A combinatorial strategy of alternative promoter use during differentiation of a heterocystous cyanobacteria

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Running title: Alternative promoters in heterocyst differentiation

Originality-significance statement

In response to nitrogen deficiency, heterocyst differentiation involves the selection of certain individual cells along cyanobacterial filaments to undergo a complete transformation into a different, nitrogen-fixing cell-type. Such transformation relies on precise regulation of transcripts that might be required, although at different levels, both in vegetative cells and in cells undergoing differentiation as heterocysts. We have noticed that some housekeeping genes are transcribed from combinations of transcriptional starts that respond to nitrogen deficiency with ostensibly opposite behavior. Here we propose that such combinations of promoters are in fact the result of combinatorial promoter use in the two cell types of cyanobacterial filaments. Using the example of the gene encoding the housekeeping sigma factor SigA, we show that at the same time its major promoter is repressed as a consequence of nutritional deprivation, a second, heterocyst-specific promoter is induced to bypass repression specifically in those cells that need to continue actively transcribing. This combinatorial strategy provides additional regulatory possibilities to achieve precise cell-specific levels of transcripts.

Summary

Heterocystous cyanobacteria such as *Nostoc* sp. are filamentous photosynthetic organisms that, in response to nitrogen deficiency, undergo a differentiation process transforming certain, semi-regularly spaced cells into heterocysts, devoted to nitrogen fixation. During transition to a nitrogen-fixing regime, growth of most vegetative cells in the filament is temporarily arrested due to nutritional deprivation, but developing heterocysts require intense transcriptional activity. Therefore, the coexistence of arrested vegetative cells and actively developing prospective heterocysts relies on the simultaneous operation of somewhat opposite transcriptional programs.

We have identified genes with multiple nitrogen-responsive transcriptional starts appearing in seemingly paradoxical combinations. For instance, *sigA*, encoding the RNA polymerase housekeeping sigma factor, is transcribed from one major nitrogen stress-repressed promoter and from a second, nitrogen stress-induced promoter. Here we show that both promoters are expressed with complementary temporal dynamics. Using a *gfp* reporter we also show that transcription from the inducible promoter takes place exclusively in differentiating heterocysts and is already detected before any morphological or fluorescence signature of differentiation is observed. Tandem promoters with opposite dynamics could operate a compensatory mechanism in which repression of transcription from the major promoter operative in vegetative cells is offset by transcription from a new promoter only in developing heterocyst.

Introduction

Cyanobacteria are considered responsible for the origin of the oxygenic atmosphere on Earth (Lyons et al., 2014). Nowadays, being the most abundant photosynthetic organisms on Earth, cyanobacteria play essential roles in the renewal of atmospheric oxygen and the production of biologically available nitrogen, both as free-living organisms and as partners in nitrogen-fixing symbioses with plants and fungi. Cyanobacteria have a wide ecological distribution that ranges from deserts to all kinds of water reservoirs, suggesting the operation of sophisticated adaptation mechanisms. Filamentous cyanobacteria are developmentally complex prokaryotes that exhibit a variety of cell differentiation processes, including the formation of spore-like cells, motile hormogonia or nitrogen-fixing heterocysts. Heterocystous cyanobacteria are filamentous photosynthetic organisms that grow either as strings of undifferentiated photosynthetic cells (called vegetative) or, upon nitrogen deficiency, as filaments containing vegetative cells and heterocysts, the latter specialized in fixation of atmospheric nitrogen. The differentiation of heterocysts is induced by environmental clues and leads to a multicellular lifestyle in which two cell types with different, complementary metabolic capabilities, cooperate to achieve growth of the filament as a whole (Flores and Herrero, 2010; Muro-Pastor and Hess, 2012). Therefore, when growth is supported by atmospheric nitrogen rather than fixed nitrogen, heterocystous cyanobacteria are obligate multicellular prokaryotes. Transition to a nitrogen-fixing regime involves a period of time in which the growth of the filament transiently stops as a consequence of nutritional deprivation. Such arrest correlates with reduced transcription of

several housekeeping genes, including those for RNA polymerase (Flaherty et al., 2011). Once mature heterocysts are fully functional nitrogen fixation begins and growth resumes.

