating at 95

ºC for 10 min and cooling from 95 ºC to room temperature. The resulting double-stranded DNA fragment was labelled and used in EMSA assays as described (Camargo *et al*., 2012), and the protein-DNA complexes were separated in native 15% polyacrylamide gels.

In vitro phosphorylation assays

Assays were performed by incubation of Strep-HetR, Strep-HetR ($S^{179}N$) or His-HetR protein with (γ-^{32}P)ATP (15-40 μM; 10 Ci/mmol) in a total volume of 40 μl in buffer C (25 mM Tris-HCl [pH 7.5], 100 mM KCl, 5 mM $MgCl_2$ and 10% glycerol) for 1 h at 30 ºC. For alkaline phosphatase treatment, phosphorylated HetR was incubated, in a total volume of 30-35 μl, with 2 U of calf alkaline phosphatase (Roche) (AP) at 37 ºC during 30-60 min. For purification of phosphorylated Strep-HetR or Strep-HetR ($S^{179}N$), 12 μg of protein that had been incubated in a total volume of 1.6 ml of buffer C with 1 mM ATP for 1 h at 30 ºC was chromatographed through a 1 ml gravity flow PhosphoProtein purification column (QUIAGEN) following the manufacturer's recommendations, except that buffer C without $MgCl_2$ was used as binding buffer and the elution buffer contained 50 mM sodium phosphate, 100 mM KCl and 10% glicerol.

Acknowledgments

We thank Sergio Camargo, Félix Ramos-León and Fernando Monje-Casas for experimental advice. Work was supported by grants BFU2010-17980 and BFU2013-44686-P from the Spanish Government, co-financed by FEDER.

Conflict of interest

The authors declare that they have no conflict of interest.

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550
551

552

553 **LEGENDS FOR FIGURES**

554

555 **Fig. 1.** Immunoblot analysis of HetR in *Anabaena***.**

556 A, B. Samples of cell-free extracts from whole filaments of wild-type *Anabaena* (WT) or
557 strain DR884a (*hetR*) grown with ammonium (0) and incubated for the indicated
558 number of hours in the absence of combined nitrogen were resolved by SDS/PAGE
559 (14% gels in A; 12% in B) and subjected to immunoblot analysis with antibodies raised
560 against HetR. Samples contained 160 μg of protein (A) or 40 μg (B).

561 C. Samples (110 μg protein) of strains CSL11 (*patS hetN*) or DR884a grown with
562 ammonium (0) and incubated for the indicated number of hours in the absence of
563 combined nitrogen resolved in 12% gels.

564 D. Samples (40 μg protein) from whole filaments or from isolated heterocysts (H) or
565 vegetative cells (V) from filaments of WT incubated for 24 h without combined nitrogen
566 resolved in 14% gels.

567 The positions of different HetR forms (bands 1, 2, 3 and 4) are indicated. Size markers
568 (in kDa) are indicated to the panel left.

569

570 **Fig. 2**. Co-elution of HetR and His-tagged RNA polymerase from *Anabaena* cell
571 extracts.

572 A. Cell-free extracts (row labeled E) from *Anabaena* expressing His-β' RNAP grown
573 with ammonium and incubated (lower panel) or not (upper panel) for 6 h in the absence
574 of combined nitrogen were passed through a Ni^{2+}-charged His-select column (Sigma).
575 Samples of the effluent resulting from washing with 3 mM imidazole (row labeled NR)
576 and of the fractions collected after elution with 3-500 mM imidazole were analyzed by
577 SDS/PAGE in 10% gels. The bands corresponding to RNAP subunits are indicated. S,
578 size standard (NuPAGE, Invitrogen); sizes are: 36, 50, 64, 98, 148, 250 kDa.

B. Samples of the indicated fractions were subjected to immunoblot analysis with HetR antibodies. Size markers (in kDa) are indicated to the panel left. HetR, 0.1 μg of purified His-HetR.

C. Cell-free extract from filaments of wild-type *Anabaena* incubated for 6 h in the absence of combined N analyzed as above.

D. Immunoblot analysis with HetR antibodies of samples of the corresponding column fractions from C.

Fig. 3. Immunoblot analysis of Strep-HetR.

