HetL provides immunity to HetR against PatS inhibition, and promotes pattern formation in the cyanobacterium *Nostoc PCC 7120*

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Abstract

Local activation and long-range inhibition are mechanisms conserved in self-organizing systems leading to biological patterns. A number of them involve the production by the developing cell of an inhibitory morphogen, but how this cell gets immune to self-inhibition is rather unknown. Under combined nitrogen starvation, the multicellular cyanobacterium *Nostoc* PCC 7120 develops nitrogen-fixing heterocysts with a pattern of a heterocyst every 10-12 vegetative cells. Cell differentiation is regulated by HetR which activates the synthesis of its own inhibitory morphogen (PatS), which diffusion establishes the differentiation pattern. Here we show that HetR interacts with HetL at the same interface as PatS, and that this interaction is required to suppress inhibition and to differentiate heterocysts. *hetL* expression is induced under nitrogen-starvation and is activated by HetR, suggesting that HetL provides immunity to the heterocyst. This protective mechanism might be conserved in other differentiating cyanobacteria as HetL homologues are spread across the phylum.
Periodic patterning of iterative forms is one of the most common features observed in developmental processes across the living kingdom. Unraveling the rules governing pattern formation helps to elucidate the genetic basis of cell differentiation. A key contribution in theoretical biology was made by Alan Turing in 1952 who demonstrated that the reaction of two molecules with different diffusion rates generates regular patterns (A. Turing, 1952). Later on, Turing equations were adapted in a new mathematical model that emphasized conspicuous features of biological development, in particular local activation linked to autocatalysis and long range inhibition (Gierer & Meinhardt, 1972) (Meinhardt & Gierer, 1974). Valuable experimental approaches showed the recurrence of this mechanism in various developmental behaviors, such as: spacing of leaves, tissue regeneration in Hydra, epidermis of insects, pigmentation in Zebra fish, embryogenesis in Drosophila, and also differentiation in some prokaryotes (for a review see (Schweisguth & Corson, 2019)). In all these developmental situations several aspects of the molecular interactions involved to generate patterning remain to be elucidated.

Among prokaryotes, several members of the Cyanobacteria phylum are able of cell differentiation. The molecular basis of differentiation has been well documented for the cyanobacterium Anabaena/Nostoc PCC 7120 (referred herein as Nostoc). Nostoc is a diazotrophic strain which can differentiate a specific cell type responsible for fixing atmospheric nitrogen. When combined nitrogen is abundant Nostoc forms long filaments consisting of a single cell type. When the filaments of Nostoc are deprived of combined nitrogen, around 10% of the vegetative cells differentiate into heterocysts. These micro-oxic cells, which provide a suitable environment for N₂-fixation, are non-dividing and semi-regularly distributed along the filaments. Nostoc differentiation follows therefore a one-dimensional pattern of heterocysts separated by ten vegetative cells. Heterocysts are unable to undergo cell division, but as vegetative cells continue dividing, the filaments grow and new heterocysts form at the middle of the intervals between preexisting heterocysts. Consequently, the pattern is dynamic and persists all along the growth (Kumar, Mella-Herrera, & Golden, 2010) (Flores & Herrero, 2010). The molecular signal inducing heterocyst differentiation is 2-oxoglutarate (2-OG), which accumulates in response to combined nitrogen starvation (Laurent et al., 2005). Among the various genes involved in the regulation
of heterocyst formation and patterning (Herrero, Stavans, & Flores, 2016), the global regulator NtcA and the specific master regulator HetR are key transcriptional factors in the cascade resulting in heterocyst development. Upon combined nitrogen starvation, NtcA interacts with 2-OG and induces heterocyst differentiation by controlling, directly or indirectly, the expression of several genes, including \textit{hetR}. (Herrero, Muro-Pastor, Valladares, & Flores, 2004; Valladares, Flores, & Herrero, 2008). HetR is essential for cell differentiation: its deletion abolishes differentiation, when its overexpression induces differentiation of multiple contiguous heterocysts under combined nitrogen-starvation and allows differentiation even under non-permissive conditions (Buikema & Haselkorn, 1991). It exists in different oligomeric states among which dimer and tetramer have been proposed to interact with DNA (Huang, Dong, & Zhao, 2004) (Valladares, Flores, & Herrero, 2016). HetR regulon includes hundreds of genes, which are either activated in response to nitrogen starvation or repressed in nitrogen-replete conditions (Flaherty, Johnson, & Golden, 2014; Mitschke, Vioque, Haas, Hess, & Muro-Pastor, 2011; Videau et al., 2014). The structure of HetR shows a unique fold comprising three domains: helix-turn-helix, flap and hood; with the latter encompassing the binding site of PatS (Kim et al., 2011) (Hu et al., 2015). Key events in pattern formation in \textit{Nostoc} is the positive autoregulation of \textit{hetR} occurring specifically in the differentiating cell (T. A. Black, Cai, & Wolk, 1993) (Rajagopalan & Callahan, 2010) and the inhibition of HetR in neighboring cells by the product of \textit{patS} (Golden & Yoon, 2003). The deletion of the \textit{patS} gene leads to the formation of multiple contiguous heterocysts, when its overexpression inhibits the differentiation process and hence induces a lethal phenotype under combined nitrogen starvation (Yoon & Golden, 1998). \textit{patS} encodes a 13-17 amino acid peptide containing at its carboxy-terminal extremity a \textbf{RGSGR} pentapeptide (PatS-5) that interacts with HetR, inhibits its DNA-binding activity (Huang et al., 2004) (Feldmann et al., 2011) and blocks cell differentiation when added to culture medium (Yoon & Golden, 1998). The hexapeptide \textbf{ERGSGR} (PatS-6) derived from PatS is also able to interact with HetR and to inhibit its activity (Hu et al., 2015). A mutant form of HetR, R223W unable to interact with PatS, forms multiple contiguous heterocysts (Khudyakov & Golden, 2004) (Hu et al., 2015). PatS-dependent signals are considered to diffuse along the filament; inhibiting HetR in the cells adjacent to the heterocyst, so preventing them from differentiating (Risser & Callahan, 2009) (Corrales-Guerrero, Mariscal, Flores, & Herrero, 2013). The \textbf{RGSGR} pentapeptide is also present
in HetN; a protein required for the maintenance of the pattern (T. A. Black & Wolk, 1994) (Higa et al., 2012) (Corrales-Guerrero et al., 2014), and recently a third protein (PatX) containing a RGTGR motif, has been reported to inhibit heterocyst differentiation when overproduced in Nostoc (Elhai & Khudyakov, 2018).