Cyanobacterial adaptation to nitrogen deficiency is mainly orchestrated at the transcriptional level by NtcA, a CRP/FNR-family transcription factor that is also required for heterocyst differentiation (Frías et al., 1994; Herrero et al., 2001). The activity of NtcA is modulated by the amount of 2-oxoglutarate (Vázquez-Bermúdez et al., 2002), which reflects the nitrogen status of the cells (Muro-Pastor et al., 2001). In the presence of combined nitrogen, the level of 2-oxoglutarate is low and NtcA has low affinity to target promoters, whereas, under nitrogen depletion, increased levels of 2-oxoglutarate stimulate binding of NtcA to its targets (Zhao et al., 2010). The consensus sequence for NtcA binding, initially described as $GTAN_8TAC$ (Luque et al., 1994), has been redefined as $GTN_{10}AC$ after genome-wide analyses involving RNASeq (Mitschke et al., 2011) and ChIP-Seq (Picossi et al., 2014). Depending on the location of the binding motif with respect to transcriptional starts, NtcA can act either as an activator or as a repressor of transcription (Mitschke et al., 2011; Picossi et al., 2014). In addition to NtcA, the developmental response that leads to heterocyst differentiation requires HetR, a regulator specifically involved in cellular differentiation (Buikema and Haselkorn, 1991; Black et al., 1993). In the context of heterocyst differentiation both factors are mutually dependent (Muro-Pastor et al., 2002) and both genes show increased transcription in developing heterocysts (Black et al., 1993; Olmedo-Verd et al., 2006). Overexpression of HetR promotes differentiation, leading to a MCH (multiple contiguous

heterocysts) phenotype (Buikema and Haselkorn, 2001). Although purified HetR has been shown to bind inverted repeats upstream of *hetP* (Higa and Callahan, 2010) or *hetZ* (Du et al., 2012), it is currently unknown how such DNA-binding activity relates to HetR-mediated regulation of transcription.

The morphological and functional transformation of a vegetative cell into a mature heterocyst requires transcription of many genes whose products are exclusively required in the context of heterocyst differentiation and function, such as those involved in synthesis and deposition of the polysaccharide and glycolipid layers of the heterocyst envelope. In addition, the transition to a different growth regime at the expense of molecular nitrogen involves a drastic change in the physiology of the organism as a whole. Because all these changes take place in filaments that contain two cell types (vegetative cells and prospective heterocysts), the transcriptional responses taking place in each of them appear combined in global analyses of the transcriptome. In order to dissect the transcriptional landscape of complex organisms, a procedure called differential RNASeq (dRNASeq) has been applied to the global transcriptional analysis of several bacteria. dRNASeq involves selection of primary transcripts that have a 5’ triphosphate (in contrast to processed RNAs having a 5’ monophosphate) and allows the identification of true transcriptional starts at single base resolution, thus providing an unprecedented view of transcriptional initiation and facilitating direct identification of promoter sequences (Sharma and Vogel, 2014).

A dRNASeq-based analysis of the transcriptome of *Nostoc* sp. strain PCC 7120 allowed the identification of all transcriptional starts along the genome as well as the classification of nitrogen-responsive promoters into different categories according to their dynamics upon nitrogen step down (Mitschke et al., 2011). The transcriptional landscape of this cyanobacterium includes many genes transcribed from complex promoter regions with several transcriptional starts. Upon inspection of the dataset we noticed that some housekeeping genes contained combinations of promoters with opposite dynamics in response to nitrogen deficiency. Here we further analyse the cases of *sigA*, encoding the housekeeping sigma factor SigA (Brahamsha and Haselkorn, 1991), and *asl4317-asl4316*, the latter encoding phospho-N-acetylmuramoyl-pentapeptide-transferase (MraY). According to RNASeq data, transcription from the major promoter of *sigA* is repressed in response to nitrogen deficiency while another promoter is induced (Mitschke et al., 2011). We show here that both promoters operate with complementary temporal (and spatial) dynamics, since the inducible promoter is expressed exclusively in developing heterocysts. Also in the case of *asl4317*, one nitrogen stress-inducible and one nitrogen stress-repressed promoter are observed. We hypothesize that the presence of such seemingly paradoxical combinations of promoters in housekeeping genes could allow increased expression in prospective heterocyst while vegetative cells are undergoing a generalized reduction of transcription as a consequence of nutritional stress.