HetR immnublotting was performed with antibodies raised against HetR and samples of purified Strep-HetR preparations (4 μg of protein) subjected to SDS/PAGE in large 8% gels. The positions of different HetR complexes (1, 2, 3 and 4) are indicated. Size markers (in kDa) are indicated to the panel left .

Fig. 4. Phosphorylation of Strep-HetR.

In vitro phosphorylation assays were performed with purified Strep-HetR, and the products were resolved by SDS/PAGE in 10% gels and transferred to PVDF membranes.

A. Time-course of (γ-^{32}P)ATP incorporation into Strep-HetR: Strep-HetR (3.2 μg; 2.1 μM) was incubated in the presence of 15 μM (γ-^{32}P)ATP for the times indicated.

B. Alkaline phosphatase treatment after Strep-HetR phosphorylation: Strep-HetR (6 μg; 3.9 μM) was incubated 1 h at 37 ºC in the presence of 20 μM (γ-^{32}P)ATP. Then, samples were treated (+) or not (-) with 2 U of AP for 1 h at 37 ºC.

C. Effect of DTT on Strep-HetR phosphorylation: Strep-HetR (4 μg; 2.6 μM) was incubated 1 h at 37 ºC with 15 μM (γ-^{32}P)ATP in the presence (+) or absence (-) of 1 mM DTT.

