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The Pkn22 Kinase of *Nostoc* PCC 7120 Is Required for Cell Differentiation via the Phosphorylation of HetR on a Residue Highly Conserved in Genomes of Heterocyst-Forming Cyanobacteria

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Hanks-type kinases encoding genes are present in most cyanobacterial genomes. Despite their widespread pattern of conservation, little is known so far about their role because their substrates and the conditions triggering their activation are poorly known. Here we report that under diazotrophic conditions, normal heterocyst differentiation and growth of the filamentous cyanobacterium *Nostoc* PCC 7120 require the presence of the Pkn22 kinase, which is induced under combined nitrogen starvation conditions. By analyzing the phenotype of *pkn22* mutant overexpressing genes belonging to the regulatory cascade initiating the development program, an epistatic relationship was found to exist between this kinase and the master regulator of differentiation, HetR. The results obtained using a bacterial two hybrid approach indicated that Pkn22 and HetR interact, and the use of a genetic screen inducing the loss of this interaction showed that residues of HetR which are essential for this interaction to occur are also crucial to HetR activity both in vitro and in vivo. Mass spectrometry showed that HetR co-produced with the Pkn22 kinase in *Escherichia coli* is phosphorylated on Serine 130 residue. Phosphoablative substitution of this residue impaired the ability of the strain to undergo cell differentiation, while its phosphomimetic substitution increased the number of heterocysts formed. Heterologous complementation assays showed that the presence of this domain is necessary for heterocyst induction. We propose that the phosphorylation of HetR might have been acquired to control heterocyst differentiation.

**Keywords:** cell differentiation, cyanobacteria, genomic conservation, Hanks-kinase, phosphorylation
INTRODUCTION

Protein phosphorylation/dephosphorylation processes play an important role in signal transduction and in regulation of physiological functions in all three domains of life. The nature of the amino-acid phosphorylated depends on the kinase family. The kinases phosphorylating proteins on Serine/Threonine or Tyrosine residues are named Hanks-type kinases (Hanks and Hunter, 1995; Stancik et al., 2018). The phosphorylation of Serine, Threonine, and Tyrosine residues catalyzed by Hanks-type kinases has long been thought to occur in Eukaryotes only. Genome sequencing and physiological studies have shown, however, that this is not the case since genes potentially encoding these kinases have been found to exist in a large number of prokaryotic genomes and to be involved in various cellular processes in several bacterial species (Pereira et al., 2011; Stancik et al., 2018). Recent studies have shown, for example, that the life cycle of *Myxococcus xanthus* is partly controlled by a network of interacting Hanks-type kinases (Munoz-Dorado et al., 1991; Nariya and Inouye, 2006). In *Bacillus subtilis*, spore development and germination are controlled by the YaT and PrkC kinases, respectively (Shah et al., 2008; Bidnenko et al., 2013). The process of morphogenesis is controlled by the StkP kinase in *Streptococcus pneumoniae* (Fleuri et al., 2014). The virulence of several bacteria such as *Mycobacterium tuberculosis* (Cowley et al., 2004), *Mycobacterium pneumoniae* (Schmidl et al., 2010) and *Yersinia pseudotuberculosis* (Galyov et al., 1993) depends on the presence of Hanks-type kinases. A recent phylogenetic analysis has suggested that the prokaryotic and eukaryotic Ser/Thr/Tyr kinases have a common evolutionary origin, which challenges the idea that the prokaryotic proteins may have originated from Eukaryotes (Stancik et al., 2018).

Cyanobacteria, the only Prokaryotes performing oxygenic photosynthesis, form a phylum of diverse bacteria colonizing a wide range of ecological environments. The availability of genome sequences covering the whole phylum (Shih et al., 2013) has made it possible to perform comparative genomic investigations on this group of prokaryotes. A genomic study has shown the presence of Hanks-type kinases in 16 of the 21 genomes analyzed. These genes range from 0 to 51 in number, and the largest numbers occur in filamentous diazotrophic strains (Zhang et al., 2007). In an overall study on the phosphoproteome of the unicellular cyanobacterium *Synechocystis* PCC 6803, which possesses seven Hanks-type kinases, 301 phosphorylation events were observed on Ser/Thr/Tyr residues when the bacterium was grown in nitrogen-rich medium, and changes in the global phosphoproteome were found to occur in response to nitrogen starvation (Spat et al., 2015). Protein modifications resulting from Ser/Thr/Tyr phosphorylation may therefore play an important role in the physiology of cyanobacteria. Relatively little is known so far, however, about the signaling pathways in which Hanks-Type kinases and their substrates are involved in cyanobacteria.

