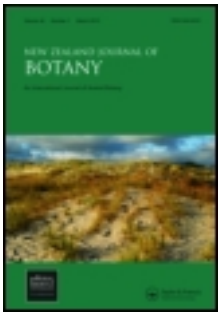

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RESEARCH ARTICLE

Transcriptional regulation of the bidirectional hydrogenase by oxygen and light in two *Anabaena* species

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Anabaena variabilis ATCC 29413 and *Anabaena* PCC 7120 were grown in batch cultures with combined nitrogen source. The *hox* genes encoding the bidirectional hydrogenase were expressed at a relatively low level in the heterocyst-free filaments. Transcriptional regulation of the bidirectional hydrogenase in response to oxygen and light was found to be similar in the two closely related strains. Low oxygen tension caused c.100-fold induction of the *hox* genes. However, when the anaerobic cultures were incubated in darkness the *hox* transcript accumulation increased by one order of magnitude compared with the illuminated anaerobic cultures. In addition, darkness could also reversibly induce *hox* expression and was found to be a positive regulator of *hox* expression even in the presence of oxygen. These results imply a specific requirement for the bidirectional hydrogenase of *Anabaena* PCC 7120 and *A. variabilis* ATCC 29413 in darkness both in the presence and absence of oxygen.

Keywords: bidirectional hydrogenase; *hox* genes; transcriptional regulation; filamentous cyanobacteria; *Anabaena* PCC 7120; *Anabaena variabilis* ATCC 29413

Introduction

Filamentous, heterocystous cyanobacteria may contain two types of [NiFe]-hydrogenases: the uptake hydrogenase and the bidirectional or Hox hydrogenase (Hallenbeck & Benemann 1978; Houchins & Burris 1981). The membrane-bound uptake hydrogenase recycles the hydrogen produced during nitrogen fixation into protons and electrons. It is found in all known diazotrophic cyanobacteria with one exception, *Synechococcus* sp. BG 043511 (Ludwig et al. 2006). In contrast, the bidirectional hydrogenase is not a universal enzyme among nitrogen-fixing strains, and the absence of this enzyme has no obvious phenotype (Tamagnini et al. 1997, 2000). The bidirectional hydrogenase has been partially purified from two closely related heterocystous strains *Anabaena variabilis* ATCC 29413 (*A. variabilis*) and *Anabaena* PCC 7120 (*Anabaena*); and characterized as an oxygen-sensitive enzyme with high affinity to hydrogen (Houchins & Burris 1981; Serebryakova et al. 1996). It consists of a catalytic subcomplex (HoxYH) that can reversibly oxidize hydrogen, and a diaphorase moiety (Hox(E)FU) that transfers electrons back and forth between $NAD(P)^+$ and the hydrogenase heterodimer (Lambert & Smith 1981; Houchins & Burris 1984; Aubert-Jousset et al. 2011). However, its physiological function is not as unambiguous as the function of the uptake hydrogenase and it seems to be different in the various model organisms. In *Synechocystis* PCC 6803 (*Synechocystis*) the Hox hydrogenase most probably assists in maintaining photosynthesis in a strongly reducing environment by acting as an electron valve for low-potential electrons (Appel et al. 2000). In *Synechococcus* PCC 7942 its proposed function is to oxidize hydrogen at the periplasmic side and allocate electrons to the

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respiratory chain (Kentemich et al. 1991; Schmitz et al. 1995). In *Gloeocapsa alpicola* the Hox enzyme was indicated to take part in glycogen degradation-based fermentative pathways (Serebryakova et al. 1998); and a similar function is proposed for it in *A. variabilis* based on the correlation between the amount of active Hox enzyme and the glycogen content of the cells (Serebriakova et al. 1994). Nevertheless, as there is no crystal structure available for the enzyme, and *hox* gene mutations did not generate explicit phenotypes (Appel et al. 2000; Masukawa et al. 2002; Pinto et al. 2012) only presumptions can be made concerning the physiological function of the bidirectional hydrogenase. Some of these assumptions are based mainly on sequence homology and cofactor incorporation similarities between the bidirectional hydrogenase and the respiratory Complex I (Appel & Schulz 1996; Schmitz & Bothe 1996; Sazanov & Hinchliffe 2006; Lauterbach et al. 2011; Horch et al. 2012).