Results and discussion

Nitrogen regulated promoters exhibiting opposite dynamics in Nostoc *sp. strain PCC 7120*

The dynamics of use of transcriptional starts in response to nitrogen deficiency has been analysed genome-wide in *Nostoc* sp. strain PCC 7120 and its heterocyst-deficient mutant derivative *hetR* by applying a dRNASeq-based procedure (Mitschke et al., 2011). The global analysis of the HetR-dependent transcriptome led to the identification of a sequence motif (DIF1, 5'TCCGGA3', around position -35 of regulated promoters), that appeared in the previously characterized heterocyst-specific promoters of several genes including the small RNA NsiR1 (Ionescu et al., 2010), *hetR* (Rajagopalan and Callahan, 2010), or *sigC* (encoding the heterocyst-specific alternative sigma factor SigC) (Brahamsha and Haselkorn, 1992; Aldea et al., 2007). Based on the available dataset of nitrogen-regulated transcriptional start sites, we have now systematically searched for promoter regions containing combinations of regulated promoters that exhibit opposite dynamics in response to nitrogen deficiency (inducible and repressed). Table 1 shows promoter regions that contain at least one nitrogen stress-repressed promoter (DEF- category) and one nitrogen stress-inducible, HetR-dependent, promoter (DIF+ category) (Mitschke et al., 2011). In the case of the DIF+ promoters, the presence of sequences matching the DIF1 motif is also included in Table 1. We decided to further characterize transcription of two genes encoding housekeeping functions, *sigA*, encoding the RNA polymerase housekeeping sigma factor, and of *asl4317-mraY*, the latter encoding MraY, involved in peptidoglycan metabolism. In both cases, repression of transcription would be consistent with

growth arrest as a consequence of nitrogen deprivation, but the identification of nitrogen stress-induced promoters was however unexpected. Interestingly, both in the case of *sigA* and of *asl4317*, the presence of sequences matching a DIF1 motif suggests heterocyst-specific transcription.

Nitrogen regulated promoters of sigA

Transcription of *sigA* had been initially analyzed by primer extension, showing transcripts with five different 5' ends located at -328 (P_1), -592 (P_2), -605 (P_3), -800 (P_4) and -868 (P_5) with respect to the translational start. P_1 was identified as the major promoter being operative both in the presence or absence of combined nitrogen (Brahamsha and Haselkorn, 1991). The use of dRNASeq later confirmed all of them except P_2 (-592) as promoters producing true primary 5' ends, suggesting the 5' ends at -592 are the result of processing of longer transcripts (Mitschke et al., 2011). According to dRNASeq data, although transcription from P_4 and P_5 appears somewhat modulated by nitrogen availability, transcription from P_1 and P_3 (see a scheme in Fig. 1A) shows the strongest response to nitrogen deprivation. Transcription from P_1 is repressed under nitrogen deprivation (log_2 fold change= -3.43 at 8 h after nitrogen step-down), while transcription from P_3 is inducible upon nitrogen deprivation in a HetR-dependent manner (log_2 fold change= 3.66 at 8 h after nitrogen step down) (Mitschke et al., 2011). Accordingly, P_1 was classified as a DEF- transcript, whereas P_3 was classified as a DIF+ transcript (Mitschke et al., 2011).