The first Hanks-Type kinase to be detected in cyanobacteria was described in the filamentous strain *Anabaena/Nostoc* PCC 7120 (referred herein as *Nostoc*) (Zhang, 1993), which possesses a total number of 48 genes potentially coding for kinases of this kind (Zhang et al., 2007). *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 called vegetative cells consisting of a single cell type. When the filaments of *Nostoc* are deprived of combined nitrogen, 5–10% of the vegetative cells differentiate into heterocysts. These micro-oxic cells are semi-regularly distributed along the filaments, which provide a suitable environment for N₂-fixation. Deprivation of combined nitrogen triggers the accumulation of 2-oxoglutarate (2-OG), the molecular signal inducing heterocyst differentiation (Laurent et al., 2005). Among the various genes involved in the regulatory cascade responsible for heterocyst formation and patterning (Herrero et al., 2016), the global regulator NtcA and the specific master regulator HetR are key transcriptional factors in the cascade resulting in heterocyst development (Herrero et al., 2016). HetR is essential for cell differentiation (Buikema and Haselkorn, 1991). It regulates hundreds of genes in response to combined nitrogen starvation (Mitschke et al., 2011; Flaherty et al., 2014; Videau et al., 2014). HetR exists in different oligomeric states among which dimer and tetramer have been proposed to interact with DNA (Huang et al., 2004; Valladares et al., 2016). The oligomerization of HetR has been shown to be regulated by phosphorylation (Valladares et al., 2016).

Based on genetic studies, the contribution of Hanks-type kinases to the differentiation process at work in *Nostoc* has been described. A mutant strain of the HepS kinase-encoding gene (*alr2760*) has been found to show an impairment focusing on the synthesis of the polysaccharide layer surrounding the mature heterocyst (Lechno-Yossef et al., 2006). The *alr1336* gene encoding the PknH kinase is required for the connections between heterocysts and vegetative cells, and also for maintaining the heterocyst pattern (Ehira and Ohmori, 2012; Fukushima and Ehira, 2018). Overproduction of the PknE kinase (*alr3732*) inhibits heterocyst development (Saha and Golden, 2011). These findings all suggest that Hanks-type kinases may play a role in several aspects of the *Nostoc* developmental program (Saha and Golden, 2011). However, the activity of these kinases, how they are regulated and the nature of their substrates still remain to be elucidated.

We have previously established that the *pkn22* (*alr2502*) gene encoding a putative Hanks-type kinase is induced by exposure to oxidative stress and to nitrogen starvation in *Nostoc*, and that this kinase connects the cellular responses to these two conditions (Yingping et al., 2015). The transcription of the *pkn22* gene is activated by NtcA when *Nostoc* is deprived of combined nitrogen (Yingping et al., 2015), and the transcription of *hetR* and *ntcA* is not under the control of Pkn22 (Yingping et al., 2015). Here we present genetic evidence that heterocyst differentiation requires the activity of the Pkn22 kinase and that there exist epistatic relationships between Pkn22 and the master regulator HetR. This makes of Pkn22 an important factor involved in regulating the physiology and the metabolism of *Nostoc*. 

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MATERIALS AND METHODS

Growth Conditions

Escherichia coli strains were grown in Luria Broth medium (Euromedex). The plasmids were maintained with Ampicillin (100 µg/ml) or Kanamycin (100 µg/ml).

Nostoc sp. PCC 7120 and its derivatives were grown in BG11 medium at 28°C in air under continuous illumination (40 µE m⁻² s⁻¹). For growth survey, strains were first cultivated in BG11 medium until OD 750 = 0.5. They were transferred either in BG11 or BG11o after a washing step in the same medium. The pkn22 insertion mutant and its growth conditions have been described previously (Xu et al., 2003). Cell cultures of Nostoc recombinant strains were supplemented with neomycin (50 µg ml⁻¹). To avoid overexpression effects of gene transcription from the petE promoter, the copper concentration used was 0.4 µM, which is below the concentration triggering a maximal induction level (Buikema and Haselkorn, 2001). Heterocyst formation was induced by transferring the cultures (OD 750 = 0.8) to BG11 without sodium nitrate. The growth was maintained for 0 hindered by transferring the cultures (OD 750 = 0.8) to BG11 without sodium nitrate until OD 750 = 0.5. They were transferred either in high carbon BG11 or BG11o medium. The pkn22 and hetR ORF were cloned into the T18 and T25 plasmids (Euromedex). The plasmids were maintained with Ampicillin (100 µg/ml) or Kanamycin (100 µg/ml).

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Plasmid Construction and Recombinant Protein Purification

The pkn22 mutant and its derivative strain expressing the pkn22 gene under the petE promoter on the pRL25 plasmid (pRLpetE-pkn22, see Table 1) have been previously described (Xu et al., 2003). The catalytic Lysine residue at position 63 of Pkn22 was changed into an Arginine residue using a megaprimer PCR strategy with the primers hetR dhfw and pkn dhfw. The resulting fragment was cloned into the T18 and T25 plasmids (Euromedex).

1. In the Two hybrid plasmid construction procedure, the hetR (alr2339) or pkn22 (alr2502) open reading frames were amplified by performing PCR on Nostoc genomic DNA using the primers hetR dhfw/hetR dhrev and pkn dhfw/pkn dhrev, respectively. After undergoing a digestion step with BamHI and EcoRI, the DNA fragments were cloned into the T18 and T25 plasmids (Euromedex).

2. To co-produce HetR and the catalytic domain of Pkn22 in E. coli, the hetR ORF was cloned under the previously described ptac promoter of the p33iat modified plasmid (Bouillet et al., 2017), and the part of the pkn22 gene encoding the kinase domain (residues 1–325) was cloned under the control of the arabinose promoter of the pBAD24 plasmid. The hetR gene was fused to a His-tag sequence. The primers used are specified in Table 3. The two recombinant plasmids were induced simultaneously using IPTG at 0.5 mM and arabinose at 0.2% overnight at 16°C. The HetR protein was then purified as described previously (Yingping et al., 2015).