The HetR/PatS regulatory loop fits the local activation (HetR)/long range inhibition (PatS) module that characterizes the Turing-adapted model of patterning (Brown & Rutenberg, 2014; A. Turing, 1952; A. M. Turing, 1990) (Gierer & Meinhardt, 1972) (Figure 1A). In addition, the action of HetN and PatX as inhibitory factors, the stochastically noisy expression of regulatory proteins (HetR and NtcA) among other features specific to cell differentiation of Nostoc, allow the emergence of more elaborated mathematical models that draw the principles governing pattern formation in cyanobacteria (Di Patti et al., 2018; Munoz-Garcia & Ares, 2016).

Because Nostoc is the simplest model to address development in a one-dimensional self-organizing system, valuable genetics and biochemistry studies have provided a fine picture of heterocyst patterning. Despite this, several questions are still unanswered and deserve investigation. In particular, how the differentiating cell, where patS is expressed at its highest level, becomes immune to self-inhibition is not fully understood yet. A genetic analysis of patS suggested that the 7 amino acids at the N-terminus of PatS protect the producing cell from inhibition likely concomitantly to the export of the active form of PatS (Corrales-Guerrero et al., 2013). Intriguingly, if patS or patS-5 are expressed specifically in the vegetative cells, or when PatS-5 is added to the culture medium, cell differentiation is abolished. However when produced in cells that have already initiated development (proheterocysts), PatS-5 is not able to inhibit differentiation (Yoon & Golden, 2001) (Wu, Liu, Lee, & Golden, 2004). These observations suggest that proheterocysts must acquire additional protection post-PatS export/processing.

The hetL gene (all3740) was unearth in a genetic screen aiming to identify factors involved in PatS signaling (D. Liu & Golden, 2002). HetL is a single domain protein composed of 40 pentapeptides (A(D/N)LXX), adopting a right-handed quadrilateral beta helix typical of an Rfr-fold common to all pentapeptide repeats containing proteins (PRPs) (Ni, Sheldrick, Benning, & Kennedy, 2009). The ectopic expression of hetL in a background of patS overexpression restores the ability of the strain to
differentiate heterocysts (D. Liu & Golden, 2002). *hetL* overexpression stimulates differentiation also when PatS-5 is added to the culture medium (D. Liu & Golden, 2002). Henceforward, HetL interferes with PatS inhibition but the molecular mechanism involved is unknown.

This study aims to further explore the function of HetL in PatS signaling. We show that HetL interacts with HetR at the same interface than PatS. This interaction is needed for HetR to escape PatS inhibition, and does not inhibit its DNA-binding activity. Analyzing *hetL* transcription, we found that this gene is induced shortly after initiation of cell differentiation and that HetR is required for its expression. Finally, we show that the expression of *hetL* in heterocysts, but not in vegetative cells, is necessary to counteract PatS inhibitory effect. We conclude that, by interacting with HetR, HetL interferes with PatS fixation and therefore provides immunity to the developing cell.
Results

HetL interacts with HetR without inhibiting its DNA binding activity

To get further insights into HetL function, we wondered whether its activity would be mediated by its direct interaction with HetR. To test this, we used the bacterial two hybrid assay (BACTH), which is based on the reconstitution of adenylate cyclase (CyA) activity by two interacting proteins that bring the T18 and T25 domains of CyA into close proximity (Karimova, Pidoux, Ullmann, & Ladant, 1998). T18 and T25 were fused to N-terminus coding sequences of HetR and HetL, and the dimerization ability of HetR was used as an internal control for this assay. The data of Figure 1B show that HetL displays a strong interaction with HetR. Interestingly, it seems that HetR-hood domain is sufficient to mediate the interaction of HetR to HetL. In addition, this experiment indicated that HetL is able to form dimers (or oligomers) (Figure 1B).

To validate this interaction, we developed a BioLayer interferometry (BLi) assay. For this purpose, HetR and HetL proteins were produced and purified using affinity chromatography (Supplementary Figure 1A). HetR was biotinylated and immobilized on streptavidin biosensors as the ligand, while HetL was used as the analyte. Upon addition of HetL, a concentration-dependent association was recorded and decreased during the dissociation step corresponding to the washing of the sensor, testifying for a direct interaction between HetL and HetR (Figure 1C). The estimated dissociation constant ($K_D$) of the HetR-HetL interaction was $6 \mu\text{M}$. The interaction between HetR and HetL observed in the BACTH assay was hence confirmed by BLi.

As HetR acts by directly binding to promoters of a subset of its target genes, we tested if the interaction with HetL would impact its DNA binding activity. To this end, we conducted electrophoretic mobility shift assay (EMSA) using the $hetP$ promoter as a target (Huang et al., 2004) (Hu et al., 2015). The previously reported ability of PatS-5 to inhibit HetR DNA binding activity was used as a control (Huang et al., 2004) (Hu et al., 2015). In the presence of HetL, HetR was still able to interact with $hetP$ promoter and the complex formed was higher than the one formed by HetR alone (Figure 1D). This result
indicates that, contrary to PatS-5 binding, the interaction between the two proteins does not inhibit the activity of HetR.

**Figure 1:** Patterning model and HetL/HetR interaction.

(A) Self-organized patterning in *Nostoc*

Heterocysts are presented in green. Vegetative cells in brown. The width of the shade around the cells represents the strength of the activation/inhibition.

Processed PatS is framed by a star.

(B) *Upper* Bacterial two hybrid assay between HetL and HetR. BTH101 strain was transformed with pKT25-hetL and pUT18C-HetR. β-galactosidase activities were measured as described in section “Materials and Methods” and were expressed in Miller units. Strains producing the T18 with T25 (control -), T25-HetR with T18 (HetR) and T25 with T18-HetL (HetL) served as negative controls. Strains producing T18-Zip and T25-Zip served as positive control (control +).

(B) *Lower* Domains organization of HetR: Helix-turn-helix domain, flap domain and hood domain.

(C) BLi assay between HetL and HetR. 2.2 µM of biotinylated HetR was loaded onto streptavidin biosensors. A 30 second baseline in PBS was performed before a 120 second association step with various concentrations of HetL at 2.5, 5, 10 and 20 µM followed by a 120 second dissociation step. Each
curve represents the average of two experiments minus the control experiment. As a negative binding control, HetL 20µM was added to the empty biosensor devoid of HetR.