We have analysed the temporal profile of transcription from both nitrogen regulated transcriptional starts, as well as the possible presence in P_1 and P_3 of sequence signatures found in previously known nitrogen-regulated promoters. Fig. 1B shows a comparison of the sequences of the P_1 promoter of *sigA* and two NtcA-repressed promoters, those of *gifA* and *rbcL* (Mitschke et al., 2011). A putative NtcA binding site ($GTGN_8TAC$) that partially overlaps the -10 box, and is therefore compatible with repression of transcription by binding of NtcA, can be identified in the case of the P_1 promoter of *sigA*. Primer extension analysis of transcripts from P_1 confirmed transient repression that was alleviated as heterocyst differentiation progressed (Fig. 1C, left panel). Such repression did not take place in an *ntcA* mutant that in fact showed increased transcription from P_1 even in the presence of combined nitrogen (Fig 1C, right panel). NtcA binding to the P_1 promoter was confirmed by electrophoretic mobility shift assays (EMSA) with purified his-tagged NtcA protein (Fig. 1D). These observations are in agreement with a previous ChIPSeq analysis showing NtcA binding to a region between coordinates 6279081-6279345 (Picossi et al., 2014). Repression of transcription from position -328 (P_1) seems therefore directly operated by binding of NtcA, suggesting repression takes place in all cells of the filament as a consequence of nitrogen deprivation.

According to dRNASeq, transcription from the P_3 promoter of *sigA* was inducible upon nitrogen step down and HetR-dependent (Mitschke et al., 2011). Fig. 1E shows an alignment of the P_3 promoter of *sigA* and the heterocyst-specific promoters of NsiR1, *hetR* and *sigC*. In the case of *sigA*, the P_3 promoter contains a DIF1-motif that appears in the same relative position with respect to

the transcriptional start site as those of the other three demonstrated heterocyst-specific promoters included for comparison. According to primer extension assays, transcription from P_3 (position -605) was already induced three hours after nitrogen step-down but decreased after a longer period of nitrogen deficiency as heterocysts differentiated (Fig. 1F, left panel). Consistent with RNASeq data, primer extension confirmed that induction of transcription from P_3 did not take place in a *hetR* mutant (Fig. 1F, right panel).

Heterocyst-specific transcription of sigA

The presence of a DIF1 motif, together with the observed HetR-dependence of P_3, suggested heterocyst-specific transcription of *sigA*. To confirm whether this was in fact the case, the *gfp* gene was fused to the sequences driving transcription from position -605 (see a scheme of the construct in Fig. 2A). Expression of GFP in cells bearing pSAM330 was analyzed by confocal fluorescence microscopy in filaments growing on top of nitrogen-free solid medium, so that different stages of heterocyst maturation could be observed in a single filament (Fig. 2B). Similar to transcription from the DIF1-containing promoter of NsiR1, also analysed by this method (Muro-Pastor, 2014), transcription from P_3 took place in developing heterocysts at a very early stage of differentiation. Quantification of the fluorescence signal along the filaments showed expression of GFP in mature heterocysts (white numbers in Fig. 2B; black arrows in Fig. 2C) as well as in pro-heterocysts (red numbers in Fig. 2B; red arrows in Fig. 2C). Furthermore, enhancement of the green signal in a portion of the image (inset in Fig. 2B) showed that increased expression was

already detected in prospective heterocysts (marked with asterisks in Fig. 2) before the characteristic signs of differentiation (increase in size and reduction of red autofluorescence) could be observed. A *gfp* fusion to the promoter of *sigC*, that is transcribed in heterocysts (Brahamsha and Haselkorn, 1992; Aldea et al., 2007), and also contains a DIF1 motif was constructed and analysed in parallel for comparison with transcription of *sigA* (Figure S1). Quantification of green (GFP) and red (autofluorescence) signals showed that transcription of *gfp* fused to the promoters of either *sigA* or *sigC* was more intense in immature heterocysts than in fully mature heterocysts. This observation, which is consistent with primer extension data shown in Fig. 1F, can be interpreted as reflecting transitory induction of both genes during early stages of heterocyst differentiation.

Nitrogen-regulated promoters of asl4317-mraY

In addition to *sigA* described above, we have also analysed the promoter region for *asl4317-all4316* (*mraY*), that contains a nitrogen stress-repressed and a nitrogen stress-induced promoter. All4316 is annotated as MraY, a protein involved in peptidoglycan synthesis and therefore fulfilling a housekeeping function in the context of cell division. In addition, because deposition of heterocyst-specific envelopes involves remodelling of the cell wall, peptidoglycan synthesis and remodelling are critical aspects of heterocyst differentiation (Lehner et al., 2011; Videau et al., 2016). In fact, several genes related to peptidoglycan metabolism are transcribed from HetR-dependent promoters (Mitschke et al., 2011). Figure 3 shows that transcription of *asl4317-*

mraY takes place from a nitrogen stress-repressed promoter whose repression, in contrast to P_1 of *sigA,* seems independent of NtcA (Figure 3B) and from a DIF1-containing promoter (presumably heterocyst-specific) that is transiently induced and depends on HetR (Fig. 3D).

