Elements of the heterocyst-specific transcriptome unraveled by co-expression analysis in Nostoc sp. PCC 7120

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There are only a few instances of cellular differentiation in bacteria. These include the formation of spores in *Bacillus*, the fruiting bodies of *Myxococcus* or the stalked cells of *Caulobacter*. A particularly striking case of cell differentiation is provided by certain filamentous cyanobacteria that can differentiate three different cell types: spore-like akinetes, motile hormogonia and N₂-fixing heterocysts.

Heterocyst differentiation is facultative. It is triggered when a source of combined nitrogen is not available and leads to a terminally differentiated cell type that cannot de-differentiate. The process is controlled by a very complex network of regulators, second messengers and peptide hormones. By construction of a co-expression network utilizing transcriptomic information from three different time course experiments performed during the course of this work and from our dataset previously obtained by differential RNA-Seq, we identify 86 novel candidate genes that were not previously related to heterocyst differentiation or had remained unknown. Comparative analysis of their promoters allows the identification of a new heterocyst-specific promoter motif (DIF2) and the assignment of a new gene to the CnfR regulon.

Our data increase the available information on HetR-dependent transcripts. In particular, the presence of antisense transcripts and trans-acting sRNAs opens new and exciting possibilities to understand the structural and metabolic transformation of a cyanobacterial vegetative cell into a N₂-fixing factory. The new elements identified in this study are of interest for understanding cell differentiation, engineering of biological N₂ fixation or production of O₂-sensitive molecules in cyanobacteria. Additionally, the approach described will be appreciated by
scientists addressing transcriptomic analyses of bacteria with alternative lifestyles or complex life cycles.
Abstract

Nitrogen is frequently limiting microbial growth in the environment. As a response, many filamentous cyanobacteria differentiate heterocysts, cells devoted to N\textsubscript{2} fixation. Heterocyst differentiation is under the control of the master regulator HetR. Through the characterization of the HetR-dependent transcriptome in Nostoc sp. PCC 7120 we identified new candidate genes likely involved in heterocyst differentiation. According to their maximum induction, we defined E-DIF (early in differentiation) and L-DIF (late in differentiation) genes. Most of the genes known to be involved in critical aspects of heterocyst differentiation or function were also classified into these groups, showing the validity of the approach. Using fusions to \textit{gfp}, we verified the heterocyst-specific transcription of several of the found genes, antisense transcripts and potentially trans-acting sRNAs. Through comparative sequence analysis of promoter regions we noticed the prevalence of the previously described DIF1 motif and identified a second motif, called DIF2, in other promoters of the E-DIF cluster. Both motifs are widely conserved in heterocystous cyanobacteria. We assigned \textit{alr2522} as a third member, besides \textit{nifB} and \textit{nifP}, to the CnfR regulon. The elements identified here are of interest for understanding cell differentiation, engineering of biological nitrogen fixation or production of O\textsubscript{2}-sensitive molecules in cyanobacteria.

Key words: heterocyst differentiation, co-expression network, cyanobacteria, hetR, DNA sequence motifs
Introduction

In the absence of combined nitrogen sources, many filamentous cyanobacteria, including *Nostoc* sp. PCC 7120 (a.k.a. *Anabaena* sp. PCC 7120), the model organism used in this work, differentiate a one-dimensional pattern of specialized cells called heterocysts, that are devoted to the fixation of atmospheric nitrogen (N$_2$) (Muro-Pastor and Hess, 2012). Heterocyst differentiation relies on a transcriptional program that is exclusive of the cells becoming heterocysts (Flaherty et al., 2011; Mitschke et al., 2011; Flores et al., 2018). Major landmarks of the process include the sequential synthesis and deposition of specific heterocyst envelopes as well as the synthesis and maturation of nitrogenase, the enzymatic complex for N$_2$ fixation. In parallel, precise coordination between nitrogen-fixing heterocysts and vegetative cells becomes established because these two cell types depend on each other in order to sustain growth of the filament as a whole. Growth at the expense of N$_2$ involves not only the morphological and metabolic transformation of selected vegetative cells to become heterocysts, but also the precise regulation of the pattern and timing of differentiation (Flores and Herrero, 2010). Thus, a high number of different regulatory factors can be expected to be involved in these processes.

Classical approaches involving transposon or chemical mutagenesis followed by screening for Fox mutants, unable to grow using only molecular nitrogen under aerobic conditions, allowed the identification of major players in the process of heterocyst differentiation, such as the master regulator HetR and genes encoding proteins involved in synthesis and deposition of envelope polysaccharides and glycolipids (*hep* and *hgl* genes, respectively), or in the synthesis and maturation of nitrogenase (*nif* genes) (see e.g. (Wolk et al., 1988; Buikema and Haselkorn, 1991b)). Some elements, such as *hetR*, were repeatedly discovered indicating the near-saturation coverage achieved in these experiments (Buikema and Haselkorn, 1991a; Black et al., 1993). Because the
approaches involving identification of Fox- mutants are based on inability to grow using
N₂, they are not suitable to identify elements that participate in more subtle aspects of
differentiation, such as patterning or time of commitment. Similarly, it is also difficult to
identify protein-coding genes or regulatory transcripts if their functions are redundant or
if they can’t be mutated because they are essential for growth even in the presence of
combined nitrogen. Therefore, it is likely that many of the involved genes and factors
have remained unidentified. Thus, alternative approaches are required to fully uncover
the genetic elements that participate in the process of differentiation.