(D) EMSA assay of HetR (1 µM) with hetP promoter (50 nM) in the presence of or PatS-5 or PatS-6 (1 µM) and HetL (4 µM). The hetP promoter incubated alone served as negative control (free DNA) and HetL plus DNA as a specific control for HetR.

HetL and PatS interact with HetR at the same interface

Since HetL has been identified on the basis of counteracting PatS inhibition and as both PatS and HetL interact with HetR at its hood domain, we hypothesized that HetL could interact with HetR at the same interface than PatS, which would therefore interfere with its inhibiting action. To test this hypothesis, we took advantage of the fact that HetR-PatS interaction involves the Hood domain of HetR and that the residue R223 of HetR is required for this interaction. Interestingly, a variant of HetR bearing a R223W substitution lost the ability to interact with HetL (Figure 2A). HetR (R223W) was still able to form dimers (Figure 2A), which indicates that this variant is correctly folded. We conclude that the absence of interaction between HetR (R223W) and HetL indicates that this residue, in addition to be involved in the interaction with PatS-5/PatS-6, is also required to the interaction with HetL. For a deeper investigation of HetR-HetL interaction, we exploited the available structures of HetL and of the Hood domain of HetR to build interaction models (Ni et al., 2009) (Hu et al., 2015). Interestingly, a group of four models among the top 10 clusters share similar orientations of HetR on HetL (supplementary figure 1C). Importantly, these models present interesting properties regarding the binding interface of HetR-Hood to HetL: (i) the residue R223 of HetR was involved in the binding interface with HetL (Figure 2B, supplementary figure 1C), (ii) HetR interaction interface with HetL matches with the one involved in PatS-5 interaction (Figure 2B). In the retained models, the HetR-Hood:HetL interaction is maintained by a large network of electrostatic interactions and hydrogen bonds. For HetR, the binding interface is composed by the surface exposed residues from strand β3 and α-helix α12’ (Figure 2B). In HetL, the large proportion of the binding interface includes the pentapeptides repeat localized in face 3 encompassed E84ADLT, K104ASLC, 124QADLR, 149YADLR, 169RANFG and 197YANLE. A small second binding interface is localized within face 2 and includes 40ADLRQ and 145ADLSY pentapeptide repeats.
To gain further information about HetR:HetL interaction, we performed a site directed mutagenesis to substitute the residue D151 from HetL to Alanine. Interestingly, this substitution impaired the binding of HetL to HetR as revealed by BACTH assay (Figure 2A). Furthermore, HetL D151A variant was still able to form dimers which is an important indication to rule out the possibility that the mutation destroyed the fold of this variant (Figure 2A). Taken together, these results indicate that HetL and PatS-5 interact with HetR at the same interface, which implies that HetL and PatS must compete for the interaction with HetR. To check this assumption, the interaction between HetR and HetL was analyzed by BLi in the presence of increasing amounts of PatS-5. The data obtained clearly indicated that the association between HetR and HetL is impaired from the addition of PatS-5 in a dose-manner response, with a total inhibition effect obtained at a concentration of 5 µM (Figure 2C). Similar results were obtained with PatS-6 (supplementary figure 2A).

The effect of PatS on HetR-HetL interaction was further analyzed by two hybrid assays. For this, a synthetic operon constituted of T18-hetL and patS-6 was constructed and used to question the interaction with HetR. While adenylate cyclase activity was restored in bacteria producing T25-HetR and T18-HetL fusions, the interaction between HetR and HetL was abolished when PatS-6 was produced (Supplementary Figure 1B), which is a further demonstration of the fact that PatS and HetL share the same interaction within HetR. The interference of PatS with the HetR-HetL complex was also observed in EMSA experiments where the addition of increasing amounts of PatS-5 abolished the binding of HetR-HetL to the hetP promoter (Figure 2D). Altogether, these data support the hypothesis of a competition between PatS and HetL for the interaction with HetR.
Figure 2: HetL-HetR interaction interface.

(A) Bacterial two hybrid assay between HetL and HetR. β-galactosidase activities were measured as described in section “Materials and Methods” and were expressed in Miller units. Strains producing the T18 and T25 served as negative control (control -). Strains producing T18-Zip and T25-Zip served as positive control (control +).

(B) Model of HetR-Hood:HetL complex. Monomers of HetR-Hood dimer structure are presented in green and light pink. HetL is presented in cyan. D151 from HetL and R223 from HetR are indicated.

(C) BLi assay between HetL and HetR in the presence of PatS-5. 10 µM of HetL was incubated 5 mins with different concentrations of PatS at 0.5, 1, 2.5 and 5 µM before bringing HetL in contact with the bound HetR. Each curve represents the average of two experiments minus the control experiment. Control experiment HetL plus different concentrations of PatS loaded onto a biosensor devoid of HetR.
**HetL does not interact with PatS**

Based on the data presented above, HetR acquired immunity against PatS inhibition can be explained by an exclusion of the inhibitor as a consequence of HetR-HetL interaction. However, an additional titration-based mechanism through a direct interaction between HetL and PatS cannot be ruled out. To test this possibility, Isothermal titration calorimetry (ITC) was used to analyze the possible interaction between HetL and PatS-5. This technique has been used previously to uncover the binding of PatS-5 to HetR (Feldmann et al., 2011). We have confirmed that HetR does indeed interact with PatS-5 with a dissociation constant ($K_D$) of 600nM similar to that of 227nM described in the literature with a stoichiometry of 1:1. The data of Figure 3 show that HetR (left panel) displays a reducing heat exchange when titrated with increasing amounts of PatS-5, indicating a saturation of HetR sites with PatS-5. This reaction is an exothermic favorable reaction with an enthalpy of $-7.43 \pm 0.67 \Delta H$ kcal/mol. On the contrary, HetL (right panel) does not show a relative heat exchange upon binding to PatS-5. The interaction between HetR and PatS-5 observed in Figure 3, validates our technical experiments and revokes the possibility that HetL could titrate PatS-5 by a direct interaction.
**Figure 3:** HetL does not interact with PatS-5.

ITC experiment to detect HetL interaction with PatS-5. The right panel shows HetR and PatS-5 interaction. The left panel shows the non-interaction of HetL and PatS-5. The respective upper panels show heat exchange upon ligand titration and bottom panels show integrated data with binding isotherms (solid line) fitted to a single-site binding model. The constant heat dilution was removed before the integrated binding isotherms. The titrant PatS-5 (800µM) was titrated into a cell containing 23µM HetR at 25°C.