Conclusion and implications

The combinatorial strategy described here for *sigA* or *asl4317-mraY* (see a schematic model in Figure 4) might represent a way to escape repression specifically in cells that are undergoing differentiation. At the whole filament level the total amount of *sigA* transcripts appears transiently reduced during adaptation to nitrogen deficiency (Flaherty et al., 2011). As shown here, such reduction is the consequence of NtcA-mediated repression of its major promoter (P_1). According to a proteomic study carried out in *Nostoc punctiforme*, the amount of NtcA protein is about eight-fold higher in heterocysts than in vegetative cells (Sandh et al., 2014). This observation, consistent with stronger transcription of *ntcA* in heterocysts of *Nostoc* sp. strain PCC 7120 (Olmedo-Verd et al., 2006) suggests that, although the P_1 promoter of *sigA* is likely repressed in all cells of filaments, repression might be stronger in differentiating cells. Even if repression by NtcA is similar in vegetative cells and (pro)heterocysts, >80% repression of P_1 is expected in developing cells according to transcriptomic data (Table 1). In this context, induction of P_3, a heterocyst-specific promoter, presumably alleviates drastic reduction in SigA levels and allows transcription to continue in those vegetative cells that are undergoing an active transformation into a functional heterocyst. Similar

conclusions can be reached for *asl4317-mraY*. Transcription data (Table 1) suggest an overall transcription repression of > 60% from the DEF- promoter, but the DIF+ promoter would increase heterocyst-specific transcription by > 3 fold, alleviating reduction of a gene product that might be required in the context of remodelling of the cell wall in differentiating heterocysts.

Complex promoters (i. e., promoter regions with several transcriptional start sites) are a common theme in bacteria with developmental alternatives (McGrath et al., 2007). Combinatorial use of promoters is also a common strategy in pathogens, with examples that range from tandem promoters with different temporal profiles (Rosario and Tan, 2016) to transcripts with different susceptibility to regulatory sRNAs depending on the 5'UTR being transcribed in free-living vs. intracellular bacteria (Quereda et al., 2014). In all those bacteria with complex lifestyles the contribution of individual promoters to the overall transcription of genes can only be assessed by methods, such as dRNASeq, able to dissect individual transcriptional starts perhaps being operative in different cell types (Sharma and Vogel, 2014).

Transcription from multiple promoters subjected to different, even opposite regulation provides additional flexibility and facilitates integration of environmental or physiological clues. In the case of *sigA* described here, both promoters are ultimately regulated by NtcA (P_1 by direct binding, P_3 via HetR). Similar complex promoters might be regulated by different transcription factors, therefore integrating different signaling pathways. Also, transcripts containing different 5'UTRs might exhibit different susceptibility to post-transcriptional

control due to secondary structure or interaction with regulatory elements including proteins or small non-coding RNAs. In this context, the observation that the heterocyst-specific transcriptome includes small RNAs (Brenes-Álvarez et al., 2016) suggests the possibility of heterocyst-specific post transcriptional regulation with yet unknown physiological relevance.

SUPPLEMENTAL MATERIAL

Table S1. Oligonucleotides used in this work

Figure S1. Comparative expression of the heterocyst-specific promoters of *sigA* and *sigC*. Schematic representation of the promoter fragments transcriptionally fused to *gfpmut2* (bearing its ribosome binding site) in pSAM330 (*sigA*, A) and pSAM332 (*sigC*, B). Confocal fluorescence images of filaments growing on top of nitrogen-free medium are shown for cells containing pSAM330 (C) or pSAM332 (D). In each case images of the red (left) or green plus red channels (right) are shown. Quantification of the signals along the filaments is shown separately for the red (upper graphs) and green (bottom graphs) channels in panels E (pSAM330) and F (pSAM332). Mature and immature heterocysts are labelled as in Fig. 2. Black rectangles indicate the signals corresponding to immature heterocysts (#6 in the case of *sigA* and #2 in the case of *sigC*).