Regulation of the expression of the bidirectional hydrogenase in cyanobacteria seems to be species specific (Bothe et al. 2010). Previously, some studies were carried out concerning the oxygen-dependent and light-dependent transcriptional regulation of the bidirectional hydrogenase of heterocystous strains. Dark–anaerobic induction of the *hox* gene clusters of *Anabaena* has been shown in parallel with increased bidirectional hydrogenase-driven methyl viologen-dependent hydrogen evolution (Sjöholm et al. 2007). In the case of *Nostoc muscorum* the *hoxH* transcript accumulation under anaerobic conditions in light was demonstrated (Axelsson & Lindblad 2002). However, the difference between the effect of low oxygen tension in light and in dark within the same strain has never been presented. In addition, although microarray experiments carried out on oxygenic cultures of *Anabaena* revealed the induction of *hox* genes upon transfer from light to dark (Ehira et al. 2005), this observation was not studied further.

This report examines the *hox* gene expression of heterocyst-free cultures of *Anabaena* and *A. variabilis* under low oxygen tension and also under atmospheric oxygen in the presence and absence of light. Our data show that under low oxygen tension the *hox* transcripts show one order of magnitude higher accumulation in darkness than in light. Moreover, in both strains the five *hox* genes were induced to different extents by darkness in the presence of oxygen.

Materials and methods

Culturing and treatment conditions

Anabaena PCC 7120, and *Anabaena variabilis* ATCC 29413 cells were propagated in a 2% CO_2-enriched atmosphere in BG-11 medium (Rippka et al. 1979) containing 350 mM $NaNO_3$ at 30 °C under 40 µmol quanta m^{-2} s^{-1} white light. Cells in the late exponential growth phase (10 µg Chl mL^{-1}) were used. Microaerobic conditions were achieved by flushing the cultures with argon gas supplemented with 2% CO_2. Under our experimental conditions the dissolved oxygen content of the cultures was below 1 µmol L^{-1} as monitored with an immersible oxygen electrode (Presens, Fibox 3; PreSens, Regensburg, Germany).

Determination of chlorophyll content

Chlorophyll *a* was extracted into methanol and its concentration was determined by applying the extinction coefficient at 665 nm as reported by Ritchie (2006).

RNA extraction and cDNA synthesis

From the cyanobacterial samples, which were normalized to equal chlorophyll contents (100 µg/sample), RNA was extracted by the phenol–guanidine–isothiocyanate–chloroform extraction protocol (Mcginn et al. 2003). Briefly, the cells were disrupted in 750 µL TRI reagent (Molecular Research Center, Cincinnati, OH, USA), snap frozen and then placed in a water bath at 60 °C for 10 min. The cell suspension was homogenized by vortexing at maximum speed (3000 RPM) twice during the heat bath. After that 150 µL chloroform was added followed by 5-min incubation at room temperature before 10-min centrifugation at 12,000 *g* at 4 °C. The same volume of phenol–chloroform–isoamyl alcohol was added to

the collected supernatant and centrifuged again for 5 min. The RNA was precipitated from the supernatant with isopropanol at room temperature for 15 min, in darkness. The pellet was collected by 25 min centrifugation at 14,000 *g* at 4 °C and washed with 70% ethanol. After the residual ethanol had evaporated the pellet was dissolved in nuclease-free water. The extracted RNA was freed from DNA contamination using a Turbo DNase Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. The quality (A_{260}/A_{280}) and quantity (A_{260}) of extracted RNA was determined with a Nanodrop (NanoDrop Technologies, Wilmington, DE, USA) spectrophotometer; 800 ng RNA/sample was reverse transcribed in a 20-µL reaction volume using random hexamer primers and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions.