Here we identified genes and sequence elements potentially involved in heterocyst
differentiation on the basis of time-resolved transcriptional profiling of the wild-type and
a hetR mutant, unable to differentiate heterocysts (Buikema and Haselkorn, 1991a),
using high-density microarrays that include coding and non-coding regions of the
genome. We found two groups of genes with temporally distinct HetR-dependent
transcriptional responses that we have named E-DIF (peaking early in differentiation)
and L-DIF (peaking late in differentiation) according to the respective maximum
expression in response to nitrogen deficiency. As proof of concept, these groups include
known hep, hgl and nif genes, but also several new elements that became clustered with
them on the basis of their transcriptional regulation. Most importantly, because our
approach uses labelled RNA directly in the hybridization of microarrays, it avoids the
pitfalls of cDNA synthesis. In addition, because the customized microarrays are not
restricted to protein-coding genes, we also identified heterocyst-specific antisense
transcripts and potentially trans-acting RNAs that might exert regulatory roles in these
differentiated cells.

Searching for sequences conserved in the promoters of either of the two DIF clusters
and in their homologs in heterocystous cyanobacteria led to the discovery of a new motif
in the E-DIF group (that we called DIF2), together with the more prevalent DIF1 motif
that has been previously identified in several HetR-dependent promoters with heterocyst-specific expression (Mitschke et al., 2011). Mutational analysis clearly demonstrated the connection between this DIF2 motif and heterocyst-specific expression. Finally, a previously defined motif mediating the CnfR-regulated expression of a promoter upstream of *nifB* (Vernon et al., 2017) or *nifP* (Tsujimoto et al., 2016) was also identified in a third transcriptional unit in the L-DIF group. Our results show that substantially more genes, elements and factors might be involved in the genetic program that leads to the differentiation or cyanobacterial heterocysts than anticipated so far.

Results

Transcriptomic analysis of the responses to nitrogen deprivation

Using differential RNA-Seq, we previously described the early (8 h) transcriptional responses of *Nostoc* sp. PCC 7120 to nitrogen deprivation (Mitschke et al., 2011). Transcriptional start sites (TSS) and their N-responsive expression dynamics in the wild type and a *hetR* mutant strain, that accumulates high levels of a non-functional HetR protein (Risser and Callahan, 2007) and is unable to differentiate heterocysts, were analyzed (Mitschke et al., 2011). Based on these transcriptomic data we designed high density microarrays that allowed the direct hybridization of labelled RNA samples. In contrast to previous approaches, these arrays contain probes for all possible transcripts originating at TSS categorized not only as driving the transcription of protein-coding genes (gTSS) but also of antisense transcripts (aTSS) and non-coding transcripts, sRNAs (nTSS) (see Supplementary methods for details). To define the HetR-dependent transcriptome as an approach to find new elements potentially involved in heterocyst differentiation and function, microarrays were hybridized to RNA samples from the wild type strain and the *hetR* mutant along a time course of nitrogen deficiency (0, 6, 12 and 24 hours after nitrogen step down). In order to construct a co-expression network, we
have also analyzed RNA samples from wild type cells growing using N\textsubscript{2} (steady state) and 8 h after addition of ammonium and another two samples from the wild type strain corresponding to the conditions of our previous dRNA-Seq analysis (0-8 hours after nitrogen step-down).

Expression data were compared between two samples as follows: every sample was compared to the absolute reference (WT-0 from the -N time-course experiment, i. e., ammonium-grown cells) and every sample was compared to its own reference (for instance hetR-12 vs. hetR-0). In order to reduce noise and false positives, only transcripts with a difference in their absolute expression values higher than log\textsubscript{2}=1.25 entered the clustering analysis of transcriptional dynamics (see Methods and Supplementary Dataset S1). The log\textsubscript{2}>1.25 threshold secured that only genes that showed a pronounced transcriptional response were included in the clustering analysis.

Ten clusters were resolved (Supplementary Dataset S2 and Fig. S1). Six clusters included elements that were induced (#3, #5, #9) or repressed (#2, #7, #10) both in the wild type and the hetR mutant, therefore suggesting these changes corresponded to global responses to nitrogen deficiency not specifically related to heterocyst differentiation. Among these, for instance, clusters #9 and #10 included previously identified responses directly regulated by NtcA (Mitschke et al., 2011), such as the inducible gene glnA (encoding glutamine synthetase; cluster #9) (Frias et al., 1994) or the repressed gene gifA (encoding the glutamine synthetase inactivating factor IF7; cluster #10) (Galmozzi et al., 2010). Two clusters contained elements that, regardless the time point, showed either a negative (#1) or positive (#8) change in the hetR mutant with respect to the wild type, and therefore were not nitrogen-regulated.

In order to identify HetR-dependent responses, we selected for further analysis the two clusters that contained elements whose expression was induced in response to nitrogen deficiency in the wild type strain but not in the hetR mutant. HetR-dependent inducible
transcripts appeared in two different clusters, that we have named E-DIF (early in differentiation, #6) and L-DIF (late in differentiation, #4) according to the temporal profile of their induction in the wild type strain (Fig. 1 and Supplementary Tables S1, S2). Information on the heterocyst-specific expression or implication in heterocyst differentiation or function existed for 27 of the 72 elements assigned to the E-DIF cluster and for 45 of the 86 elements of the L-DIF cluster. Interestingly, 14 of the E-DIF members and 5 of the L-DIF members were classified as putative antisense or trans-acting sRNAs (Fig. 1 and Supplementary Tables S1, S2). The enrichment of previously characterized heterocyst-related genes in these two clusters supports the idea that the previously unknown elements could be relevant. The finding of several non-coding RNAs suggests a frequent role of these versatile regulatory molecules in heterocyst differentiation.