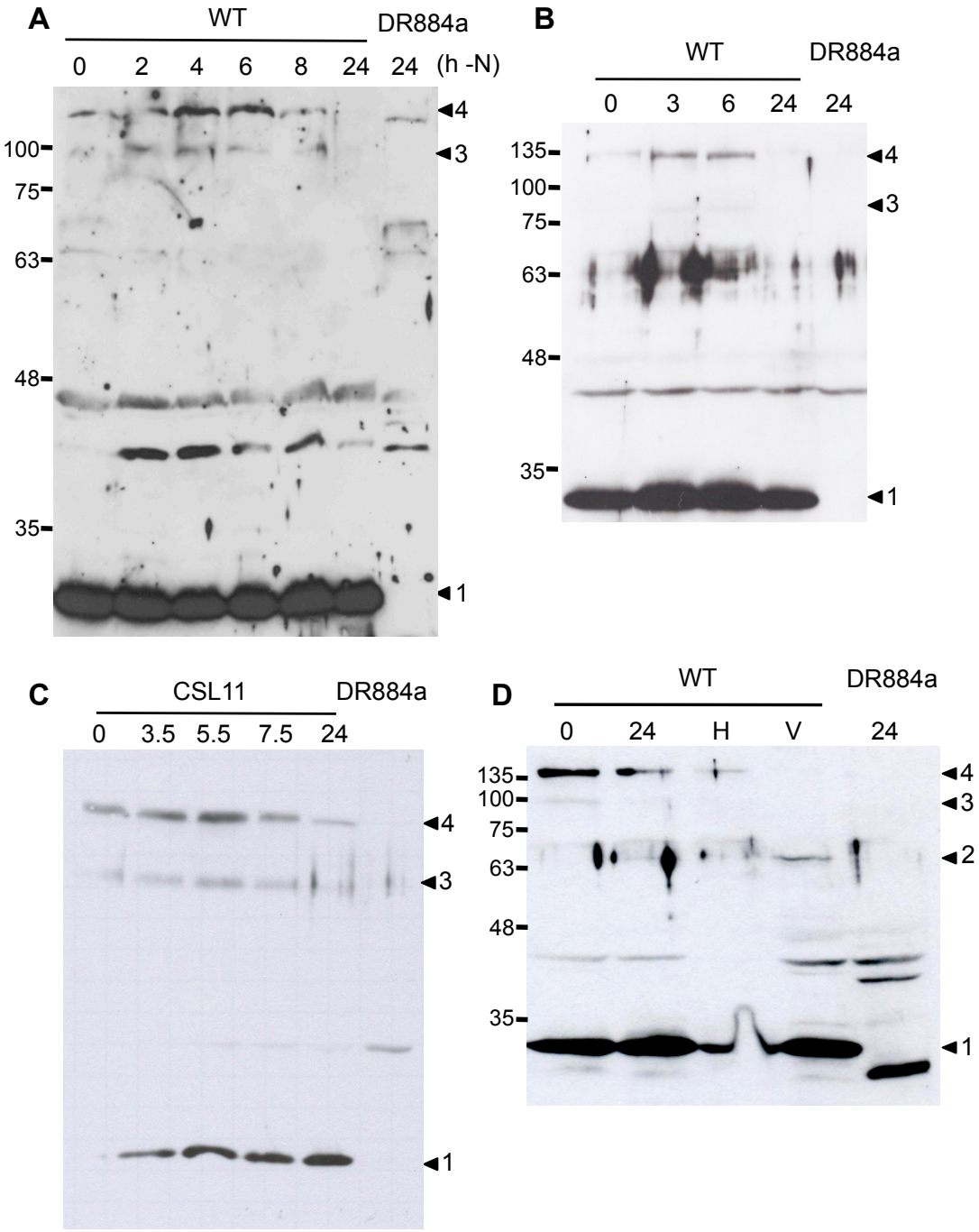
605 Upper panels in A, B, C, images of radioactive membranes obtained with a Cyclone
606 storage phosphor system; lower panels, immunoblotting of radioactive membranes
607 using antibodies against HetR. The positions of different HetR complexes (1, 2, and 3)
608 are indicated. Size markers (in kDa) are indicated to the panel left.
609
610 **Fig. 5.** Phosphorylation of His-HetR.
611 A. HetR immunoblotting with a sample of the protein preparation (2 μg) used for the
612 phosphorylation assays run in a large 8% gel. The positions of different HetR
613 complexes (1, 2, 3 and 4) are indicated. Size markers (in kDa) are indicated to the
614 panel left.
615 B. In vitro phosphorylation assays were performed with purified His-HetR (2 and 4 μg
616 protein) and (γ-^{32}P)ATP (40 μM), and the products were resolved by SDS/PAGE in
617 10% gels and transferred to a PVDF membrane. Upper panel, image of radioactive
618 membrane obtained with a Cyclone storage phosphor system; lower panel,
619 immunoblotting of radioactive membrane using HetR antibodies.
620
621 **Fig. 6.** Effect of phosphorylation on HetR oligomerization.
622 A. HetR immnublotting was performed with HetR antibodies and samples of purified
623 Strep-HetR preparation (2.2 μg of protein) incubated for 1 h with the specified
624 concentration of ATP under phosphorylation assay conditions and subjected to
625 SDS/PAGE in large 8% gels. The positions of different HetR complexes (1, 2, 3 and 4)
626 are indicated.
627 B. Protein amounts, estimated after scanning, in the different bands of the membrane
628 shown in A, indicated as the fraction of the amount of the corresponding band in the
629 sample treated with no ATP.
630
631 **Fig. 7.** Effect of phosphorylation and PatS-5 on the binding of HetR to DNA.