Gene expression assessment

The relative gene expression levels were determined by reverse transcription–quantitative polymerase chain reaction (RT-qPCR) using cDNA prepared from total RNA. Each reaction mixture contained 10 µL Maxima SYBR Green/Rox Master Mix (Thermo Scientific, Waltham, MA, USA), 3 µM of each primer, 1 µL cDNA (dilution 1/20) and RNase-free sterile water to a final volume of 20 µL. RT-qPCR was performed using the ABI 7000 detection system. The RT-qPCR conditions were as follows: (i) an initial denaturing step at 95 °C for 10 min; (ii) 40 cycles, with one cycle consisting of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. Standard dilutions of the cDNA were used to check the relative efficiency and quality of primers (Higuchi et al. 1993). Genomic DNA contamination was ruled out by including RT-negative controls (not reverse-transcribed RNA) in the assays. Negative controls (no template cDNA) were also included and melting curve analysis was used to test the specificity of the reactions. Additionally, the PCR products were subjected to gel electrophoresis on 2% agarose gels stained with GelRed Nucleic Acid Stain (Biotium, Hayward, CA, USA) to confirm the absence of non-specific products. RT-qPCRs were performed with three biological replicates and technical duplicates of each cDNA sample. In each gene expression experiment the expression level of the *rnpB* gene—which is widely used in cyanobacterial gene expression studies (Navarro et al. 2000; Fujimori et al. 2005; Sjöholm et al. 2007; Oliveira & Lindblad 2008)—was determined and used as reference. The gene-specific primers used in the reactions as well as the amplicon sizes and parameters derived from the analyses of RT-qPCR data are listed in Table 1. The data obtained were analysed using the 7000 System SDS Software 1.2.3. (Applied Biosystems), Microsoft Office Excel and Origin 8.6. According to the comparative Cq method, also referred to as the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak 2008), the Cq values of the genes of interest were double normalized, first to the respective Cq values of the reference gene, second to their respective initial values (Cq of the gene of interest at the 0 time-point of the experiment). Statistical significance of the results was assessed using Student's *t*-test.

Results

Effect of light on hox *gene expression under low oxygen tension*

Expression of the five structural genes of the bidirectional hydrogenase were monitored by RT-qPCR in oxygen-deprived cultures of *A. variabilis* (Figs 1A, 1B) and *Anabaena* (Figs 1C, 1D) that were either illuminated (Figs 1A, 1C), or kept in darkness (Figs 1B, 1D) for 2 h. The *hox* genes showed the highest relative induction at the first time-point of the anaerobic incubation, which was taken 30 min after the dissolved oxygen content of the medium dropped below 1 µM. At this time-point in the illuminated *A. variabilis* cultures the *hoxE* transcripts accumulated up to 70 ± 25-fold with a highly significant *P* value of 0.001. This level only slightly decreased during the 2 h of measurements. In these cultures the other four *hox* genes showed relative expressions similar to the *hoxE* gene (Fig. 1A). In the 30-min light–

Table 1 Oligonucleotide sequences used as gene-specific primers in the reverse transcription–quantitative polymerase chain reaction; and parameters of the resulting amplicon and the amplification.