Transcripts in the E-DIF cluster were induced at an earlier time after nitrogen deprivation (6 hours) than transcripts in the L-DIF cluster, which appeared induced in the 12 or even the 24 hours samples (Fig. 1A and B). The transcriptional profiles of hepA, hglE, hetN and nifP, four previously characterized genes, illustrate this different timing of maximum expression (Fig. 1C). Consistent with their known sequential roles in the differentiation process (reviewed in (Flores et al., 2018)) the hep (heterocyst polysaccharide) genomic islands appeared in the E-DIF cluster, while hgl (heterocyst glycolipids) and nif genes appeared in the L-DIF cluster (Fig. 1B). Expression data for the two previously characterized hep and hgl genomic islands are visualized as genome plots in Supplementary Fig. S2, together with the positions of several transposon insertions that yielded a Fox phenotype (Fan et al., 2005; Huang et al., 2005). The complete results of the microarray analyses are presented as whole genome plots in Supplementary data files 1-4.

The E-DIF cluster of HetR-dependent early responses to nitrogen deprivation
Elements that appeared in the E-DIF cluster are shown in Fig. 1B (left panel). In addition to hep genes (hep island alr2822 to alr2841 and genes alr3698 to alr3700) and devH, encoding a transcriptional regulator involved in synthesis of the hgl layer (Ramírez et al., 2005) the genes encoding NtcA (global nitrogen regulator) and HetR (specific differentiation regulator), both previously described as induced in heterocysts (Black et al., 1993; Olmedo-Verd et al., 2006), also appeared in the E-DIF cluster, indicating they both were similarly induced in a HetR-dependent way in early stages of differentiation. In fact, when integrated in plasmid alpha in single copy, a gfp fusion to the promoter driving transcription from position -271 with respect to the hetR open reading frame, the major inducible TSS of hetR corresponding to genomic position 2821366f (Rajagopalan and Callahan, 2010; Mitschke et al., 2011), was transcribed in prospective heterocysts before any morphological sign of differentiation was observed (Fig. S3). Quantification of the green fluorescence indicated that expression of GFP was already strong in cells that were only initiating their transformation into heterocysts, as indicated by the presence of chlorophyll-derived red autofluorescence, characteristic of vegetative cells and immature prospective heterocysts (Fig. S3B). Such early expression is consistent with previous observations for other elements, such as NsiR1 (Muro-Pastor, 2014) or sigC (Muro-Pastor et al., 2017) that also appeared in the E-DIF cluster.

Initial stages of heterocyst differentiation involve the establishment of a precise pattern of one heterocyst every ten to fifteen vegetative cells in the filaments. One gene involved in such patterning is patS, encoding a small peptide (that includes the sequence RGSGR) produced in heterocysts that represses differentiation of neighboring vegetative cells (Yoon and Golden, 1998). Consistent with its role in early inhibition of differentiation, patS appeared in the E-DIF cluster. Recently, another gene encoding a RGTGR-containing peptide that would act as a second, PatS-like inhibitor of heterocyst differentiation, has been described and named patX (Elhai and Khudyakov, 2018). According to our data, asl2332, the gene encoding PatX, appears also within the E-DIF
cluster, suggesting that the, perhaps redundant, inhibitory effects of both PatS and PatX would take place in early stages of differentiation. *patC*, described as participating in patterning of heterocysts (Corrales-Guerrero et al., 2014) also appears in the E-DIF cluster.

Transcription of *alr1174* and *alr3808*, encoding ruberythrin A and a Dps protein respectively, has been previously described to be induced in heterocysts (Zhao et al., 2007; Li et al., 2015). According to our data, these two genes, likely involved in protection against reactive oxygen species, appear in the E-DIF cluster, and therefore are transcribed during initial stages of heterocyst differentiation.

The E-DIF cluster (as well as the L-DIF cluster, see below) contains several nitrogen-starvation inducible non-coding RNAs, including NsiR1, an early marker of heterocyst differentiation (Ionescu et al., 2010; Muro-Pastor, 2014), NsiR2 (Mitschke et al., 2011), whose heterocyst-specific expression is shown here by *gfp* fusion to its promoter (Fig. S4) or NsiR8 (Brenes-Álvarez et al., 2016), as well as several transcripts produced in antisense orientation with respect to annotated genes. As an example of an N-regulated, HetR-dependent, antisense transcript, we have further analyzed transcription from the aTSS at position 6031913r, antisense to *alr5059* encoding a putative peptidyl prolyl cis-trans isomerase (Fig. 2). Northern blot analysis demonstrated that this HetR-dependent antisense transcript accumulated already 6 h after nitrogen removal (Fig. 2B). Its heterocyst-specific transcription from position 6031913r was verified by construction of a fusion to *gfp*. Quantification of GFP signal and red autofluorescence indicated that the strongest GFP expression took place in cells that, according to the presence of red autofluorescence, were still at an early stage of differentiation (Fig. 2C-E), consistent with the appearance of this antisense transcript in the E-DIF cluster.

*The L-DIF cluster of HetR-dependent late responses to nitrogen deprivation*
Elements that appeared in the L-DIF cluster of transcriptional responses are shown in Fig. 1B (right panel). Whereas the dynamics of expression of genes in the E-DIF cluster was very homogeneous, with induction already in the 6 h sample (Fig. 1A), expression of transcripts in the L-DIF cluster appeared in several, slightly different, temporal profiles. Although in all instances the expression was low in the 6 h sample, highest expression was observed in the 12 h sample, the 24 h sample, or both as illustrated by the examples in Fig. 1C.

Similar to the E-DIF cluster, previously characterized genes appeared in the L-DIF cluster. These included hgl and nif genes, as well as hetN, encoding a putative ketoacyl reductase involved in maintenance of the heterocyst pattern along the filaments (Callahan and Buikema, 2001). In addition, genes flv1B and flv3B encoding two heterocyst-specific flavodiiron proteins (Ermakova et al., 2013), and the cox2 and cox3 operons encoding cytochrome oxidases (Valladares et al., 2007), all associated to oxygen protection in heterocysts, were assigned to the L-DIF cluster.