3. To express and purify HetR and its variants, the hetR ORF was amplified by PCR from Nostoc genomic DNA using the primers hetR pet78 fw/hetR pet78 rev. After a digestion step with NdeI and BamHI, the DNA fragment was cloned into pET28a plasmid (Novagen), giving hetR fused at its N-terminus to a histidine-tag. This plasmid was then used as a template to transform the Serine residues at positions 127, 130, and 179 into Alanine using the megaprimer PCR strategy with the primers hetR S127G

| Strain name | Description/ Antibiotic resistance | Origin |
|-------------|-----------------------------|--------|
| Wild type   | Nostoc/Anabaena PCC 7120 wild type strain | Pasteur Cyanobacterial Collection |
| pkn22 mutant | Nostoc insertion mutant of the pkn22 gene (Sp+Sm) | Xu et al., 2003 |
| pkn22/petE-pkn22 | containing the pRL-petE-pkn22 plasmid (Sp+Sm and Neo) | Yingping et al., 2015 |
| pkn22/hetR | pkn22 mutant containing the pRL-petE-hetR plasmid (Sp+Sm and Neo) | This study |
|ΔhetR | Nostoc deletion mutant of the hetR gene | Borthakur et al., 2006 |
|ΔhetR/hetR | ΔhetR mutant containing the pRL-petE-hetR plasmid (Neo) | This study |
|ΔhetR/hetR[S127A] | ΔhetR mutant containing a pRL-petE plasmid harboring a hetR mutated gene encoding for the amino acid substitutions indicated between brackets (Neo) | This study |
|ΔhetR/hetR[S127G] | ΔhetR mutant containing a pRL-petE plasmid (Neo) | This study |
|ΔhetR/hetR[S127A] | ΔhetR mutant containing a pRL-petE plasmid (Neo) | This study |
followed by 1 h of incubation at 45°C with 1 µl.

RNA was extracted as previously described (Xu et al., 2003). Quantitative RT-PCR protocols were applied with pRLpetE as a reference. The production of HetR proteins in Nostoc?hetR strain (Borthakur et al., 2005). The pRLpetE plasmid using hetR pRLfw/hetR pRLrev strategy with the primers . Likewise, Serine 130 was mutated with the primers hetR S127A/D, hetR S130A/D and hetR S130D/D. The PCR fragments were then cloned into the pT25 plasmid with the primers hetR/hetRS130D. The resulting library of pT25-hetR variants in Nostoc, the hetRS127A/D, hetRS130A/D and hetRS179A/D sequences were amplified from the corresponding genes from Rivularia PCC 7116 (Riv7116_3691) and Oscillatoria nigroviridis (Osc7112_0139) were synthesized by Eurofins (Novagen) expression strain. The purification of HetR was performed as described previously (Hu et al., 2015).

To produce the phosphoablative HetR variants in Nostoc, the hetRS127A/D, hetRS130A/D and hetRS179A/D sequences were amplified from the corresponding plasmids using hetR pRL-fw/hetR pRL-rev as primers. After undergoing digestion with Apal and BamHI, the PCR fragments were cloned into the pRLpetE plasmid and conjugated in the Nostoc hetR strain (Borthakur et al., 2005). The hetR genes from Rivularia PCC 7116 (Riv7116_3691) and Oscillatoria nigroviridis (Osc7112_0139) were synthesized by Eurofins and cloned into the pRLpetE plasmid using the BamHI and EcoRI restriction sites. The production of HetR proteins in Nostoc was checked by Western blot using anti-HetR antibodies.

Quantitative RT-PCR
RNA was extracted as previously described (Xu et al., 2003). Chromosomal DNA was removed by treating RNA preparations with 1 µl of DNase (at 2 U/µl) (Ambion) for 1 h at 37°C. The concentration of RNA was determined spectrophotometrically. Reverse transcription: for each reaction, 1 µl of RNA was used. The number of the filaments analyzed was 60–100 in average; The conditions were kept for further analysis.

TABLE 2 | Percentage of heterocysts formed by different strains used in this study after combined nitrogen starvation.

| Strain | % of heterocysts, 24 h after nitrogen starvation | % of heterocysts, 96 h after nitrogen starvation |
|--------|-----------------------------------------------|-----------------------------------------------|
| Wild type | 10–12 | 10–12 |
| pkn22 mutant | 0 | 1–2 |
| pknC | 8–10 | 10–12 |
| pkn22/hetR | 12* | 10 |
| ΔhetR | 0 | 0 |
| ΔhetR/hetR | 10* | 10–12* |
| ΔhetR/hetRS130A | 0 | 0 |
| ΔhetR/hetRS130D | 22* | 22–25* |

The number of the filaments analyzed was 60–100 in average; The conditions were kept for further analysis.