Gene ID	Gene symbol	Primer sequence (5′–3′)	Size (bp)	T_M (°C)	NTC (Cq)	E (%)	Slope	R^2	*y*
Ava_4653 & *alr0751*	*hoxE*	CCCCAGGGAGTCCATAGTTG CCGTGCCGTTAGCAGTGATA[1]	150	79.2	38[2]	93	−3.5	0.99	20.1
Ava_4654	*hoxF*	CTGGTGTTGGTTGTATGCGC TGTGCCGCTTTCCCTCTT[1]	150	80.3	N.d	91	−3.57	0.99	18.6
alr0752	*hoxF*	CAAGCTTATATTGCGGCTCAGGG CACCGTCGCCCATTTCAAG[1]	149	79.6	N.d	94	−3.48	0.99	19.1
Ava_4657 & *alr0762*	*hoxU*	TCCGACACTGTGCCATTTAG TGTAGGCGATCGCTATTTGT[1]	150	80.2	N.d.	99	−3.35	0.99	18.7
Ava_4659 & *alr0764*	*hoxY*	GAAGTTAGCAACAGTATGGTTAGGTGG AGCCATTCGTCCAAATCAAGAA[1]	71	71.9	N.d.	90	−3.6	0.99	19.3
Ava_4661 & *alr0766*	*hoxH*	GAATCGTCATCGACCCCGTTAC GGCATTTCCCACAAAGGACGAC[1]	153	79.9	38[2]	95	−3.45	0.99	18.5
Ava_R005	*rnpB*	CGTGAGGATAGTGCCACAGAAA AATACTGCTGGTGCGCTCTTACC[1]	150	75.2	39[2]	94	−3.48	0.99	21.2
rnpB	*rnpB*	GAGCGATCGTGAGGATAGTG CTCTTACCGCACCTTTGCA[1]	150	80.4	N.d.	95	−3.44	0.99	20.5

Gene IDs are derived from Cyanobase (Kazusa DNA Research Institute (KDRI) 2012).
Size, amplicon size; T_M, amplicon melting temperature; NTC, Cq value of the No Template cDNA Control; E, amplification efficiency; *y*, *y*-intercept; N.d., Not detected.
[1]Reverse primer.
[2]Non-specific amplicons (unstable at the T_M of the specific amplicon).

anaerobic samples of *Anabaena* the *hoxY* and the *hoxH* genes showed the highest, 400 ± 100-fold and 200 ± 80-fold, induction relative to their respective control values (P = 0.03). At this timepoint the relative expression levels of the genes encoding the diaphorase subunits were induced to a lower extent compared with the genes encoding the hydrogenase moiety of the enzyme complex (Fig. 1C). Transferring the oxygen-deprived cells into darkness for 30 min caused three orders of magnitude higher transcript accumulation compared with the growth conditions (P = 0.001). After 1 h this induction decreased slightly, and after 2 h, two orders of magnitude relative induction could be measured (P = 0.01). These dark–anaerobic inductions of the *hox* genes were analogous in the case of *Anabaena* and *A. variabilis* (Figs 1B, 1D).

Reversible effect of light on the transcriptional regulation of the bidirectional hydrogenase

A. variabilis and *Anabaena* cultures were incubated in darkness under atmospheric oxygen (Fig. 2). In the first 30 min all of the *hox* genes were induced. *hoxE* showed the highest induction 21 ± 2-fold (P = 0.008) and 11 ± 5-fold (P = 0.01) in *A. variabilis* and in *Anabaena*, respectively. In *A. variabilis* during the 2 h of dark incubation the *hoxE*, *hoxF* and *hoxU* genes encoding the diaphorase moiety remained at significantly high relative transcript levels with a P value of 0.008, while the *hoxY* and *hoxH* genes encoding the catalytic subunit of the hydrogenase moiety showed a considerably lower level of induction (P = 0.02). After the dark treatment the cells were transferred to light, which reverted their expression towards their initial levels (P = 0.25) (Fig. 2A). In the case