**The interaction between HetL and HetR is required to HetR function in vivo**

As *hetL* was discovered on the basis of its capacity, when overexpressed, to suppress the inhibitory effect of PatS, we used this approach to evaluate the impact of the interaction between HetR and HetL on the differentiation process. The wild type version of *hetL* or the mutated gene encoding HetL (D151A) were expressed in *Nostoc* cells under the control of the *petE* promoter, and quantitative RT-PCR analyses were carried out to check the accurate overexpression of these genes upon induction. Results revealed an over 30-fold higher expression of the *hetL* and the *hetL* (D151A) genes in the recombinant strains compared to the wild type (**Figure 4A**), indicating that the two versions of *hetL* are actually overexpressed. PatS effect was analyzed either by the overexpression of *patS* gene from the *petE* promoter or by the addition in the medium of PatS-5 (or PatS-6). The cultures were transferred into
a nitrate-depleted medium during 48 hours to assess heterocyst development. In agreement with published data, the overproduction of PatS or the addition of PatS-5 inhibited the differentiation process, while the overexpression of *hetL* restored the ability of the PatS overproducing strain to form heterocysts (Figure 4B). HetL overproduction also allowed the ability of the strain to form heterocysts when PatS-5 was added in the culture (Supplementary figure 2). The percentage of the heterocysts formed in the *hetL* overexpressing strain was equal to that of the wild type strain (Table 1). Interestingly, the recombinant strain overproducing HetL (D151A) was not able to form heterocysts when PatS was overproduced or upon addition of PatS-5 in the growth medium (Figure 4B, Supplementary figure 2). The interaction of HetL with HetR is therefore needed to counteract PatS inhibition.
Figure 4. HetL-HetR interaction is required for heterocyst differentiation.

(A) *hetL* or *hetLD151A* in the indicated strains was overexpressed from the *petE* promoter. Gene expression was induced with 3 μM copper. qRT-PCR experiments were undergone to determine *hetL* or *hetLD151A* expression level.

(B) Microscopic bright field images (upper) and auto-fluorescence images (lower) of indicated *Nostoc* strains after nitrogen stepdown in addition of 3 μM copper. White arrows point to heterocysts. Images with heterocysts are framed in red.

**hetL** expression in the heterocyst is required for providing HetR immunity against PatS

**inhibition**

*hetL* expression level is too low to be detected by Northern blot or fluorescent gene fusions (D. Liu & Golden, 2002). We therefore chose quantitative RT-PCR approach to analyze *hetL* transcription during the differentiation process. The *hetP* gene whose transcription is activated by HetR 8 hours after nitrogen step-down was used as an internal control for this experiment (Mitschke et al., 2011). RNAs were collected from the wild type strain and the ΔhetR mutant at different times after nitrogen starvation and the transcript levels of the two genes were expressed relatively to their amount at time zero. Results reveal that *hetP* expression was, as expected, strongly induced starting from 8 hours after nitrate depletion. Contrary to the 10-fold induction in the wild type strain, the expression of *hetP* did not significantly increase in the ΔhetR strain which is consistent with the activation of this gene by HetR (Figure 5A). The *hetL* gene showed a similar transcription profile to that of *hetP*, yet its expression level was much lower. In the wild type strain, a 3.5-fold increase of *hetL* transcripts was observed 8 hours after nitrogen step-down and was maintained up to 24 hours. In the ΔhetR mutant, no induction of *hetL* transcription was observed. The region including 500 nucleotides upstream of the start codon of *hetL*, which must include the promoter of this gene, was analyzed searching for the two binding sites consensus reported for HetR: the high affinity consensus (GTAGGCGAGGGTCTAACCCCTCATTACC), and the low affinity one (GCTTATGGTGCCAATGCCCACCATAATA) (Videau et al., 2014). None of these sequences are present in the *hetL* promoter. We concluded that, even if low, the transcription of *hetL* gene is induced
early during the differentiation program and that HetR is required for hetL activation but its action is likely indirect and mediated by another factor.

From the results presented above, it can be deduced that HetL acts in the heterocyst. To further confirm this assumption, we expressed hetL either from the rbcL promoter, which is specific to vegetative cells, or from the patS promoter which is expressed in the heterocysts early after nitrogen stepdown. The ability of HetL to suppress heterocyst inhibition triggered by the addition of PatS-5 was analyzed. Results show that the strain expressing the PpatS-hetL gene was able to form heterocysts even in the presence of PatS-5, but when expressed from the rbcL promoter, hetL was unable to prevent the inhibitory effect of PatS-6 (Figure 5B). This result is in favor of HetL acting specifically in the heterocyst.
Figure 5. *hetL* expression is under the control of HetR

(A) *hetL* expression is induced in response to nitrogen starvation and is dependent on HetR. qRT-PCR was made to determine the *hetL* expression in WT and Δ*hetR* strains. *hetP* expression was determined as inner positive control.

(B) Effect of *hetL* expression from patS promoter or *rbcL* promoter. Microscopic bright field images (left) and auto-fluorescence images (right) of indicated *Nostoc* strains after 24 hours of nitrogen stepdown are shown. White arrows point to heterocysts. Images with heterocysts are with red frames.

HetL provides immunity to PatX-derived pentapeptide

In addition to the RGSGR pentapeptide, heterocyst pattern formation has been recently shown to involve the HRGTGR peptide derived from the PatX protein (Elhai & Khudyakov, 2018). To further characterize HetL function, we wondered whether it would be involved in PatX signaling as well. Bli experiments showed that the HRGTGR peptide, like PatS-5, inhibited the interaction between HetR and HetL (Figure 6A). In addition, the strain expressing the *PpetE-hetL* gene was able to form heterocysts even in the presence of the HRGTGR peptide (Figure 6B), while the overproduction of HetL (D151A) did not allow to bypass PatX-6 inhibition since heterocysts were not observed (Figure 6B). From these results, we conclude that HetL provides immunity to the developing cells against the two inhibitory peptides involved in pattern formation.
**Figure 6** HetL provides protection against PatX-derived pentapeptide.

(A) BLi assay between HetL and HetR in the presence of PatX-6 (HRGTGR). 10 µM of HetL was incubated 5 mins with different concentrations of PatX6 at 0.05, 0.1, 0.5 and 2.5 µM before bringing HetL in contact with the bound HetR. Each curve represents the average of two experiments minus the control experiment. Control experiment HetL 10µM loaded onto a biosensor devoid of HetR.

(B) WT/PpetE-hetL strain was able to form heterocysts even in the presence of the PatX-6 peptide. Microscopic bright field images (upper) and auto-fluorescence images (lower) of indicated *Nostoc* strains after 24 hours of nitrogen stepdown in addition of 3 µM copper are shown. White arrows point to heterocysts. Images with heterocysts are with red frames.