632 A. EMSA assays were performed with a 29-bp DNA fragment of the *hetP* promoter
633 region and increasing amounts (0.036, 0.07, and 0.108 μg) of purified Strep-HetR that
634 had been incubated under phosphorylation assay conditions with 1 mM ATP and then
635 passed through a PhosphoProtein purification column (HetR-P) or incubated without
636 ATP (HetR). (In the former case, protein amount was quantified after elution from the
637 column.)
638 B. EMSA with Strep-HetR or Strep-HetR $S^{179}N$ incubated without (lanes 2, 5 and 7) or
639 with 1 mM ATP and then passed (lanes 4, 6 and 8) or not (lane 3) through a
640 PhosphoProtein purification column. PatS indicates assays in the presence of 150 μM
641 PatS-5.
642 The first lane in A and B shows assays in the absence of added proteins. The
643 percentage of retarded DNA with regard to the total DNA amount in each lane is
644 indicated. Open triangles point to the free DNA fragment, and closed triangles to DNA-
645 HetR complexes.
646
647 **Fig. 8.** Effect of PatS-5 on HetR oligomerization.
648 A. D. HetR immnublotting was performed with HetR antibodies and samples of purified
649 Strep-HetR preparation (1.08 μM in A; 0.83 μM in D) incubated for 1 h with the
650 specified concentration of PatS-5 and subjected to SDS/PAGE in large 8% gels. The
651 positions of different HetR complexes (1, 2, 3 and 4) are indicated.
652 B. E. Protein amounts, estimated after scanning, in the different bands of the
653 membranes shown in A and D, respectively, indicated as the fraction of the amount of
654 the corresponding band in the sample treated with no PatS-5.
655 C. Immunoblotting performed with PatS-5 antibodies of the membrane used in D.
656 (Immunoblotting with the HetR antibodies was performed after erasing the signals due
657 to the PatS-5 antibodies, which were used first.)
658