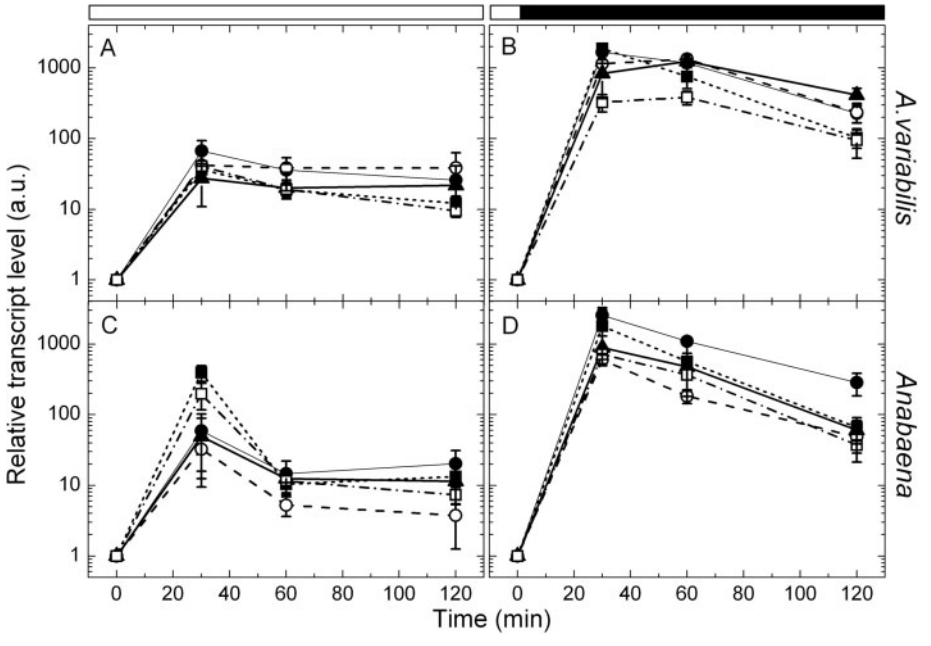

Figure 1 Effect of light on the induction of *hox* genes under oxygen deprivation. The oxygen content of the medium of **A, B**, *Anabaena variabilis* and **C, D**, *Anabaena* PCC 7120 cells were kept below 1 μM during 2 h of **A, C**, illumination or **B, D**, darkness. The relative expression levels of the *hoxE* (thin line, solid circle), *hoxF* (dashed line, open circle), *hoxU* (thick line, solid triangle), *hoxY* (short dashed line, solid square) and *hoxH* (dash dotted line, open square) genes are shown after normalization to their respective initial values represented by 1. Error bars correspond to standard deviation derived from three independent experiments. The white and black bars indicate the light and dark periods.

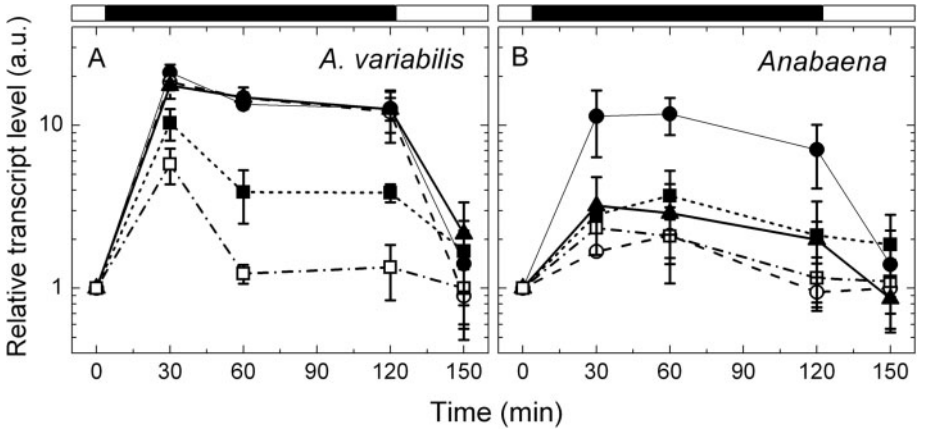

Figure 2 Dark induction of *hox* genes in aerobic cultures. During the dark incubation, **A**, *Anabaena variabilis* and **B**, *Anabaena* PCC 7120 cells were kept under atmospheric oxygen supplemented with 2% CO_2 and transferred from light to dark for 2 h followed by 30 min recovery period in light. The relative expression levels of the *hoxE* (thin line, solid circle), *hoxF* (dashed line, open circle), *hoxU* (thick line, solid triangle), *hoxY* (short dashed line, solid square) and *hoxH* (dash dotted line, open square) genes are shown after normalization to their respective initial values represented by 1. Error bars correspond to standard deviation derived from three independent experiments. The white and black bars indicate the light and dark periods.