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Table 1. Genes identified in *Nostoc* sp. strain PCC 7120 with at least one TSS in the DEF- category and one TSS in the DIF+ category.

			Reads RNASeq[a]					
			WT		*hetR*			
Gene	**TSS[a]**	**Class[a]**	**0 h**	**8 h**	**0 h**	**8 h**	**DIF1 motif[b]**	**Annotation[c]**
asr1775	2134104f	DEF -	104	42	386	16		Unknown protein
	2134180f	DIF +	7	164	1	1	**T**A**CGGA**	
all2736	3333806r	DEF -	737	445	493	357		Hypothetical protein
	3333817r	DIF +	17	519	1	1		
asr3342	4038302f	DEF -	8614	3003	4732	1953		Acyl carrier protein
	4038311f	DIF +	1	22	8	0		
alr3402	4113609f	DIF +	15	289	1	4		Nucleoside diphosphate kinase
	4113655f	DEF -	87	23	46	26		
all3652	4408394r	DEF -	430	251	315	150		Phosphoribosylformyl glycinamidine synthetase II
	4408666r	DIF +	6	26	9	6		
asl4317 - mraY	5171243r	DEF -	1318	438	1575	344		Hypothetical protein - MraY
	5171277r	DIF +	47	428	11	7	T**G**C**GGA**	
alr4485	5369226f	DEF -	33	4	11	18		ABC transporter, permease protein
	5369311f	DIF +	5	71	2	1		
all4884	5821721r	DEF -	37	13	43	14		Hypothetical protein
	5821959r	DIF +	16	719	3	1		
all5263	6279167r (P_1)	DEF -	1116	145	432	528		Transcription initiation factor; SigA
	6279444r (P_3)	DIF +	2	53	1	1	**TCCGGA**	

[a] Position, classification and number of reads for each TSS are shown according to RNASeq data (Mitschke et al., 2011).

[b] Presence of sequences matching a DIF1 motif (TCCGGA) (Mitschke et al., 2011).

[c] According to Cyanobase (http://genome.microbedb.jp/cyanobase)

FIGURE LEGENDS

Fig. 1. Nitrogen-regulated promoters P_1 and P_3 of the *sigA* gene. A. Scheme of the promoter region of *sigA* showing the positions of two nitrogen-regulated transcriptional starts, located at positions -605 (P_1) and -328 (P_3) with respect to the translational start (genomic coordinates 6279167r and 6279444r, respectively). B. Sequences around the nitrogen stress-repressed transcriptional starts of *sigA* (bold, underlined, and marked with a bent arrow), *gifA* and *rbcL* (bold, underlined) are aligned with respect to their -10 boxes shown in grey. Putative NtcA binding sites are shown underlined with the nucleotides matching the palindromic consensus $GTAN_8TAC$ shown in red. C. Primer extension analysis of transcripts from P_1 in RNA isolated from the wild type strain and the *ntcA* derivative CSE2 (Frías et al., 1994), after the cells were transferred to nitrogen-free medium for the number of hours indicated in each case. D. Electrophoretic mobility shift assays with purified NtcA and the DNA fragment comprising P_1 (positions -484 to -227 with respect to the translational start of *sigA*), P^{32}-labelled using γ-dATP and T_4 polynucleotide kinase. The empty triangle points to the free fragment whereas the filled triangle indicates the position of the retarded fragment. E. Sequences upstream of the nitrogen stress-induced transcriptional start of *sigA* (bold, underlined, and marked with a bent arrow), are shown together with those upstream the heterocyst-specific transcriptional starts of NsiR1, *hetR* and *sigC* (bold, underlined). Sequences matching the consensus for the DIF1 motif TCCGGA are shown in red. F. Primer extension analysis of transcripts from P_3 in RNA isolated from the wild type strain (WT) or the *hetR* derivative 216 (Buikema and Haselkorn, 1991)

after the cells were transferred to nitrogen-free medium for the number of hours indicated in each case. Nitrogen step-down, RNA isolation, primer extension and EMSA with a purified his-tagged version of NtcA were carried out as previously described (Muro-Pastor et al., 1999). All oligonucleotides used in this work are shown in Supplemental Table S1.