Concerning the heterocyst-specific uptake hydrogenase, in addition to genes hupS-hupL, genes asr0689 to alr0692, located between all0688 (hupS) and the hyp operon (hypF to hypB) encoding maturation proteins, all appeared in the L-DIF cluster. This suggests that the products of these genes might be functionally related and is further consistent with the definition of an “extended” hyp operon including genes asr0689 to alr0693 (Agervald et al., 2008). The predicted products of asr0689, asr0690 and alr0691 are annotated as unknown or hypothetical proteins. However, alr0692 would encode a protein with homology to NfuA involved in maturation of Fe-S proteins in E. coli under oxidative stress and iron starvation conditions (Angelini et al., 2008).
As an example of a previously undescribed gene whose expression with an L-DIF profile suggests a role in heterocyst differentiation, we have validated HetR dependent, heterocyst-specific expression of *alr0645* (Fig. 3). *Alr0645* possesses four predicted transmembrane domains within a phage holin superfamily IV domain involved in transport of proteins across the cytoplasmic membrane. Northern blot analysis identified transcripts of about 1 kb with increased abundance in the 12 hours sample and strongest accumulation 24 hours after combined nitrogen removal (Fig. 3B). We verified the HetR-regulated expression from position 748886f by primer extension analysis (Fig. 3C), which is consistent with a previously annotated gTSS (Mitschke et al., 2011). Heterocyst-specific transcription of *alr0645* was analyzed in a promoter fusion to *gfp*. Quantification of GFP signals and red autofluorescence indicated that the strongest expression of GFP took place in cells that, according to the absence of red autofluorescence, were already at an advanced stage of differentiation (Fig. 3D-F), consistent with assigning *alr0645* to the L-DIF cluster.

Similar to the E-DIF cluster, the L-DIF cluster contains several non-coding transcripts, including NsiR9 (Brenes-Álvarez et al., 2016). Consistent with its position in the L-DIF cluster, heterocyst-specific transcription from the promoter of NsiR9, analyzed in a fusion to *gfp*, took place in heterocysts at an advanced stage of differentiation (Fig. S4).

### Conserved sequences associated to heterocyst-specific expression in the E-DIF and L-DIF clusters

The promoter regions (750 bp) upstream from all genes included in each of the two DIF clusters and their homologs in genomes of heterocystous cyanobacteria (Shih et al., 2013) were searched for conserved motifs using DREME (see Methods for details). The position-specific occurrences of sequences matching the conserved motifs identified by DREME were analyzed by MAST in the promoters of each cluster (from -300 to +50 with
respect to the corresponding TSS). Only the promoter for the first gene in each transcriptional unit was addressed (see Methods for details on selection of TSS to be included in the analysis, and Supplementary Tables S1 and S2).

For the E-DIF group, sequences corresponding to the DIF1 motif previously defined as TCCGGA (Mitschke et al., 2011) were found around position -35 with respect to the TSS in 30 out of 45 promoters (two mismatches allowed) (Fig. S5), including perfect matches in the promoters of patS (-580), hetR (-271, Fig. S3), or the promoter for the antisense transcript to alr5059 described above (Fig. 2). The phylogenetic conservation of DIF1 motifs across genomes from heterocystous cyanobacteria is illustrated by the occurrence of the sequence TCCGGA around position -35 in the promoters for one sRNA (NsiR1) and one previously unknown inducible gene (alr3676) in the E-DIF group (Fig. S6). Consistent with recent observations (Elhai and Khudyakov, 2018; Wegelius et al., 2018), a conserved G (likely associated to the -10 box of these promoters) is located 16-18 nt downstream of the DIF1 motif (Fig. S5, S6).

In addition to the DIF1 motif that is prevalent in the promoters of the E-DIF cluster, a second motif appeared slightly upstream from position -35. This motif was identified twice by MAST in the promoters of two sRNAs, NsiR2 (Mitschke et al., 2011) and NsiR8 (Brenes-Álvarez et al., 2016), as well as in the promoters corresponding to open reading frames alr2463, asr1314, and to as_all4999 (Fig. 4A). The motif, that we have named DIF2, is a hexameric direct repeat separated by two nucleotides, with the consensus sequence TTGGCAWWTTGGCA. The DIF2 motif is phylogenetically conserved in the promoters of all homologs of NsiR2, NsiR8 and alr2463 chosen as examples (Fig. S7).

To test the functional relevance of the DIF2 motif, two mutations were introduced that altered the first repeat (pSAM321) or increased the spacing between repeats (pSAM322) in the promoter of NsiR2 that was fused to gfp in pSAM320, bearing a promoter fragment
from position -117 to +19 with respect to the TSS (Supplementary Table S4 and Fig. S8). Each variant was introduced into wild type Nostoc sp. PCC 7120 and integrated into plasmid alpha by homologous recombination. Hence, the resulting strains bear two copies of the NsiR2 promoter, the native one in the chromosome, driving transcription of NsiR2, and one of the variants fused to gfp integrated in plasmid alpha. Transcription from both NsiR2 promoters (that starts at two closely spaced positions) was analyzed simultaneously by primer extension using two different primers, one specific for the chromosomal copy, and another specific for the gfp fusion integrated in plasmid alpha (Fig. S8). The results of primer extension (Fig. 4B) and confocal fluorescence microscopy (Fig. 4C) showed that the 135-nt DNA fragment fused to gfp in pSAM320 was sufficient to promote early heterocyst-specific expression, and that either the mutation of the upstream repeat or the increase in the spacing between the two repeats led to lack of GFP expression.