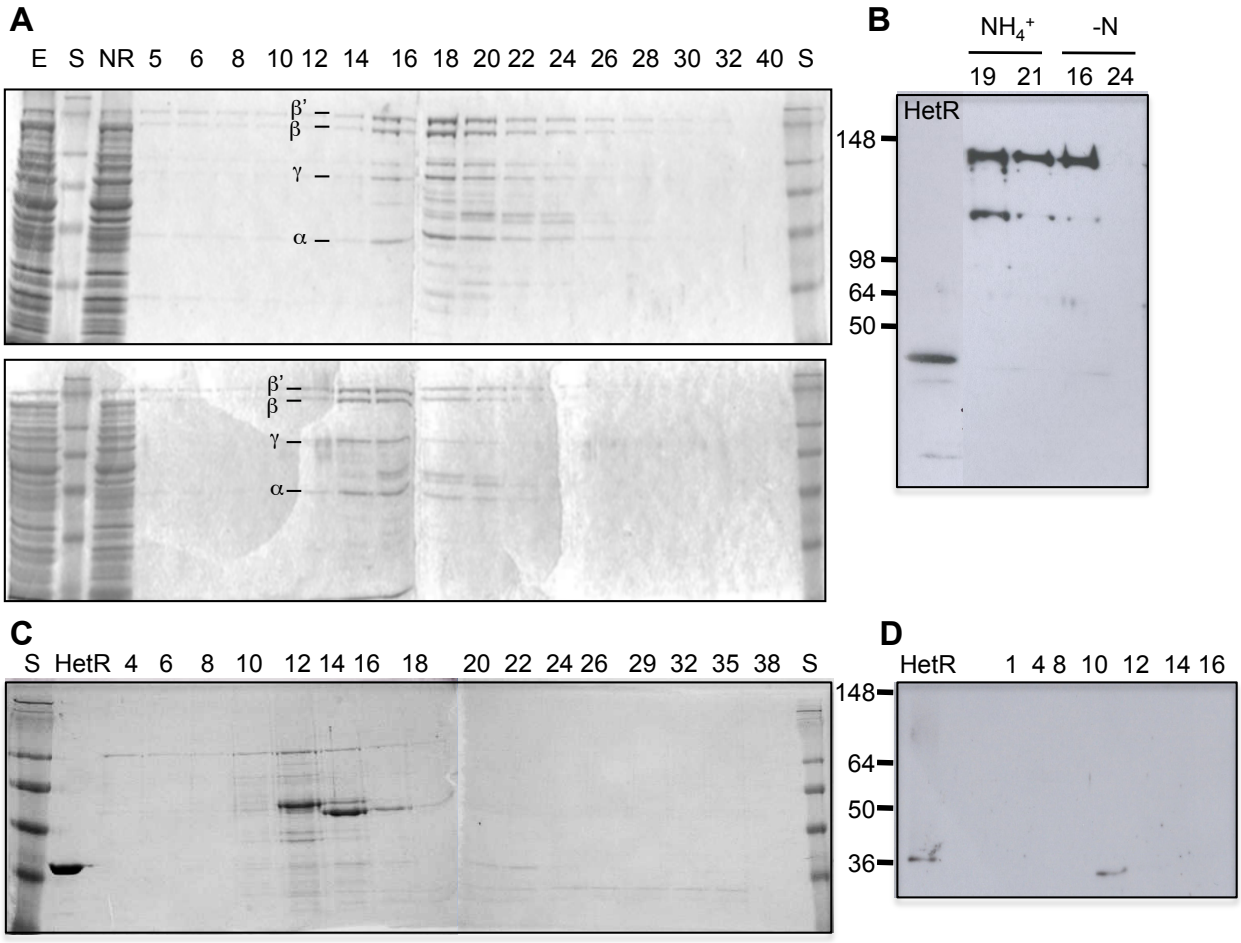
659 **Fig. 9.** Immunoblot analysis and in vitro phosphorylation of HetR protein from
660 *Anabaena* mutant strain 216.
661 A. Total cell extracts from filaments of the indicated strains grown with ammonium (0)
662 or grown with ammonium and incubated for the indicated times in the absence of
663 combined nitrogen (50 μg protein from WT; 20 μg from strain 216) were resolved by
664 SDS/PAGE on 12% gels and subjected to immunoblot analysis with HetR antibodies.
665 B. Purified Strep-HetR and Strep-HetR $S^{179}N$ proteins (4 and 6 μg in each case, left
666 panels; 4 μg, right panels), as indicated, were incubated for 1 h with 15 μM (γ-^{32}P)ATP
667 in the presence or absence of 1 mM DTT, and the products were resolved by
668 SDS/PAGE in 10% gels and transferred to PVDF membranes. Upper panels, images
669 of radioactive membranes; lower panels, immunoblotting of radioactive membranes
670 with HetR antibodies.
671 The positions of different HetR complexes (1, 2, 3 and 4) are indicated. Size markers
672 (in kDa) are indicated to the right (A) or the left (B) of panels.
673
674 **Fig. 10.** Model of the spatiotemporal regulation of HetR by phosphorylation and PatS.
675 In the presence of ammonium the undifferentiated *Anabaena* filament bears a low level
676 of HetR tetramer as the result of a low level of expression of the *hetR* gene and of *patS*
677 (encoding the PatS inhibitor). All the cells are represented as equivalent, which doesn't
678 preclude some coupling in *hetR* (Corrales-Guerrero *et al*., 2015) and *patS* (Yoon and
679 Golden, 2001) expression fluctuations. After N step-down, the HetR tetramer increases
680 in abundance, consistent with induction of *hetR,* which reaches a maximum after ca.
681 12-18 h. *hetR* induction takes place initially in small clusters of contiguous cells and
682 later in single cells (see Risser *et* al., 2012; Corrales-Guerrero *et al*., 2015). During this
683 time, *patS* is induced in differentiating cells, but processed to generate the active
684 peptide, which is transferred to the neighbouring cells. We propose that during
685 heterocyst differentiation the increase in HetR tetramer is also patterned. When