HetR interacts with a pentapeptide repeat protein homolog to HetL

In addition to HetL, the genome of *Nostoc* contains 31 genes potentially coding for PRPs of various sizes. This large family includes proteins predicted to be located either in the cytoplasm, in the membrane or in the periplasm (Ni et al., 2009). Five of them display PRs among other domains, while the others, such HetL, are integrally formed by PR domains (Supplementary Table 1). Given the high sequence identity shared by these PRPs (32% in average), predicting functional specificity based on
sequence similarity is not possible. Because hetL mutant does not show any specific differentiation phenotype (D. Liu & Golden, 2002), we wondered if this could be due to a cross-complementation with another PRP. In this regard, we analyzed the capacity of HetR to interact with some HetL homologs. For this, we chose All3256 and All4303 because they share closest features with HetL. They have a similar size (237, 268, 213 amino acids respectively), a similar organization of the PRs domains, and the three are predicted to be cytosolic (Figure 7A). Figure 7B shows the results of a BACTH assay questioning the putative interaction of these proteins with HetR. Only All4303 displayed interaction with HetR, and even if this interaction is two-fold weaker than that of HetR-HetL it is significant compared to the negative control. This experiment indicates that at least one among the 31 PRPs is able to interact with HetR, which makes a cross-complementation of the hetL mutation with all4303, or another PRP coding gene a possible scenario.
Figure 7. HetR interacts with a HetL homolog and patterning model including HetL.

(A) HetL, All3256 or All4303 PR domains organization pentapeptide repeat domains as HetL.

(B) Bacterial two hybrid assay between HetR and HetL paralogs. BTH101 strain was transformed with pKT25-hetR and pUT18C-all4303 or pUT18C-all3256, β-galactosidase activities were measured as described in section “Materials and Methods” and were expressed in Miller units. Strains producing the T18 and T25 served as negative control. Strains producing T18-Zip and T25-Zip served as positive control.

(C) Schematic model integrating HetL in the patterning system of Nostoc.

R: HetR, L: HetL or HetL homolog, IM: inhibiting morphogen (Providing from the processing of PatX, PatS or HetN).

Local activation/protection: once activated, HetR activates the transcription of hetR, hetL, patS/hetN. The HetR/HetL network is favored. HetL provides immunity against the inhibiting morphogens produced in situ or entering the cell by diffusion. HetR is active, heterocyst develops.

Long range inhibition: the diffusion of the morphogens creates an inhibition gradient. In the HetR/Morphogen network, HetR is inactive and the concentration of HetL is below the protective threshold. Differentiation is inhibited.

Discussion

Eighteen years ago, the hetL gene was discovered on the basis of its ability, when overexpressed, to bypass the inhibitory effect of patS overexpression on cell differentiation (D. Liu & Golden, 2002). In their conclusion, the authors of this study speculated that PatS and HetL might act by modulating HetR activity. This speculation reveals to be an accurate prediction as demonstrated by the results of the experiments presented in this manuscript, added to all the knowledge accumulated on HetR and PatS functions from previous valuable studies that are fundamental to our investigation. In particular, as the unique structural fold of HetR with its two exposed domains (flap and hood) was proposed to favor protein-protein interactions, we questioned the possible interaction of HetR with proteins involved in patterning. In this context, HetL was found to interact strongly with HetR without abolishing its DNA-binding activity, which suggests that HetR-HetL complex may be active regarding gene regulation in vivo (Figure 1). A possible mechanism to explain the role of HetL in PatS signaling is the titration by HetL of the inhibiting peptide. This hypothesis was ruled out since ITC assay did not show any interaction between HetL and PatS (Figure 3). Alternatively, a site-exclusion mechanism can be proposed for HetL. The observation that HetL and PatS-5 (or PatS-6) interact with HetR at the same
interface is in agreement with this suggestion (Figure 2B). In Bli assays, increasing concentrations of PatS-5 interfered with HetR-HetL interaction, and in EMSA experiments addition of PatS-5 abolished the formation of HetR-HetL complex in a concentration-dependent manner (Figure 2C-D). In addition to confirming the docking model predicting a same HetR-interaction interface for PatS and HetL, these data imply that the concentration of HetL in the (pro)heterocyst must be higher than that of PatS peptide, which is plausible because (i) PatS peptide is diffusible (Wu et al., 2004; Yoon & Golden, 2001), (ii) HetL features do not include any motif or domain that predicts a putative translocation/secretion or association with membranes that could decrease its amount in the producing cell. Moreover, as hetL transcription is activated by HetR (Figure 4), HetL protein is likely to accumulate in the cytoplasm of differentiating cells.

Alternatively, it can be assumed that in the (pro)heterocyst the affinity of HetR for HetL must be higher than that for PatS. Bli and ITC experiments showed that in vitro, the affinity of HetR to PatS is 10-fold higher than for HetL. (compare data of Figure 2C and Figure 3). One might speculate that in vivo, and especially in the (pro)heterocyst, the affinity of HetR for HetL increases either due to a modification of HetR or to its interaction with another factor. HetR has been shown to be regulated by phosphorylation (Valladares et al., 2016) (Roumezi et al., 2019), if this posttranslational modification occurs only in the developing cell it could explain a high affinity of HetR for HetL specifically in the heterocyst. In addition, the observation that the overexpression of hetL in vegetative cells, from petE or rbcL promoters, did not lead to their differentiation into heterocysts is rather in favor of a mechanism promoting the association between HetR and HetL specifically in the heterocyst. Resolving the structure of HetR-HetL complex and following the behavior of HetL protein and PatS in vivo will help getting more information about the dynamics and the nature of HetR complexes in the two cell types through the differentiation process.

A deletion mutant of hetL differentiates heterocysts as well as the wild type strain (D. Liu & Golden, 2002). Function redundancy or overexpression-dependent action are the two hypotheses that have been presented to explain hetL mutant phenotype. The observations that hetL gene is expressed after nitrogen
starvation, under the positive control of HetR, and that at least one homolog of HetL (All4303) interacted with HetR, are rather in favor of functional redundancy. A global transcription study using RNA sequencing has shown that all4303 transcription is induced in response to nitrogen starvation (Flaherty, Van Nieuwerburgh, Head, & Golden, 2011). The hetL gene has also been reported in this study to be induced after nitrogen starvation, which comforts our data. The other PRPs coding genes whose transcription has also been reported to be regulated in this condition are reported in Supplementary Table 1. At this stage of our investigation, we cannot rule out that homologs of HetL might also be part of PatS signaling, but the fact that the genetic suppressor screen for elevation of PatS inhibition selected only hetL would rather point it as the principal factor involved in the differentiation process (D. Liu & Golden, 2002). Because of the presence of multiple homologs, cross-complementation might occur when hetL happens to be mutated.