Bacterial Two Hybrid Assays
Bacterial two-hybrid assays were performed as described by 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. 3 ml of LB medium supplemented with ampicillin, kanamycin and 0.5 mM IPTG (Sigma Aldrich) were inoculated and grown at 30°C overnight. β-Galactosidase activity was determined as previously described (Zubay et al., 1972). The values presented are means of five independent assays on samples containing 10 independent clones each.

hetR Mutagenesis
Random mutagenesis was performed on the hetR gene ORF using the GeneMorph II Random Mutagenesis Kit from Stratagene with the primers hetdhfw/hetRdhrev and pkn dhfw/pkn dhrev. The PCR fragments were then cloned into the pT25 plasmid (Zubay et al., 1972) using BamH1 and EcoRI restriction sites. The strain BTH101 previously transformed by pT18-pkn22 was transformed here by the resulting library of pT25-hetR mutants and plated onto LB plates containing Ampicillin and Kanamycin. After being incubated for 2 days at 30°C, colonies were replicated on MacConkey petri dishes and incubated again for 2 days at 30°C. Minipreps of DNA were prepared from the white colonies and used to transform an MC4100 strain to isolate the pT25-hetR mutant alone. A further two-hybrid assay was performed between the isolated T25-hetR mutant and T18-pkn22 in the strain BTH101 strain in order to check the loss of the previous interactions. The production of the recombinant T25-HetR mutant proteins was then checked by performing Western blot analysis using antibody directed against HetR. The DNA of clones showing full-length T25-HetR proteins were sequenced.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot Analysis
Proteins were fractionated by performing SDS-PAGE (12% except where indicated) stained with Coomassie blue (Euromedex, Souffleweyshim, France). For electrophoresis under non-reducing conditions, β-mercaptoethanol was omitted.
from the SDS-PAGE gels, and the proteins were not heated before loading into the gel. For immunoblot analysis, the proteins were transferred to nitrocellulose membranes before being revealed with specific polyclonal antibodies. Immune complexes were detected with anti-rabbit peroxidase-conjugated secondary antibodies (Promega) and enhanced chemiluminescence detected with anti-rabbit peroxidase-conjugated secondary antibodies (Promega) and enhanced chemiluminescence reagents (Pierce, Illich, France). Anti-HetR antibodies were developed by Covab and used at a 1:1000 dilution.

**Phosphorylation Assays**

HetR (50 μM) or BSA (50 μM) were incubated either under the same experimental conditions used in Valladares et al. (2016) (incubation with 30 μM ATP, 15 μCi [γ-32P]-ATP, 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM MgCl2 and 10% glycerol) or submitted to a kinase assay as follows: the proteins were incubated in a phosphorylation buffer (20 mM HEPES (pH 7.2), 100 mM KCl, 5 mM MgCl2, 1 mM DTT, 50 μg cold ATP). The reaction was initiated by adding 2 μCi [γ-32P]-ATP. As phosphatase inhibitor, PhosStop (Roche) was added to all the phosphorylation assays following the manufacturer indications. The mixtures were incubated during 60 min at 30°C. To examine the ability of HetR to phosphorylate the myelin basic protein (MBP, Sigma), MBP (1 μg) was incubated in the phosphorylation buffer with or without HetR (10 μM). The kinase domain of PrkC of *B. subtilis* (3 μM) was used as a positive control. The reactions were stopped by adding the Laemmli buffer and the proteins were separated by SDP-Page under non-reducing conditions (without heating and without reducing agent in the loading buffer). Radioactive signals from phosphorylated proteins were revealed by autoradiography using a FUJI phosphomager. The experiment was repeated three times with independent protein purifications and one representative result is shown.

**Electrophoretic Mobility Shift Assays (EMSA)**

The promoter region of the *hetP* gene (*alr2818*) was obtained by PCR using *hetP* RT-forward and *hetP* RT-reverse primers (Table 3). The forward primer was modified at its 5’ end by adding the 6-carboxyfluorescein (6-FAM) dye. Purified HetR protein was incubated with the promoter fragments (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 diithiothreitol and 1 μg DiDC competitor (poly(2'-deoxyinosinic-2'-deoxyctydilic acid) sodium salt), at 4°C for 30 min. The electrophoresis was performed at 250 V for 60 min. 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.

**Mass Spectrometry and Data Analysis**

A total of 15 μg of purified HetR were separated by performing SDS-PAGE electrophoresis under non-reducing conditions. Protein-containing bands were subjected to trypsin digestion after several steps: Blue Coomassie stained gel bands were washed with 100 mM acetonitrile/ammonium bicarbonate pH 7.5 for two
The pkn22 mutant is unable to sustain diazotrophic growth. (A) Growth curve of Nostoc strains grown in either BG11 (nitrate-containing medium) or BG110 (nitrate free medium). Each sample was measured in triplicate and error bars give the standard deviations. (B) Microscope images of Nostoc strains grown for 24 h in BG110. Heterocysts are indicated by arrows. PknC stands for the pkn22 complemented strain and pkn22/pkn[K36A] for the pkn22 strain complemented with the pkn22 gene bearing the K36A substitution. (C) Quantitative RT-PCR analysis of the pkn22 transcripts in presence (BG11, white bars) or absence of combined nitrogen (BG110, gray bars). Data are expressed as fold-change between normal and starvation conditions. Each sample was measured in triplicate and the standard deviation is indicated by error bars. Values were normalized to the mpB transcript. The value obtained for the Wild type strain in BG11 was set to 1.