686 differentiation is completed, *hetR* expression decreases, and the PatS effect decays.
687 The abundance of total HetR tetramer also decreases, and it is restricted to
688 heterocysts. We propose that both PatS, mainly during differentiation, and
689 phosphorylation, in mature heterocysts, negatively affect the HetR tetramer
690 conformation. Blue 4-ring structures, HetR tetramers; Red curves, processed PatS.
691 Thickness is intended to coarsely represent protein or peptide levels.
692

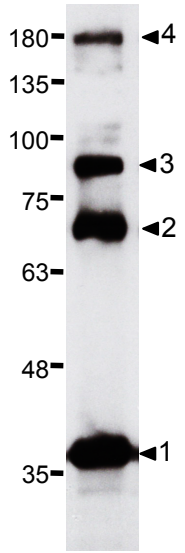
Valladares *et al*. Figure 1



Valladares *et al*. Figure 2



Valladares *et al*. Figure 3



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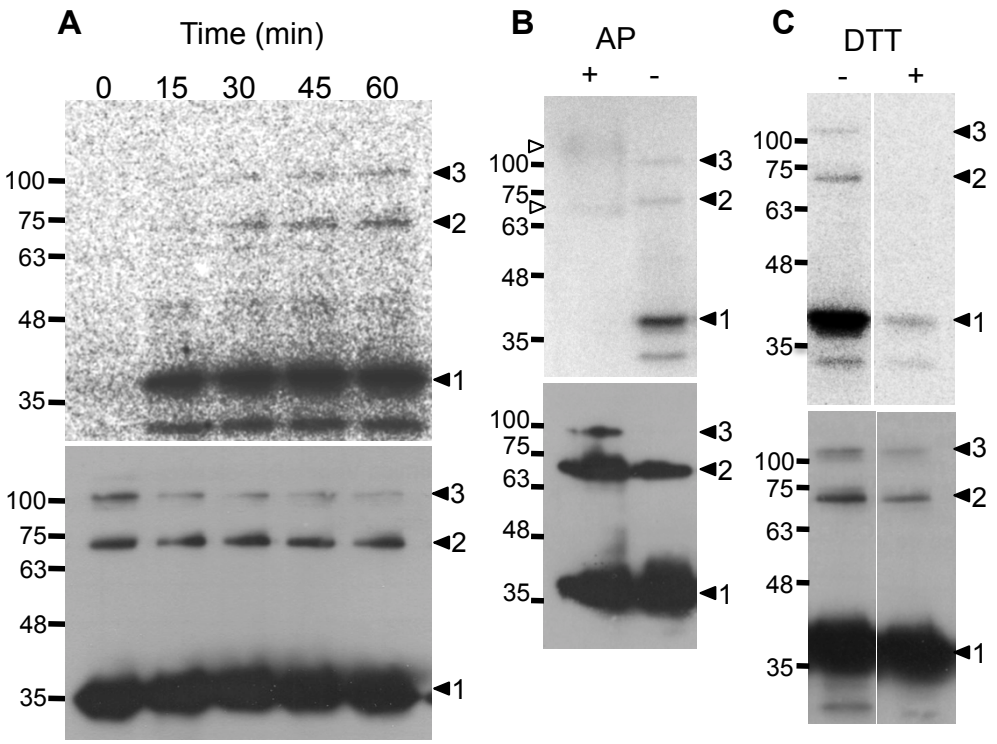

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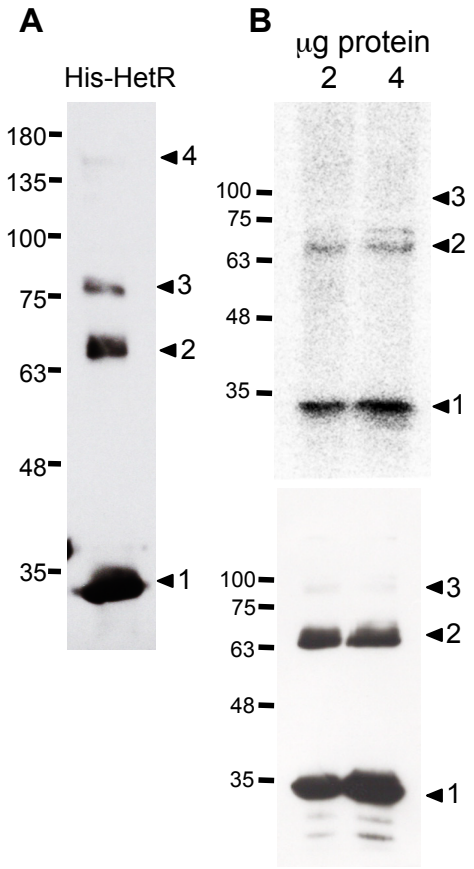
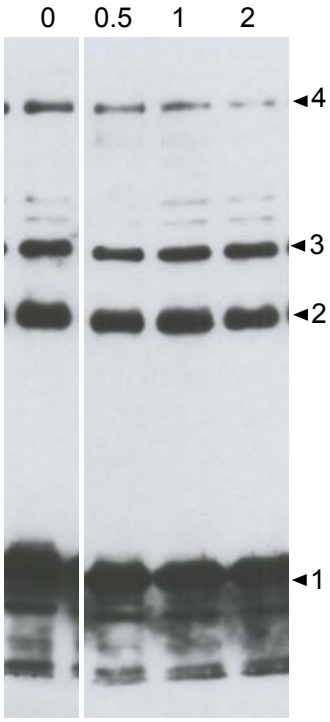