Fig. 2. Expression of P_{sigA}-*gfp* along nitrogen fixing filaments of *Nostoc* sp. strain PCC 7120 bearing pSAM330. A. Schematic representation of the location of the transcriptional starts corresponding to P_1 and P_3 (bent arrows) and the P_3 promoter segment transcriptionally fused to *gfpmut2* (bearing its ribosome binding site) in plasmid pSAM330, a derivative of pCSEL24 that is integrated in the alpha megaplasmid of *Nostoc* sp. strain PCC 7120 as described previously (Olmedo-Verd et al., 2006). B. Confocal fluorescence image of a filament growing on top of nitrogen-free medium is shown for the red and green channels. A region of the filament is enhanced for better observation of green fluorescence and shown as an inset on the left side. Fluorescence was analysed and quantified using a Leica TCS SP2 confocal laser-scanning microscope. GFP and red fluorescence were imaged using the 488 nm line supplied by an argon ion laser. Fluorescent emissions were monitored by collection across windows of 500-538 nm (GFP imaging) and 630-700 nm (chlorophyll fluorescence). Quantification of the signals along the filament is shown separately for the red (C) and green (D) channels. Mature heterocysts (numbers 1, 2, 3, 5, 6, 8 and 9) are indicated in white (B) or black (D). Immature heterocysts (numbers 4 and 7) are indicated in red. Prospective heterocysts are indicated with single asterisks. Black arrows indicate the positions of mature

heterocysts, whereas red arrows indicate immature heterocyst. The segment of the graphs corresponding to the inset in B is framed with a black rectangle. 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/, 1997-2016).

Fig. 3. Nitrogen-regulated promoters of *asl4317-all4316 (mraY)*. A. Scheme of the two genes and the promoter region showing the positions of the two nitrogen regulated transcriptional starts, located at positions -39 and -73 with respect to the translational start. B. Primer extension analysis of transcripts starting at position -39 in RNA isolated from the wild type strain (WT) or the *ntcA* mutant strain CSE2 after the cells were transferred to nitrogen-free medium for the number of hours indicated in each case. C. Sequences upstream of the nitrogen stress-induced transcriptional start of *asl4317* (bold, underlined, and marked with a bent arrow), are shown together with those upstream the heterocyst-specific transcriptional starts of *sigA*, NsiR1, *hetR* and *sigC* (bold, underlined). Sequences matching the consensus for the DIF1 motif are shown in red. D. Primer extension analysis of transcripts starting at position -73 in RNA isolated from the wild type strain (WT) or the *hetR* mutant strain 216 after the cells were transferred to nitrogen-free medium for the number of hours indicated in each case.

Fig. 4. Schematic representation of the combined operation of P_1 and P_3 promoters of the *sigA* gene upon nitrogen step down. In the presence of combined nitrogen P_1 is operative in vegetative cells (thick grey arrow under

sigA), while transcription from P_3 does not take place. Upon nitrogen step down leading to increased levels of 2-oxoglutarate (6-12 h), transcription from the major P_1 promoter is repressed by NtcA in all cells (thin grey arrow under *sigA*) while P_3 is induced only in prospective heterocysts (thick red arrow under *sigA*). Because the amount of NtcA is higher in developing heterocysts, repression of P_1 is expected to be stronger in those cells. Once the heterocysts are mature (20-24 h) the transcription from P_3 is reduced (thin red arrow under *sigA*). Concerning P_1 we can only speculate that repression by NtcA is alleviated as the transient limitation in nitrogen availability is reverted by fixation of atmospheric nitrogen. Similarly, expression from P_3 is highest during early stages of development but is reduced as the heterocysts become mature. The result of such combination is that, while there is a transient reduction in expression in vegetative cells, expression remains somewhat constant in developing heterocysts that are actively differentiating. [2-OG], concentration of 2-oxoglutarate.