In contrast to these findings for the E-DIF cluster, the comparison of promoters in the L-DIF cluster only led to the identification of conserved motifs that corresponded to sequences associated to the CnfR (a.k.a. PatB, alr2512, also in the L-DIF cluster) -regulated expression of the nifB promoter of Anabaena variabilis ATCC 29413 (Vernon et al., 2017) and the nifB and nifP promoters of the non-heterocystous cyanobacterium Leptolyngbya boryana (Tsujimoto et al., 2016) (Fig. S9A). In addition to nifB and nifP, we identified the CnfR-associated sequence motif also in the promoter driving transcription of a tricistronic operon (alr2522 to alr2524) with unclear function. alr2522 has been assigned to the core genome of heterocyst-forming cyanobacteria (Simm et al., 2015) and is up-regulated in a mutant overexpressing the sigma factor SigJ that confers resistance to desiccation by activating genes involved in polysaccharide synthesis (Yoshimura et al., 2007). asr2523 and alr2524 appeared among genes downregulated in mutants of sigma factors SigC or SigE (Ehira and Miyazaki, 2015).

Phylogenetic conservation of CnfR-associated sequence motifs in the promoter regions
of *nifB*, *nifP* and *alr2522* homologs across genomes of heterocystous cyanobacteria is shown in Fig. S9B together with the TSS determined for *nifB* in *Nostoc* sp. PCC 7120 (Mulligan and Haselkorn, 1989; Mitschke et al., 2011) and in *Nodularia spumigena* CCY9414 (Voβ et al., 2013) as well as the TSS determined for the *nifP* homolog in *Nodularia spumigena* CCY9414 (Voβ et al., 2013).

**Discussion**

Heterocyst differentiation in filamentous cyanobacteria involves substantial transcriptome remodeling. Most of these changes take place specifically in the cells that start differentiating as heterocysts. In contrast to general responses to nitrogen deficiency, mainly aimed at nitrogen scavenging and under control of NtcA, transcriptional responses specifically involved in heterocyst differentiation additionally depend on the master regulator HetR. In this study, we have identified two groups (early and late) of HetR-dependent (DIF) genetic elements (genes, sRNAs, antisense transcripts) involved in heterocyst differentiation and/or function. The approach we have followed is based on the clustering of similar transcriptional profiles with changes above a certain threshold. This approach allows the identification of essential elements which are not possible to mutate and of functionally redundant or accessory elements that would not yield a distinct phenotype when mutated. Nevertheless, such elements can have a role in heterocyst differentiation or function.

Using GFP as a reporter, we demonstrate that transcripts included in the DIF clusters defined here are expressed specifically in heterocysts. This is consistent with their dependence on HetR and corroborates the different temporal profiles that led to their inclusion in the E-DIF or L-DIF groups. Both groups include previously known genes but also genes of unknown function. A schematic representation of all elements identified in both DIF groups defined here is shown in Fig. 5. Genes previously related to heterocyst
differentiation and function are shown in the left side of the scheme, whereas elements for which previously no function has been defined in the context of heterocyst differentiation are shown to the right.

NtcA and HetR are mutually dependent in the context of differentiation (Muro-Pastor et al., 2002). Both genes are transcribed from complex promoter regions that include multiple, constitutive and inducible TSS (Olmedo-Verd et al., 2006; Rajagopalan and Callahan, 2010; Mitschke et al., 2011). The observation that their transcripts accumulate with E-DIF dynamics suggests their heterocyst-specific promoters make a major contribution to the overall transcriptional response to the absence of combined nitrogen. In both cases the regulated TSS (position -271 for hetR and -180 for ntcA) is controlled by a promoter containing a DIF1 motif (Fig. S5). The observation that the expression of ntcA itself correlates with the expression of heterocyst-specific genes is consistent with the higher amount of NtcA protein observed in heterocysts vs. vegetative cells (Sandh et al., 2014). Increased amounts of NtcA in developing heterocysts might contribute to the differential regulation of NtcA-dependent genes in heterocysts vs. vegetative cells. In this context, increased transcription of NtcA-induced genes, such as glnA or nblA, in developing heterocysts, could lead to higher levels of certain proteins that are otherwise not heterocyst-specific, resulting, for instance, in higher glutamine synthetase activity or phycobilisome degradation in those specialized cells. Similarly, regulatory loops involving sRNAs regulated by NtcA, such as NsrR1 (Álvarez-Escribano et al., 2018) could operate more strongly in heterocysts vs. vegetative cells.

The patS and hetN genes appeared in the E-DIF and L-DIF clusters, respectively. This is consistent with recent observations concerning the relative timing of their expression (Di Patti et al., 2018), and the roles in early establishment (PatS) or maintenance (HetN) of the pattern (Yoon and Golden, 1998; Callahan and Buikema, 2001). Recently, it has been demonstrated that patS and hetN are not functionally equivalent when their
promoter regions are switched, therefore suggesting their respective timing of expression is critical for proper function (Rivers et al., 2018). Two other genes described as participating in patterning of heterocysts, \textit{patC} (Corrales-Guerrero et al., 2014) and \textit{patX} (Elhai and Khudyakov, 2018), also appeared in the E-DIF group. Therefore, according to the transcriptional profiles described here, up to three elements would act as negative regulators during initial steps of differentiation in \textit{Nostoc} sp. PCC 7120. This observation illustrates possible functional redundancy among critical players governing cell differentiation processes.

Timing of expression of genes encoding proteins related to oxygen protection suggests an early role during development for elements like RbrA, a cyanobacterial rubrerythrin (Zhao et al., 2007) or the Dps protein encoded by \textit{alr3808} (Li et al., 2015), both in the E-DIF group (Fig. 1B). In contrast, the heterocyst-specific flavodiiron proteins encoded by \textit{flv1B} and \textit{flv3B} (Ermakova et al., 2013; Ermakova et al., 2014) or the cytochrome oxydases encoded by \textit{coxBAC2} and \textit{coxBAC3} (Valladares et al., 2007), all in the L-DIF cluster, would likely be involved in functional aspects of mature heterocysts.