Phylogenetic Analysis

The genomic set analyzed in this study included 160 genomes present in the NCBI database². The complete list of the genomes selected to build the tree is given in Supplementary Table S1. To build the phylogenetic tree of HetR, the genomes cited above were analyzed with BlastP (Altschul et al., 1990) using the sequence form Nostoc as a query and an e-value < e⁻⁹⁵. Multiple alignments of the proteins were generated using the Constraint-based multiple Alignment tool (COBALT) (Papadopoulos and Agarwala, 2007). The phylogenetic tree was constructed using the

²https://blast.ncbi.nlm.nih.gov/Blast.cgi
FIGURE 2 | The pkn22 mutant is not able to develop heterocysts. Heterocyst pattern formation in the wild type, pkn22 mutant, and pkn22 derivative strains. Strains were grown in BG11 medium to an OD$_{750}$ of 0.4 and induced to form heterocysts by transfer to BG-110 medium. Vegetative cells and heterocysts were scored microscopically at indicated times after heterocyst induction. The data shown are representative of three independent experiments.

RESULTS

The pkn22 Mutant Was Impaired in Heterocyst Differentiation

Since the transcription of the pkn22 gene is induced in response to combined nitrogen starvation and depends on the global transcriptional cellular differentiation activator NtcA (Yingping et al., 2015), we wondered whether this kinase might contribute to the growth under combined nitrogen starvation, and hence to the process of heterocyst development. To answer this question, the growth of the mutant was compared with that of the wild type strain in BG11 medium (with nitrogen) and BG11$_0$ medium (without any combined nitrogen). The results presented in Figure 1 show that the growth of the mutant was impaired in the absence of a combined nitrogen source. The introduction of the pkn22 gene into a replicative plasmid in the mutant (pknC strain) (Yingping et al., 2015) partially restored the ability of the mutant to grow in the presence of N$_2$ as the sole nitrogen source (Figure 1A). These data indicate that the growth of Nostoc requires the presence of the Pkn22 kinase under nitrogen starvation. The heterocyst differentiation process was then analyzed in the pkn22 mutant in comparison with the wild type strain. Interestingly, the heterocyst formation process was delayed in comparison with what occurred in the wild type strain. No heterocysts were observed during the first 24 h after the nitrogen step-down (Figure 1B and Supplementary Figure S2) in the mutant strain, and a few heterocysts began to appear only 96 h after the step-down (Supplementary Figure S2 and Table 2). These heterocysts did not allow the strain to grow in the absence of combined nitrogen starvation, since the growth of the mutant did not resume after several days in BG11$_0$. In order to further examine the pkn22 mutant phenotype, the pattern of
heterocyst occurrence along the filament was analyzed at various times after the nitrogen step-down. The wild type strain showed a mean number of 12 vegetative cells between two heterocysts, 24 h after combined nitrogen starvation, whereas the pkn22 mutant did not form any heterocysts at all, as mentioned above. Ninety-six hours after the onset of nitrogen starvation, the pkn22 mutant did not form any heterocysts at all, as mentioned above.

The Catalytic Residue of Pkn22 Contributed Importanty to Cell Differentiation

To investigate how Pkn22 is involved in the differentiation process, we examined how the process of heterocyst development was affected by catalytic residue substitutions. Multiple alignment of the amino acid sequence of Pkn22 with those of other Ser/Thr kinases showed that the Lysine at position 63 corresponds to the conserved residue responsible for ATP binding in this class of kinases (Hanks and Hunter, 1995; Li et al., 1995; Supplementary Figure S1A). The Lysine 63 was substituted to Arginine, the mutated gene was expressed in the pkn22 mutant and the resulting strain was called pkn22/pkn[K63R]. Quantitative RT-PCR analysis were undergone to check that the mutated gene was actually expressed. The data obtained indicated that the pkn22 transcripts were expressed at similar levels in the PknC and the pkn22/pkn[K63R] strain (Figure 1C). Contrary to what was observed with the wild type pkn22 gene, the ectopic expression of a mutated gene encoding a protein with a K63R substitution did not restore the ability of the pkn22 mutant to develop heterocysts, or to grow under combined nitrogen starvation conditions (Figure 1B and Supplementary Figure S1B), conceding that the mRNA level of the pkn[K63R] reflects the protein level. It was therefore concluded that normal cellular differentiation requires the kinase activity of Pkn22.

hetR Overexpression Compensated for the pkn22 Mutation

Since the differentiation process was initiated later in the pkn22 mutant than in the wild type strain, we wondered whether this process might be impaired in the mutant during the initiation of the developmental program. We therefore examined the effects of the ectopic expression of regulatory genes controlling the initiation of heterocyst differentiation in the mutant. The ntcA and hetR genes encoding the global and specific regulators of heterocyst differentiation, respectively, were expressed in the mutant. The transcription of ntcA and hetR genes are subjected to autoregulation (Black et al., 1993; Cai and Wolk, 1997; Buikema and Haselkorn, 2001) and are mutually dependent (Muro-Pastor et al., 2002), which might bias the conclusions drawn. We therefore chose to express them from the copper-inducible petE promoter that has been used for hetR ectopic expression from a replicative plasmid (Buikema and Haselkorn, 2001). Although the overproduction of ntcA had no effect (Supplementary Figure S2), the introduction of hetR restored the wild-type phenotype, since heterocysts were observed after 24 h and the recombinant strain pkn22/hetR was able to grow in the absence of combined nitrogen (Figures 3A,B). The hetR gene also corrected the defective pkn22 mutant pattern, since heterocysts had formed in every 10 or 12 vegetative cells in the majority of the filaments 72 h after the nitrogen step-down (Figure 2 and Table 2). The overexpression of hetR in the wild type strain has been reported to increase the number of heterocysts along the filaments and to result in contiguous heterocysts (Buikema and Haselkorn, 2001). During the present experiments, we also overexpressed hetR in the wild type strain and in the ΔhetR
strain, and observed the same phenotype as previously described indicating that the hetR gene was actually overexpressed under our experimental conditions (Supplementary Figure S2). It was therefore concluded that heterocyst formation requires the presence of the Pkn22 kinase, and that the overexpression of hetR can compensate for the absence of this kinase (see section “Discussion”).