Valladares *et al*. Figure 6

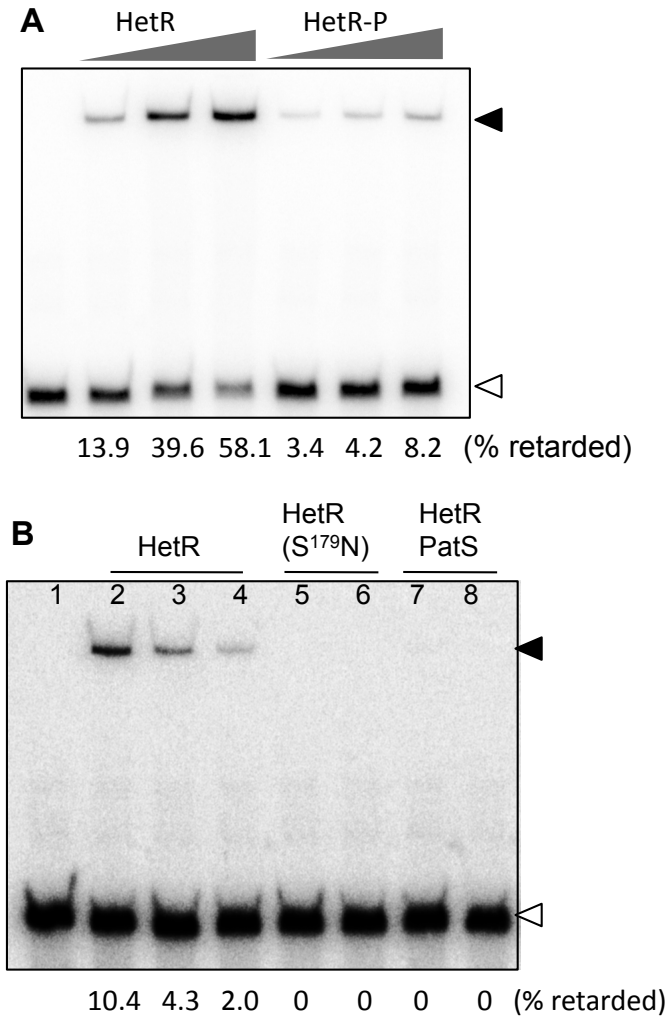
A



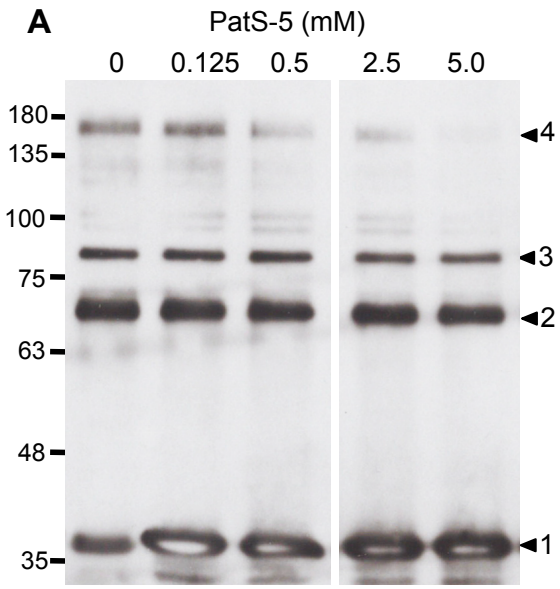
B

HetR oligomer	ATP (mM)		
	0.5	1.0	2.0
Band 4	0.58	0.52	0.08
Band 3	0.70	0.93	1.04
Band 2	0.90	1.08	0.84