The relatively homogeneous temporal dynamics observed for genes in the E-DIF cluster points to a limited number of participating regulatory factors. Accordingly, the search for regulatory sequences that appear statistically enriched in the E-DIF cluster led to the identification of the DIF1-motif (TCCGGA) (Mitschke et al., 2011) previously associated to early heterocyst-specific expression (Muro-Pastor, 2014; Muro-Pastor et al., 2017). A new motif (TTGGCAWWTTGGCA) that we have called DIF2, is present in the promoters of several transcripts (including sRNAs NsiR2 and NsiR8) that also belong to the early heterocyst-specific transcriptome. The slightly different temporal profiles observed for induction of L-DIF elements, likely due to the participation of several different regulators, is consistent with a limited identification of conserved promoter elements in this group. Nevertheless, the array of conserved sequences defined as CnfR-regulated promoters
for \textit{nifB} (Vernon et al., 2017) and \textit{nifP} (Tsujimoto et al., 2016) could also be identified in the promoter driving transcription of another gene cluster with late DIF profile (\textit{alr2522- alr2524}), suggesting these genes might also be under control of CnfR in \textit{Nostoc} sp. PCC 7120. The presence of DIF1, DIF2 or CnfR motifs in the corresponding promoters is indicated with asterisks in the elements shown in Fig. 5.

Phylogenetic conservation of the occurrence of DIF1, DIF2 or CnfR motifs in certain genes suggests conservation of the transcriptional machinery involved in regulated transcription of the corresponding promoters across heterocystous cyanobacteria. Whether specialized sigma factors, repressors or activators are involved in the regulation of heterocyst-specific promoters described here is currently unknown. Genes encoding sigma factors SigC, SigE and SigG are transcribed heterocyst-specifically (Aldea et al., 2007), although the enhanced transcription of \textit{sigG} in heterocysts might be a consequence of cell envelope stress during differentiation (Bell et al., 2017). Furthermore, \textit{sigC} is transcribed in very early stages of differentiation (Muro-Pastor et al., 2017). Although \textit{sigC} or \textit{sigE} mutants show a slight delay in differentiation (Khudyakov and Golden, 2001; Mella-Herrera et al., 2011) and both SigC and SigE are required for normal expression of heterocyst-specific genes (Mella-Herrera et al., 2011; Ehira and Miyazaki, 2015), no single sigma factor is exclusively associated to heterocyst development, likely due to functional redundancy. Identification of the proteins involved in heterocyst-specific regulated transcription is a challenge for future research.

The N-responsive transcriptome of \textit{Nostoc} sp. PCC 7120 includes abundant non-coding transcripts (Flaherty et al., 2011; Mitschke et al., 2011). Both DIF clusters defined here include, in addition to protein-coding genes, several antisense and putative sRNAs, suggesting that cell-specific post-transcriptional regulation is relevant to properly adjust expression levels of certain genes specifically in developing heterocysts. Both trans-acting sRNAs and antisense RNAs have been described as regulators of cyanobacterial
metabolism, including acclimation to changing iron availability (Georg et al., 2017), carbon supply (Eisenhut et al., 2012) or light intensity (Georg et al., 2014). Nitrogen-regulated sRNAs are involved in feed-forward regulatory loops providing additional levels of response to nitrogen deficiency (Klähn et al., 2015; Álvarez-Escribano et al., 2018).

Similarly, heterocyst-specific sRNAs (NsiR1, NsiR2, NsiR8 or NsiR9) and antisense transcripts identified in the E-DIF and L-DIF groups of transcriptional responses could modulate the stability and/or translation of their corresponding target transcripts specifically in developing heterocysts, thus contributing to the metabolic rearrangements that take place in these specialized cells.

Classical approaches involving transposon mutagenesis had identified major players of the process of heterocyst differentiation. Some other genes, such as nitrogenase genes, can be identified on the basis of their similarity to genes previously described in other nitrogen-fixing organisms. However, genes that remain annotated as unknown have not been identified in the context of mutagenesis experiments or by homology to genes with known function. And yet, the transcriptional profiles of the new elements identified in this work strongly suggest they participate in some, perhaps subtle, aspects of heterocyst differentiation or function of the nitrogen-fixing machinery. Knowledge of these additional factors might be crucial to optimize experimental approaches to engineer biological nitrogen fixation using nitrogenases of cyanobacterial origin (Liu et al., 2018) or to use cyanobacteria as hosts for the production of O2-sensitive molecules (Avilan et al., 2018).

**Experimental procedures**

**Strains and growth conditions**

Cultures of *Nostoc* sp. PCC 7120 WT and the *hetR* mutant 216 bearing point mutation S179N (Buikema and Haselkorn, 1991a) were bubbled with an air/CO₂ mixture (1% v/v).
and grown photoautotrophically at 30°C in BG11C medium (Rippka et al., 1979) lacking NaNO₃ but containing 6 mM NH₄Cl, 10 mM NaHCO₃, and 12 mM N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid-NaOH buffer (pH 7.5). Additionally, cells were grown in the same medium but in the absence of any combined nitrogen source (BG11₀C). To induce nitrogen deficiency, cells were filtered, washed with and resuspended in nitrogen-free medium (BG11₀C). RNA samples were isolated from cells taken at T = 0 h (WT₀ and hetR₀) and T = 6, 8, 12, 24 h after removing combined nitrogen from the media. It should be noted that precise timing of heterocyst differentiation depends on laboratory conditions and the previous growth state of cells. All nitrogen step-down experiments shown here were performed with exponentially growing cells. Additionally, for the experiment involving addition of ammonium to N₂-fixing cells, 10 mM NH₄Cl, and 20 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid-NaOH buffer (pH 7.5) were added to cells previously grown in the absence of combined nitrogen. Construction of strains bearing plasmids is described in Supplementary methods. Plasmids and oligonucleotides used are described in Supplementary Tables S3, S4 and S5.