Genes encoding proteins containing tandem pentapeptide repeats are abundant in all cyanobacterial genomes, which render the study of their function by gene-deletion approaches precarious. Interestingly, despite their high amino acid homology, their structures possess distinctive features that can help investigating their function. Exploring these structural features and searching for potential binding-partners can be a successful strategy in their study. The gyrase-binding PRP is the first protein of this family whose partner was identified (Tran, Jacoby, & Hooper, 2005). PPRs from this family are largely conserved among bacteria, where number of them have been, based on their ability, proposed to provide resistance to quinolone-type antibiotics. Structural studies revealed the distribution of large contiguous patches of negative electrostatic potential resembling DNA which gave insights in their function as mimicking DNA structure for binding to gyrase (Xiong, Bromley, Oelschlaeger, Woolfson, & Spencer, 2011). The structure of HetL does not show such a large distribution of negative charges, but instead contiguous patches of positively and negatively charged surfaces are distributed at the surface of the structure. These patches have been suggested to mediate the binding to unknown potential partners, other than DNA (Ni et al., 2009). In docking simulation analyses reported in this study, these charged surfaces especially those localized in face 2 and face 3 mediate the interaction with the HetR-Hood domain. At the best of our knowledge, the interaction of HetL with HetR constitutes the second case of
protein-protein complex identified for a PRP member. In *Nostoc* two other integral PRPs (PatL (J. Liu & Wolk, 2011) and FraF (Merino-Puerto, Herrero, & Flores, 2013)) and a pentapeptide repeat domain-containing protein (HglK, (K. Black, Buikema, & Haselkorn, 1995)) are involved in cell differentiation, but their mechanism of action is not fully uncovered. Analyzing their interaction with their physiological partners will provide a better understanding of the role played by PRPs in the developmental program of *Nostoc*.

Genetic experiments showed that HetL interaction with HetR is critical for escaping PatS overproduction and addition to the culture medium of the (E)RGSGR and (H)RGTR inhibiting peptides (Figure 4, 6) which suggests that HetL provides immunity to the developing cells all along the differentiation process. HetL might therefore be important for pattern initiation and establishment, exerted by PatX and PatS, and also for pattern maintenance mediated by HetN and PatS. We propose that HetR can be engaged in two different networks depending on cell types: a HetR-HetL positive network in the (pro)heterocyt as a consequence of hetL expression, and a negative HetR-inhibiting peptide complex taking place in the HetL-free vegetative cells. The HetR/HetL network implements the local-activation and long-range inhibition model with a third module consisting on local protection which explains how a differentiating cell gets immune to self-inhibition (Figure 7C). An interesting particularity of patterning in *Nostoc* is that future heterocysts do not emerge as isolated cells. Rather, strings of 3-4 developing cells appear before resolving into a single proheterocyst (Wilcox, Mitchison, & Smith, 1973). These clusters of cells “competent” for differentiation were suggested to be correlated to the stochastic fluctuation in hetR expression (Corrales-Guerrero et al., 2015). In addition, we propose that a stochastic variation of hetL expression among the string of competent cells might also be part of the resolving mechanism fixing the decision to differentiate in the cell that inherits enough HetL to be protected from self-inhibition. Microfluidic approaches probing multiple and integrated aspects of heterocyst formation along with mathematical models should shed light on how patterning emerges, resolves and persists in *Nostoc*. An interesting evolutionary aspect resulting from the conservation of PRP in cyanobacterial genomes is that the function of HetL described here might be accurate for other heterocyst-forming cyanobacteria. It is also likely that understanding all the parameters and factors

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- **References:**
  - Corrales-Guerrero et al., 2015
  - Wilcox, Mitchison, & Smith, 1973
governing heterocyst-pattern formation will be helpful in discovering the molecular mechanisms underlying patterning in more complex biological systems.
**Materials and methods**

**Strains, plasmids and primers:**

The strains, plasmids and primers used in this study are listed in Supplementary file 1. All the cyanobacterial strains are derivative from *Nostoc* PCC 7120 (Pasteur Cyanobacterial Collection, https://www.pasteur.fr/fr/sante-publique/crbip/les-collections/collection-cyanobacteries-pcc).

**Growth conditions, conjugation and heterocyst induction:**

*Nostoc* and derivatives were grown in BG11 medium at 30 °C under continuous illumination (40 µE m⁻²s⁻¹). When appropriate, media were supplemented with antibiotics at the following concentrations: neomycin (50 µg mL⁻¹), erythromycin (200 µg mL⁻¹). Heterocyst formation was induced by transferring the exponentially growing cultures (OD 750 = 0.8) to BG110 (BG11 without sodium nitrate) medium. The presence of heterocysts was confirmed by microscopy.

Conjugation of *Nostoc* was performed as described in reference (Cai & Wolk, 1990). Briefly, *E. coli* strains (bearing the replicative plasmid and the RP-4 conjugative plasmid) grown to exponential growth phase, were mixed to an exponentially grown *Nostoc* culture. The mixture was plated on BG11 plates and antibiotics were added 24 hours later for plasmid selection.

**Plasmid construction:**

All the plasmids used in this study are listed in Supplementary file 1.

The strategy used for plasmid construction is briefly described below. All the recombinant plasmids were analyzed by sequencing.

*pXX1*: pKT25-*hetR*

The open reading frame of *hetR* gene (alr2339) was amplified using the *hetR* dh fw T25 and *hetR* dh rv primers and cloned into the PstI and EcoRI restriction sites of the pKT25 expression plasmid.

*pXX2*: pKT25-*hetL*
The open reading frame of \textit{hetL} gene (all3740) was amplified using the \textit{hetL} dh fw T25 and \textit{hetL} dh rv primers and cloned into the PstI and EcoRI restriction sites of the pKT25 expression plasmid.

\textbf{pXX3: pKT25-\textit{hetL}D151A}

The \textit{pXX2} plasmid was used as template to substitute the D151 residue of HetL to Alanine. For this the primers Mut \textit{hetL}D151A fw and Mut \textit{hetL}D151A rv were used as primers in a megapriming PCR assay.