Interactions Between Pkn22 and HetR

The existence of a genetic link between Pkn22 and HetR described above suggests that these two proteins may be functionally linked, and it was therefore proposed to determine whether they may interact with each other. For this purpose, bacterial two hybrid assays based on the reconstitution of adenylate cyclase activity were performed in an E. coli cya mutant (Karimova et al., 1998). Pkn22 and HetR were fused to the N-terminus of the T18 and T25 domains of Bordetella pertussis adenylate cyclase, using the two compatible plasmids pUT18 and pKT25, respectively. In the cya strain BTH101, adenylate cyclase activity was restored when the T18-HetR and T25-HetR proteins were produced together (Figure 4A). HetR protein is known to form dimers/tetramers (Kim et al., 2011; Valladares et al., 2016) and the interaction observed between HetR monomers in the bacterial two hybrid assays confirmed the validity of this approach. Pkn22 monomers were not found to interact with each other in our tests (Figure 4A). Adenylate cyclase activity was restored when the T18-Pkn22 and T25-HetR were produced together, and the β-galactosidase activities obtained were almost at the same level as in the positive control sample [the T18 and T25 fused to the leucine zipper region of the yeast protein GCN4 as used in the original system (Karimova et al., 1998), consisting of the proteins from the original system] (Figure 4A). This finding indicates that HetR and Pkn22 interacted with each other in our assays.

In order to select HetR variants that could no longer interact with Pkn22, random mutagenesis was performed on the hetR coding sequence, which was then cloned into the pT25 plasmid. The resulting library of pT25-hetR mutants was then screened using two hybrid method against pT18-pkn22, and clones devoid of adenylate cyclase activity were identified. Expression of the recombinant T25-hetR mutant genes was then checked by performing Western blot using an antibody directed against HetR. Forty variants of the HetR protein with one or two amino acid substitutions were obtained in all. Clones giving full-length T25-HetR proteins were kept for further analysis (Figure 4B). The plasmids of these clones were extracted and sequenced, and the loss of interactions with Pkn22 was again quantified by performing β-galactosidase assays (Figure 4C), the
mutations harbored by the three clones obtained introduced the following substitutions: S127G, P202L, S179G. With a view to characterizing the physiological consequences of disrupting the Pkn22-HetR interactions, and to avoid the input of hetR transcriptional regulation, the hetR mutated genes were expressed in a replicative plasmid under the petE promoter in a ΔhetR strain. The synthesis of these variants in the hetR mutant was checked by Western blot analysis (Figure 5A) and the ability of the recombinant strains to develop heterocysts was analyzed (Figure 5B). The expression of the wild type version of hetR from the same promoter and the same plasmid was used as a control. The substitutions that affected HetR function and abolished the differentiation process were S179G and P202L. The mutant harboring the S127G substitution was not able to form heterocysts after 24 h, and only a few heterocysts were observed 48 h after the nitrogen step-down (Figure 5B and Table 2). The finding that amino acids involved in the interaction with Pkn22 were also required for HetR to function normally supports the idea that there exists a functional relationship between this Hanks kinase and the master cell differentiation regulator.

A Phosphorylated Serine Residue Was Crucial to HetR Activity

Since Pkn22 belongs to a class of kinases which are potentially able to phosphorylate proteins on Serine or Threonine residues, we wondered whether Pkn22 might phosphorylate HetR, and therefore proposed to test this hypothesis by performing in vitro phosphorylation assays. Unfortunately, however, despite a large panel of purification methods tried, we did not succeed in
In the presence of Pkn22, HetR is phosphorylated on a Serine residue. (A) Immunoblot of purified HetR (50 µM) used in the mass spectrometry analysis. The concentration of the gel used was 4–20%. The electrophoresis was undergone on SDS-Page under non-reducing conditions. Several oligomeric forms of HetR were obtained; the monomer, dimer, trimer, and tetramers are indicated by arrows. Same results were obtained when HetR was produced in the absence or presence of Pkn22. (B, C) Mass spectrometry analysis of HetR produced in E. coli in the presence (B) or absence of the kinase domain of Pkn22 (C). After purification, the recombinant HetR protein was subjected to trypsin digestion. The spectra show the fragmentation pattern of the phosphopeptides. The mass increment of the fragment Y4, due to the phosphorylation of Ser 130, is indicated by the red arrow. The blue arrow indicates the fragment Y4 without the mass increment. The data presented in the figure are representative of five independent experiments with the monomer form. Similar data were obtained with the dimeric and trimeric forms. The yield of the tetrameric form obtained was below the concentration needed for mass spectrometry analysis.