**RNA extraction, microarrays, Northern blots and primer extension assays**

Total RNA was isolated using hot phenol as described (Mohamed and Jansson, 1989) with modifications (see Supplementary methods). Probes in the microarrays were designed in antisense orientation allowing the direct hybridization to labeled RNA, without conversion to cDNA. The microarray contains multiple probes for every protein-coding region, and for every previously detected transcript with more than 50 reads in the dataset of (Mitschke et al., 2011). In addition, all intergenic regions without known transcription were covered by probes on both strands. Details of the labeling and hybridization protocol can be found in (Voβ and Hess, 2014) and in Supplementary methods. Microarrays were hybridized with samples from two experiments. The first one
contains two biological replicas of a time course (0, 6, 12 and 24 hours) after removal of combined nitrogen using ammonium-grown cells of the wild type and the hetR mutant. The second experiment is a single time point experiment after removal of combined nitrogen from cells growing in the presence of ammonium (0, 8 hours) or after addition of ammonium to cells growing at the expense of atmospheric nitrogen, N$_2$ (0, 8 hours). The microarray data can be accessed in the GEO database under the accession GSE120377. Northern blot hybridization and primer extension analysis of 5´ ends was performed as previously described (Muro-Pastor et al., 1999; Steglich et al., 2008).

**Computational methods**

Raw data were processed with the limma (Ritchie et al., 2015) R package to extract the differentially expressed probes. We performed the following comparisons of expression between two samples: every condition vs. the absolute reference (WT 0 h from the -N experiment = ammonium-grown cells) and every condition vs. its own reference (for instance, hetR-12 vs. hetR-0). A total of 8919 genetic elements (genes, putative sRNAs, putative asRNAs, etc.) that exhibited a log$_2$FC (fold change) >1.25 in at least one of the comparisons were selected for further analysis (Supplementary Dataset S1). A co-expression network was constructed based on the microarray data. We used a cutoff of 0.92 of the absolute value of Pearson correlation between the expression profile of one feature and the expression profiles of any other feature in the microarray. If two features have a correlation >0.92 (absolute value) they are connected by an edge in the network. Using these parameters, the network contains 7134 features. Finally, we performed a clustering analysis of the 7134 features in the co-expression network using the mfuzz R package (Kumar and Futschik, 2007) and obtained ten clusters using a “fuzzifier” (m parameter) of m=1.25. We selected a strict confidence threshold of 0.85 to consider a gene placed within the core of any given cluster. Cytoscape was used for the visualization and edition of the network (Supplementary data file 5). We ran DREME
(Bailey, 2011) with default parameters, except e-value (e=10) and maximum number of motifs to find (m=12) to search for conserved sequence motifs in each of the two clusters of HetR-dependent responses (#4 and #6), while the other two clusters bearing HetR-independent responses (#9 and #10) served as negative control dataset. In each prediction, we used the pool of sequences corresponding to one cluster as input, while sequences of the remaining 3 clusters served as negative control dataset. The resulting motifs were then searched in a window from -300 to +50 of the respective TSS in the E-DIF (#6) or L-DIF (#4) clusters using MAST (Bailey and Gribskov, 1998) with the parameter settings ev=1000, -remcorr and –mt 2e-3. To map the identified motifs relative to the set of TSS, TSS positions were taken from reference (Mitschke et al., 2011) (E-DIF cluster), reference (Flaherty et al., 2011) (L-DIF cluster), as well as from the expression of probes in the microarrays. In addition, some TSS were experimentally determined by primer extension. Only the TSS of the first gene was considered for transcriptional units involving several genes. Additional details on computational methods can be found in Supplementary methods.

Fluorescence microscopy

Images of filaments subjected to nitrogen deficiency on plates were taken four days after plating in media without combined nitrogen. The accumulation of GFP was analyzed and quantified using a Leica TCS SP2 confocal laser scanning microscope as described (Muro-Pastor, 2014). GFP was excited at 488 nm by an argon ion laser, and the fluorescent emission was monitored by collection across windows of 500-538 nm (GFP imaging) and 630-700 nm (cyanobacterial autofluorescence). Images were treated with ImageJ 1.45s software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2018).

Supporting information
Appendix S1: Supporting pdf file, including supplementary methods, supplementary Figures S1 to S9, supplementary Tables S1 to S5 and description of supplementary data files 1 to 5 (to be downloaded from http://hdl.handle.net/10261/179430).

Dataset S1: 8919 elements with a log₂FC larger than 1.25 (in any of the comparisons made).

Dataset S2: 2463 elements that enter the 10 clusters (confidence >0.85) obtained after mfuzz analysis.

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Figure legends

Fig. 1. The E-DIF and L-DIF clusters of HetR-dependent inducible transcripts. A. Expression changes of transcripts classified as early in differentiation (E-DIF; upper panel) and late in differentiation (L-DIF; lower panel) along the 20 samples analyzed by microarray hybridization. Samples corresponded, from left to right, to a time course after nitrogen depletion (\(-N\); 0, 6, 12 and 24 hours after nitrogen removal; two biological replicates for each sample) of the wild type and the hetR mutant, as well as one nitrogen step down experiment (\(-N\); 0 and 8 hours after nitrogen removal) and a nitrogen addition experiment (+N; 0 and 8 hours after addition of ammonium to N2-growing cells) of the wild-type strain. Expression changes are indicated as Z-scores. B. Expression of elements included in both clusters (E-DIF, left panel; L-DIF, right panel) along the time course (indicated in hours) of nitrogen depletion in the wild type and the hetR mutant. Several previously described transcriptional units and clusters of related genes are indicated by vertical black bars. C. Expression of four selected previously known transcripts exhibiting different temporal profiles of induction after nitrogen depletion. In B-C, color key represents row Z-scores for expression of each listed element, therefore the values are not absolute but relative.