of *Anabaena* the *hoxF*, *hoxU*, *hoxY* and *hoxH* genes were moderately induced compared with *hoxE* with *P* values of 0.04 and 0.01, respectively. The reversible effect of darkness was more apparent in the case of *hoxE* and *hoxU* than in the case of other *hox* genes of *Anabaena* (Fig. 2B).

Discussion

In a previous study oxygen level has been found to affect mainly the enzyme activity of the bidirectional hydrogenase in *A. variabilis*, with only a slight effect on *hox* gene expression (Serebriakova et al. 1994). In contrast, under our experimental conditions we have observed, by the highly sensitive RT-qPCR method, a significant induction of *hox* genes, whose extent was up to 70 ± 25-fold and 400 ± 100-fold in *A. variabilis* and *Anabaena*, respectively (Figs 1A, 1C). This considerably high level of induction could be further increased (up to three orders of magnitude compared with growth conditions) when low oxygen tension was combined with darkness.

Darkness and low oxygen tension applied either separately or in combination, caused an elevated *hox* transcript accumulation in both strains. The discrete, positive effect of darkness on *hox* gene expression suggests the involvement of the bidirectional hydrogenase in metabolic pathways that are especially active in darkness. Under low oxygen tension the *hox* gene induction in darkness is likely to be due to the requirement for higher Hox enzyme levels, which are involved in dark fermentation, as was previously proposed (Serebriakova et al. 1994). However, it is more complicated to connect the positive effect of darkness on *hox* gene expression to a possible physiological function under atmospheric oxygen. Although the dissolved oxygen content of the cell suspensions did not change during the hours of dark incubation when measured in the extracellular medium (data not shown), some decrease in the intracellular oxygen content—due to the absence of photosynthetic oxygen evolution and the simultaneous oxygen consumption via respiration—cannot be excluded. However, this effect is unlikely to cause a significant difference between the oxygen levels of the medium and the intracellular compartment because oxygen equilibration between the cells and the medium is fast and efficient. Therefore, the induction of *hox* gene

expression in darkness should occur independently of decreased oxygen level.

Primarily based on sequence similarities the Hox enzyme was proposed to be involved in electron transfer towards respiration (Appel & Schulz 1996; Schmitz & Bothe 1996). This hypothetical function assumes that the Hox enzyme can perform hydrogen uptake under physiological conditions, an idea that is supported by the very low K_m of Hox for hydrogen (Houchins & Burris 1981), as well as by enzyme activity assays showing Hox-dependent hydrogen uptake, especially in darkness (Spiller et al. 1983). In an apparent contrast with the above idea the bidirectional hydrogenase isolated from *Synechocystis* was shown to preferably perform hydrogen production over hydrogen oxidation (McIntosh et al. 2011). However, the *in vivo* Hox hydrogenase activity strongly depends on the physiological condition of the cell, especially on the availability of reducing equivalents for hydrogen production, which is strain dependent. In addition, the bidirectional hydrogenase of different strains can potentially have partly different catalytic properties, as indicated by the different amino acid sequences of the catalytic subunit of the enzyme (Ludwig et al. 2006). Therefore, the bias of the Hox enzyme can be different in various cyanobacterial strains.

The dark–aerobic induction of the *hox* genes in *Anabaena* and *A. variabilis* strongly indicates that the bidirectional hydrogenase enzyme can take part in aerobic respiration in these cyanobacteria. However, further investigations in these and other nitrogen-fixing strains are needed for corroboration of this idea.

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