\textbf{pXX4: pUT18C-\textit{hetR}}

The open reading frame of \textit{hetR} gene (alr2339) was amplified using the \textit{hetR} dh fw T18 and \textit{hetR} dh rv primers and cloned into the PstI and EcoRI restriction sites of the pUT18C expression plasmid.

\textbf{pXX5: pUT18C-\textit{hetR}R223W}

The \textit{pXX4} plasmid was used as template to substitute the R223 residue of HetR to Tryptophan. For this the primers Mut \textit{hetR}R223W fw and Mut \textit{hetR}R223W rv were used as primers in a megapriming PCR assay.

\textbf{pXX6: pUT18C-\textit{hetL}}

The open reading frame of \textit{hetL} gene (all3740) was amplified using the \textit{hetL} dh fw T18 and \textit{hetL} dh rv primers and cloned into the PstI and EcoRI restriction sites of the pUT18C expression plasmid.

\textbf{pXX7: pUT18C-\textit{hetL}-RBS-\textit{patS}}

The open reading frame of \textit{patS} gene (asl2301) with extra 350 bp after the stop codon, was amplified using the RBS-\textit{patS} dh fw T18 and RBS-\textit{patS} dh rv T18 primers and cloned into the SalI and XhoI restriction sites of the \textit{pXX6} expression plasmid. The RBS is the one from \textit{petE} promoter.

\textbf{pXX8: pUT18C-\textit{hetL}-RBS-\textit{patS6}}

The open reading frame of \textit{patS6} (encoding for PatS6 peptide: ERGSGR) with extra 350 bp after the stop codon, was amplified using the RBS-\textit{patS6} dh fw T18 and RBS-\textit{patS6} dh rv T18 infusion primers and cloned into the SalI restriction site of the \textit{pXX6} expression plasmid. The RBS is the one from \textit{petE} promoter.
The open reading frame of *hetR*hood (encoding for HetRhood from Y215 to R296) was amplified using the *hetR*hood dh fw and *hetR*hood dh rv primers and cloned into the PstI and EcoRI restriction sites of the pUT18 expression plasmid.

The open reading frame of *all3256* gene was amplified using the *all3256* dh fw T18 and *all3256* dh rv primers and cloned into the PstI and EcoRI restriction sites of the pUT18C expression plasmid.

The open reading frame of *all4303* gene was amplified using the *all4303* dh fw T18 and *all4303* dh rv primers and cloned into the PstI and EcoRI restriction sites of the pUT18C expression plasmid.

The open reading frame of *hetL* gene (all3740) was amplified using the *hetL* pET28 fw and *hetL* pET28 rv infusion primers and cloned into the XhoI and NcoI restriction sites of the pET28a expression plasmid using the In-Fusion technology (Takara In-Fusion® HD Cloning kit).

The open reading frame of *patS* gene (asl2301) with extra 700 bp after the stop codon, was amplified using the *patS* pRL fw and *patS* pRL rv infusion primers and cloned into the BamHI restriction site of the pRL1272-PpetE replicative plasmid in *Nostoc* (Takara In-Fusion® HD Cloning kit).

The open reading frame of *hetL* gene (all3740) was amplified using the *hetL* pRL fw and *hetL* pRL rv infusion primers and cloned into the BamHI restriction site of the pRL25T-PpetE replicative plasmid in *Nostoc* (Takara In-Fusion® HD Cloning kit).
The open reading frame of *hetL* gene mutated to encode for a D151A substitution was amplified using the *hetL* pRL fw and *hetL* pRL rv infusion primers with pKT25-*hetLD151A* as template and cloned into the BamHI restriction site of the pRL25T-PpetE replicative plasmid in *Nostoc* (Takara In-Fusion® HD Cloning kit).

pSC1: pRL25T-P*patS*-*hetL*

The promoter of *patS* gene was amplified using *PpatS* fw and *PpatS* rv, the *hetL* coding region was amplified using *hetL* P*patS* fw and *hetL* P*patS* rv. *Nostoc* genomic DNA was used as template for both amplifications; The two amplicons were cloned into the BamHI restriction site of the pRL25T plasmid using the In-Fusion technology.

pSC2: pRL25T-P*bcL*-*hetL*

The promoter of *rbcL* gene was amplified using *PrbcL* fw and *PrbcL* rv, the *hetL* coding region was amplified using *hetL* P*bcL* fw and *hetL* P*bcL* rv. *Nostoc* genomic DNA was used as template for both amplifications. The two amplicons were cloned into the BamHI restriction site of the pRL25T plasmid using the In-Fusion technology.

**Protein purification:**

For HetL purification, BL21DE3 strain containing the pXX12 plasmid was grown until an optical density (OD 600nm) of 0.6. *hetL* induction was achieved by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG, SIGMA) of 0.4 mM over night at 16 °C. Cells were harvested at 8000 rpm at 4°C during 2 min. The pellet was re-suspended in 25 mL of lysis buffer (50 mM Tris HCl (pH 8), 0.3 M NaCl), and cells were disrupted using French press. After centrifugation at 8000 rpm for 30 min at 4°C, the supernatant was loaded onto a column containing 1 mL of NiNTA agarose resin (Qiagen, Hilden, Germany) pre-equilibrated with lysis buffer containing 10 mM Imidazole. The column was rinsed with 10 mM and 35 mM Imidazole, both prepared in lysis buffer. Fractions were collected (in 200 mM Imidazole buffer, prepared in lysis buffer). The Imidazole was eliminated using the PD10 columns (GE Healthcare). The proteins were concentrated using Vivaspin columns (SIGMA) and
quantified using the Bradford assay (SIGMA). HetR purification was undergone as previously described (Hu et al., 2015).

**Electrophoretic mobility shift assays (EMSA):**

The promoter region of the hetP gene (alr2818) was obtained by PCR using PhetP fw and PhetP rv primers. The forward primer was modified at its 5’ end by adding the 6-carboxyfluorescein (6-FAM) dye. Purified HetR (1uM) and HetL (2-4uM) proteins, were incubated with the hetP promoter (50 nM) in a buffer containing 10 mM Tris (pH 8), 150 mM potassium chloride, 500 nM EDTA, 0.1% Triton X-100, 12.5% glycerol, 1 mM dithiothreitol and 1 µg DiDC competitor (poly(2’-deoxyinosinic-2’-deoxycytidylic acid) sodium salt), at 4 °C in dark for 30 minutes. The electrophoresis was performed at 250 volts for 60 minutes. The DNA was revealed using Typhoon FLA 9500 (GE Healthcare Life Sciences). The experiment was repeated three times with independent protein purifications and one representative result is shown.