Fig. 2. as_alr5059, an N-regulated, HetR-dependent antisense transcript in the E-DIF cluster. A. Scheme of the chromosomal region covering all5058 to all5060. gTSS annotated for the three genes according to (Mitschke et al., 2011) are indicated by bent arrows, as well as the aTSS inside alr5059. A scheme of the fragment fused to gfp in pMBA76 is shown, as well as the fragment used as RNA probe in Northern blot (red bar). B. Northern blot analysis of as_alr5059 in Nostoc sp. PCC 7120 and mutant strain 216 (hetR) (upper panel) at different time points (indicated in hours) after nitrogen removal. Hybridization to a probe for 5S rRNA was used as loading control (bottom panel). C. Expression of P_\text{as_alr5059} \_gfp along nitrogen-fixing filaments of Nostoc sp. PCC 7120 bearing pMBA76. Confocal fluorescence image (red and green channels merged) of a
filament growing on top of nitrogen-free medium is shown together with quantification of the signals for the red (autofluorescence, D) and green (GFP, E) channels. Mature heterocysts (#1 and #3) are indicated in white (C) or black (E). Immature heterocyst (#2) is indicated in red. Black arrows in (D) indicate positions of gaps in red autofluorescence, indicative of mature heterocysts.

**Fig. 3.** *alr0645*, an N-regulated, HetR-dependent gene in the L-DIF cluster. A. Scheme of the chromosomal region covering *alr0644* to *all0646*. gTSS for *alr0645* according to (Mitschke et al., 2011) is indicated by a bent arrow. The fragment fused to gfp in pMBA28 is shown, as well as the fragment used as DNA probe in Northern blot (red bar). B. Northern blot analysis of *alr0645* in Nostoc sp. PCC 7120 and mutant strain 216 (*hetR*) at different times after combined nitrogen removal (upper panel). Hybridization to a probe for *rnpB* was used as a loading control (lower panel). C. Primer extension analysis (oligonucleotide alr0645_3, Table S3) of transcripts starting at position 748886f in RNA isolated from the wild type strain (WT) or the *hetR* mutant strain after the cells were transferred to nitrogen-free medium for 24 hours. D. Expression of *P*<sub>alr0645-gfp</sub> along nitrogen-fixing filaments of *Nostoc* sp. strain PCC 7120 bearing pMBA28. Confocal fluorescence image (red and green channels merged) of a filament growing on top of nitrogen-free medium is shown. Quantification of the signals along the filament is shown separately for the red (E) and green (F) channels. Mature heterocysts (#1 and #3) are indicated in white (D) or black (F). Immature heterocyst (#2) is indicated in red. Black arrows in (E) indicate positions of gaps in red autofluorescence, indicative of mature heterocysts. Sizes are indicated in nt.

**Fig. 4.** DIF2 motif associated to heterocyst-specific expression of promoters of the E-DIF group. A. Scheme of HetR-dependent promoter sequences in the E-DIF group that contain a putative DIF2 motif. Sequences matching the TTGGCA repeats are indicated in red, and putative -10 sequences are indicated in blue. B. Primer extension analysis of the TSS of NsiR2 in strains bearing plasmids pSAM320, pSAM321 or pSAM322.
integrated in plasmid alpha. Schemes of the wild-type chromosomal nsiR2 region and
the different mutant variants of the additional nsiR2 promoter fused to gfp (integrated in
plasmid alpha) are shown on the left. The positions of the oligonucleotides used as
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Primer extension analysis with primer NsiR2 (upper panel) or GFP4 (lower panel) was
carried out using RNA isolated from strains SAM320, SAM321, and SAM322 after the
cells were transferred to nitrogen-free medium for the number of hours indicated. C.
Expression pattern of GFP from the wild-type promoter of NsiR2 (strain SAM320), or
from the two mutated variants in strains SAM321 or SAM322. Images correspond to
merged red (autofluorescence) and green (GFP) fluorescence.

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elements defined here are summarized according to their assignment to the E-DIF
(orange) and L-DIF (green) groups defined in this work. Genes appearing in the two
groups are shown along a temporal sequence of differentiation and according to their
previously known (left side) or unknown (right side) implication in the differentiation of
heterocysts. Elements bearing promoters with a recognizable conserved sequence motif
are indicated with blue (DIF1), red (DIF2), or black (CnfR) asterisks. Note that this
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B. Northern blot analysis of as_alr5059 in Nostoc sp. PCC 7120 and mutant strain 216 (hetR) (upper panel) at different time points (indicated in hours) after nitrogen removal. Hybridization to a probe for 5S rRNA was used as loading control (bottom panel). C. Expression of P_{as_alr5059}gfp along nitrogen-fixing filaments of Nostoc sp. PCC 7120 bearing pMBA76. Confocal fluorescence image (red and green channels merged) of a filament growing on top of nitrogen-free medium is shown together with quantification of the signals for the red (autofluorescence, D) and green (GFP, E) channels. Mature heterocysts (#1 and #3) are indicated in white (C) or black (E). Immature heterocyst (#2) is indicated in red. Black arrows in (D) indicate positions of gaps in red autofluorescence, indicative of mature heterocysts.
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