Supplementary Figure S4). The monomer, dimer and trimeric forms were separately submitted to spectrometry analysis. The results of mass spectrometry analysis showed that when HetR was co-produced with Pkn22, it was phosphorylated on the Ser 130 residue (compare Figures 6B,C). The phosphorylation was detected on the three oligomeric forms of HetR. To characterize the impact of a potential phosphorylation of the Ser 130 residue on HetR activity, phosphoablative (Serine to
Alanine) and phosphomimetic (Serine to Aspartate) substitutions were introduced instead of this Ser residue and the mutated genes obtained were expressed in the hetR mutant. The impact of these mutations on the differentiation process was deduced from the comparison of the phenotypes of these mutants with that of the hetR strain complemented with the wild type version of hetR expressed in the same manner than the mutated versions. The data presented in Figure 7A indicate that the HetR S130A phosphoablative variant was not able to complement the hetR strain, since no heterocysts were observed. On the other hand, the phosphomimetic variant was found to induce the formation of approximately twofold more heterocysts than the wild type strain (Figures 2, 7A and Table 2) and multiple contiguous heterocysts were observed (Figure 7A). The production of these variants in the ΔhetR strain was checked by Western blot using anti-HetR antibodies (Figure 8B). The S179A substitutions abolished the capacity of HetR to induce heterocyst formation (Figure 8A) and to bind to hetP promoter in vitro (Figure 8C). To establish whether this residue is actually phosphorylated in vivo, or whether the effect observed was due to the similarity between this residue and the DNA binding motif, further investigations are required. In the case of Ser 127, the phosphoablative substitution performed here did not affect the DNA binding activity of HetR or its ability to induce heterocyst differentiation (Figures 8A,C), which suggests that this residue is probably not phosphorylated in vivo.

**Conservation of Serine 130 Residue in Cyanobacteria Forming Heterocysts**

Since the Ser 127 and Ser 179 residues were identified in the genetic screen for the loss of HetR ability to interact with Pkn22, the impact of their phosphoablative substitutions was analyzed. Their synthesis in the ΔhetR strain was checked by Western blot using anti-HetR antibodies (Figure 8B). The S179A substitutions abolished the capacity of HetR to induce heterocyst formation (Figure 8A) and to bind to hetP promoter in vitro (Figure 8C). To establish whether this residue is actually phosphorylated in vivo, or whether the effect observed was due to the similarity between this residue and the DNA binding motif, further investigations are required. In the case of Ser 127, the phosphoablative substitution performed here did not affect the DNA binding activity of HetR or its ability to induce heterocyst differentiation (Figures 8A,C), which suggests that this residue is probably not phosphorylated in vivo.

Sequence alignment and phylogenetic analysis of the HetR sequence across the cyanobacterial phylum showed that Ser 130 belongs to a sequence [TSLTS] which is highly conserved in the cyanobacterial strains which are able to differentiate heterocysts (Nostocales and Stigonematales) (Figures 9A,B and Supplementary Figure S3). The phosphorylation of HetR might therefore occur in diazotrophic cyanobacteria other than...
The data presented in this paper indicate that the Pkn22 kinase is required for normal heterocyst development to occur via the phosphorylation of the master regulator HetR. In line with this conclusion, an insertion mutant of the pkn22 gene was unable to form heterocysts within 24 h, as well as being unable to grow under N₂-regime. We have previously established that the transcription of the pkn22 gene is under the control of the couple NtcA-2OG. It is therefore likely that the activity of this kinase might occur during the early steps of the developmental program, making it a new player in the initiation cascade that triggers heterocyst formation (Figure 10A).

The genetic and biochemical data obtained in this study brought to light the existence of a connection between Pkn22 and the master regulator of heterocyst differentiation: HetR. Pkn22 seems to be epistatic to HetR, since the overexpression of the hetR gene was able to compensate for the absence of Pkn22. In bacterial two component systems, where signal transduction is mediated by the phosphorylation of the response regulator protein by the sensor kinase, the overproduction of the response regulator compensates for the kinase activity of the sensor (for a recent review on the subject, see Jacob-Dubuisson et al., 2018). A similar mechanism may explain why increasing the amount of HetR triggered cell differentiation in the pkn22 mutant background. In this context, the ability of the two proteins to interact and the fact that residues present in HetR...
which are required for the interaction with the kinase are also required for the differentiation process point to the existence of physical and functional relationships between them. The fact that two Serine residues (S127 and S179) were found to be required for HetR and Pkn22 interaction raised the question whether HetR might be phosphorylated on Serine residues. The phosphoablative substitution of S179 was found to inhibit the heterocyst differentiation process. If the residue S179 was actually phosphorylated in vivo, it would be interesting to establish whether Pkn22 is responsible for this phosphorylation event or whether it is mediated by another kinase. Three other S/T kinases have been reported to be involved in heterocyst differentiation (Lechno-Yossef et al., 2006; Saha and Golden, 2011; Ehira and Ohmori, 2012). It would be worth determining whether any crosstalk among them and Pkn22 might contribute to the functional role of HetR.
HetR has been shown to be phosphorylated when incubated with radioactive adenosine triphosphate (ATP) (Valladares et al., 2016). Consequently, before analyzing the possible phosphorylation of HetR by Pkn22, we wondered whether HetR would be able to act itself as a kinase by analyzing its ability to catalyze phosphotransfer in vitro. The data presented in Supplementary Material, where the PrkC kinase from B. subtilis was used as a positive control (Shah et al., 2008), indicated that HetR does not possess a kinase activity similar to that of Ser/Thr/Tyr kinases (Supplementary Figure S3B) and can consequently be used as a substrate in a phosphorylation test in vitro to analyze its putative phosphorylation by Pkn22. HetR was found to be phosphorylated on the S130 residue only when it is co-produced with Pkn22 in E. coli (Figure 6B). Phosphoablative substitution of S130 inhibited the process of heterocyst formation, while strains harboring phosphomimetic substitution of this residue formed larger numbers of heterocyst compared to the strain expressing the wild type version of HetR (Figures 2, 7A and Table 2). This finding suggests that the phosphorylation of this residue is required for HetR to function in vivo. The fact that the phosphoablative substitution of S130 did not abolish the DNA binding activity (Figure 7C) suggests that the phosphorylation of this serine may be necessary for interactions to occur between HetR and the RNA polymerase or other protein partners. Indeed, this residue is located in the Flap domain, which is exposed in the structure and has been thought to be required for protein-protein interactions to be possible (Kim et al., 2011; Hu et al., 2015; Figure 10B). On the other hand, S179 is located near the DNA binding motif (Figure 10), which may explain the impact of a post-translational modification of this residue on the interaction of the protein with the promoter. We attempted to purify HetR from Nostoc at various times during the differentiation process with a view to analyzing its phosphorylation state, but the amount of protein obtained was below that required for mass spectrometry purposes. It is conceivable that the whole population of HetR proteins does not have to be phosphorylated to initiate differentiation, which would also be a limiting factor for the mass spectrometry analysis of HetR purified from Nostoc. It is planned to use other approaches such as phosphoproteome analysis in the future in order to study more closely how HetR phosphorylation contributes to the differentiation process.