**RNA preparation, reverse transcription, and quantitative Real-Time-PCR:**

RNAs were prepared using the Qiagen RNA extraction kit (Qiagen) following the manufacturer’s instructions. An extra TURBO DNase (Invitrogen) digestion step was performed to eliminate the contaminating DNA. The RNA quality was assessed by tape station system (Agilent). RNAs were quantified spectrophotometrically at 260 nm (NanoDrop 1000; Thermo Fisher Scientific). For cDNA synthesis, 1 µg total RNA and 0.5 µg random primers (Promega) were used with the GoScript™ Reverse transcriptase (Promega) according to the manufacturer instructions. Quantitative real-time PCR (qPCR) analyses were performed on a CFX96 Real-Time System (Bio-Rad). The reaction volume was 15 µL and the final concentration of each primer was 0.5 µM. The qPCR cycling parameters were 95°C for 2 min, followed by 45 cycles of 95°C for 5 s, 55°C for 60 s. A final melting curve from 65°C to 95°C was added to determine the specificity of the amplification. To determine the amplification kinetics of each product, the fluorescence derived from the incorporation of BRYT Green® Dye into the double-stranded PCR products was measured at the end of each cycle using the GoTaq® qPCR Master Mix 2X Kit (Promega). The results were analysed using Bio-Rad CFX Maestro software, version 1.1 (Bio-Rad,
France). The RNA 16S gene was used as a reference for normalization. All measurements were carried out in triplicate and a biological duplicate was performed for each point. The amplification efficiencies of each primer pairs were 80 to 100%. All of the primer pairs used for qPCR are reported in Supplementary file 1.

**Bacterial two hybrid assays:**

Bacterial two-hybrid assays were performed following the procedure described by Karimova et al (1998) (Karimova et al., 1998). Briefly, after co-transforming the BTH101 strain with the two plasmids expressing the T18- and T25- fusions, LB plates containing ampicillin and kanamycin were incubated at 30°C for 2 days. For each assay, 10 independent colonies were inoculated in 3 ml of LB medium supplemented with ampicillin, kanamycin and 0.5 mM IPTG, and incubated at 30°C overnight. ß-galactosidase activity was determined as previously described (Zubay, Morse, Schrenk, & Miller, 1972). The values presented are means of 3 independent assays.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE):**

Proteins were fractionated by performing SDS-PAGE (12% except where indicated) stained with Coomassie blue (Euromedex, Souffelweyersheim, France). For immunoblot analysis, the proteins were transferred to nitrocellulose membranes before being revealed with anti-Histidine monoclonal antibodies (Qiagen). Immune complexes were detected with anti-rabbit peroxidase-conjugated secondary antibodies (Promega) and enhanced chemiluminescence reagents (Pierce, Illkirch, France).

**Synthetic peptides:**

PatS-5 (RGSGR), Pat-6 (ERGSGR) and PatX-6 (HRGTGR) peptides were synthesized by Genecust (https://www.genecust.com/en/).

**Isothermal titration calorimetry (ITC):**

ITC was performed to demonstrate the interaction of HetR and HetL with PatS peptides. The working buffer for both proteins and peptides was PBS pH 7.4 to avoid buffer mismatch. The experiments were performed at 25°C using the MicroCal PEAQ-ITC (Malvern UK) with 19 injections, first with an initial
injection of 0.4 µL followed by 18 injections of 2 µL. The protein ligands were in the cell and the peptide analytes were in the syringe. The reaction was performed with a constant stirring speed of 750 rpm, each injection lasted for 4 seconds with a 150 second space between each injection. A constant heat control (offset) was removed from the raw data to account for heat dilution before integration. The data were fitted using a ‘One Set of Sites’ model in the PEAQ-ITC Analysis Software.

**Bio-Layer interferometry assays:**

The BLi machine (BLItz) from FortéBio was used to perform biolayer interferometry to determine the interaction between HetR and HetL. 2.2 µM of biotinylated HetR was loaded onto streptavidin biosensors in PBS. A 30 second baseline in PBS was performed before a 120 second association step with various concentrations of HetL at 2.5, 5, 10 and 20 µM followed by a 120 second dissociation step. To determine if the PatS-5 and PatS-6 peptides compete with HetL for HetR binding, 10 µM of HetL was incubated 5 mins with different concentrations of PatS at 0.05, 0.1, 0.5 and 2.5 µM before adding HetL to the bound HetR. All binding was performed in triplicate, the dissociation constant was obtained using the BLItz Pro Data Analysis software using a global 1:1 fit model.

**Molecular docking simulation:**

The available atomic coordinates of the hood domain of *Nostoc* HetR (PDB ID: 4YNL) and HetL (PDB ID: 3DU1) were used as templates for molecular docking simulations. Molecular docking study of the HetR with HetL was performed using HADDOCK2.2 webserver (http://milou.science.uu.nl/services/HADDOCK2.2/) (van Zundert et al., 2016). The goal was to identify critical residues involved in HetR-HetL complex formation. To define Haddock run restraints, the surface exposed residues of both HetR and HetL were considered as active residues directly involved in the interaction. For both structures, the surface exposed residues were selected manually using PyMol (Supplementary Table 2). Two independent docking simulations were performed. For each run, ten clusters were generated, and classified based on their HADDOCK score. The best model of each cluster was analyzed by PDBePISA to explore their binding interfaces.

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Author contributions

AL conceived and designed the study. XX, VR and SC performed the research. DB supervised Blais assays. BD performed docking analysis. AL wrote the manuscript.

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Table 1

Percentage of heterocysts formed by different strains used in this study after combined nitrogen starvation. The number of the filaments analyzed was 60-100 in average.

| Strain and condition                        | % of heterocysts, 24H after nitrogen starvation | Mean interval between 2 heterocysts |
|---------------------------------------------|------------------------------------------------|------------------------------------|
| Wild type                                   | 9.1±1.6                                        | 10.7±1.3                           |
| WT/PpetE-patS                               | 0                                              | ND                                 |
| WT and PatS-5 addition                      | 0                                              | ND                                 |
| WT/PpetE-hetL                               | 9.±1.6                                         | 10.7±1.3                           |
| WT/PpetE-patS PpetE-hetL                    | 9.±1.6                                         | 10.7±1.3                           |
| WT/PpetE-hetL and PatS-5 addition           | 9.±1.6                                         | 10.7±1.3                           |
| WT/PpetE-patS PpetE-hetL[D151A]             | 0                                              | ND                                 |
| WT/PpetE-patS PpetE-hetL[D151A]             | 0                                              | ND                                 |