Valladares *et al*. Figure 7

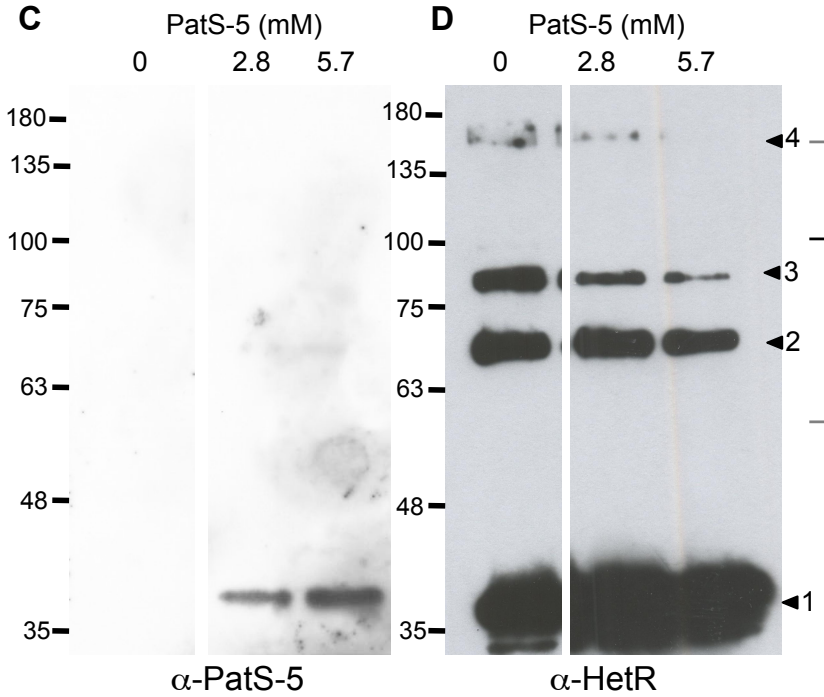


Valladares *et al*. Figure 8



B

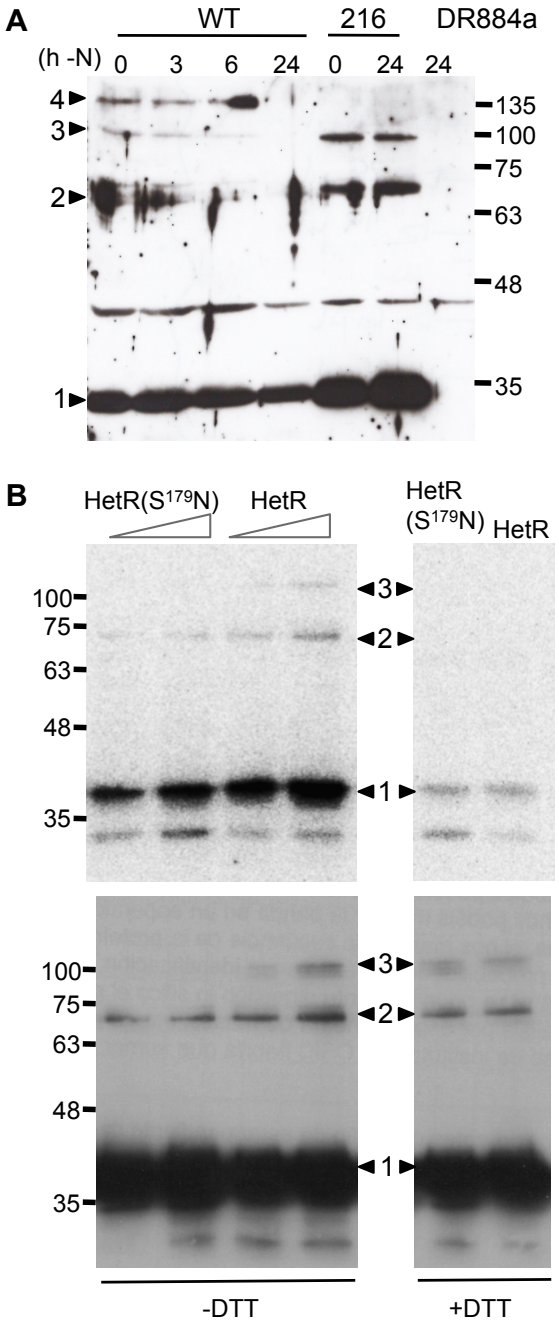
HetR oligomer	PatS-5 (mM)			
	0.125	0.5	2.5	5.0
Band 4	1.07	0.42	0.21	0.00
Band 3	1.10	1.19	0.86	0.83
Band 2	0.99	0.95	0.93	0.89



E

HetR oligomer	PatS-5 (mM)	
	2.8	5.7
Band 4	0.24	0.01
Band 3	0.46	0.20
Band 2	0.92	0.67
Band 1	1.33	1.15

Valladares *et al*. Figure 9



Valladares *et al*. Figure 10