Previous RNA seq and ChIP Seq analyses have shown that the transcription of genes expressed in the heterocysts and vegetative cells requires the presence of HetR (Mitschke et al., 2011; Flaherty et al., 2014). It is tempting to imagine that a post-translational HetR modification might constitute one of the mechanisms responsible for the cell-type specificity of this regulator. In this respect, it has been reported that shortly after the onset of nitrogen starvation, HetR protein shows a higher isoelectric point than under combined-nitrogen conditions (Zhou et al., 1998); phosphorylation may be involved in this post-translational modification in response to combined nitrogen starvation. In line with this idea, HetR has been found phosphorylated when incubated with radioactive ATP in vitro (Valladares et al., 2016). Moreover, this phosphorylation has been reported to inhibit the accumulation in vitro of the tetrameric form which has been
postulated to be the active regulatory form in the heterocyst (Valladares et al., 2016). This in vitro phosphorylation has been suggested to be either catalyzed by HetR or by a kinase form E. coli which could be co-purified with HetR (Valladares et al., 2016). The regulation of heterocyst differentiation is a spatio-temporal regulated process during which a dynamic of HetR phosphorylation could be proposed to occur based on Valladares et al. (2016), study and the data presented in our work: in response to combined nitrogen starvation, NtcA activation by 2-OG, induces pkn22 expression which leads to HetR phosphorylation and initiation of the developmental program (Figure 10B). When the heterocyst reaches maturity, the stimulation of the autophosphorylation activity of HetR, or the activation of another kinase, limits its regulatory action. Analyzing the phosphorylation/dephosphorylation of HetR separately in the vegetative cells and in the heterocysts through the developmental program will give more insight in the mechanism of this master regulator.

Interestingly, the residue S130 that was found to be phosphorylated in E. coli only when HetR was co-produced with Pkn22 belongs to a motif [TSLTS] which is conspicuously highly conserved in the Nostocales and Stigonematales strains (Figure 9). The phosphorylation of HetR described in the present study might therefore also occur in diazotrophic cyanobacteria other than Nostoc PCC 7120. In addition, the genomes of strains belonging to the Nostocales and Stigonematales species contain at least one copy of the Hanks-type kinase gene (Zhang et al., 2007). The acquisition of the [TSLTS] motif by HetR sequences and its phosphorylation might therefore be an evolutionary step toward the occurrence of heterocyst differentiation and diazotrophy. The fact that HetR from Rivularia, which harbors the [TSLTS] sequence, complemented the ΔhetR Nostoc mutant, while that from Oscillatoria strain which does not contain this sequence did not (Figure 9) is in agreement with this hypothesis. Further studies on the phosphorylation state of HetR in heterocyst-forming strains other than Nostoc in comparison with unicellular strains will yield deeper insights into the functional role of HetR and its speciation in the course of evolution.

HetR can therefore be added to the hitherto rather short list of transcriptional regulators (other than two-component systems) that are phosphorylated by Hanks-type kinases. The other known examples on this list are the global gene regulator AbR (Kobir et al., 2014) and the fatty-acid-displaced regulator FatR (Derouiche et al., 2013) in B. subtilis, and the anti-sigma RseA of M. tuberculosis (Barik et al., 2010). The interplay between response regulators and Hanks-type kinases is definitely a topic worth investigating, since studies on these lines will shed further light on how the phosphorylation process serves in bacteria to detect and transduce environmental signals.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

AL conceived and designed the study and wrote the manuscript. BR, XX, VR, and YF performed the research. AL and RL supervised the research. AL, BR, and RL analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.03140/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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