Identification of hox genes and analysis of their transcription in the unicellular cyanobacterium Gloeocapsa alpicola CALU 743 growing under nitrate-limiting conditions

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Abstract

The unicellular non-N2-fixing cyanobacterium Gloeocapsa alpicola CALU 743 contains a bidirectional hydrogenase. Parts of all structural genes, encoding the hydrogenase, were identified, cloned and sequenced. When comparing the sequences with analogous sequences from other cyanobacteria the highest similarity was observed with hox genes from Synechocystis sp. PCC 6803. The hydrogenase activity increased considerably when the cells were grown aerobically in a medium with limiting concentrations of nitrate. However, the relative abundances of hoxH and hoxY transcripts, detected by RT-PCR, did not change significantly, demonstrating that the increase in the activity of G. alpicola hydrogenase was not a result of the increase of the transcription. In contrast, in Anabaena variabilis the induction of a bidirectional hydrogenase activity correlated with the relative level of hoxH and hoxY transcripts. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Cyanobacteria; Bidirectional hydrogenase; hox genes; Transcription; Nitrogen starvation

1. Introduction

The bidirectional hydrogenase of cyanobacteria can either produce or oxidize H2 in the presence of suitable electron donor or acceptor [1–6]. It is [NiFe]-enzyme, consists of two moieties: a hydrogenase (HoxHY) and a diaphorase (HoxFU). The presence of third diaphorase subunit (HoxE) is proved for Anacystis nidulans (=Synechococcus sp. PCC 6301) and Synechocystis sp. PCC 6803 [6]. Identified and sequenced cyanobacterial hox genes (Anabaena variabilis ATCC 29413 [2], Anabaena sp. PCC 7120 [7], A. nidulans [3], Synechocystis sp. PCC 6803 [4] and Chroococcidiopsis thermalis CALU 758 [8]) are similar but the overall gene arrangement is different [3–5]. Transcriptional studies evidence that all structural hydrogenase genes are transcribed together with accessory genes as a unit in A. variabilis, whereas in A. nidulans, two transcriptional units for the hox gene cluster have been detected [9]. Recently, circadian control of hox genes expression has been shown in Synechococcus sp. PCC 7942 [10].

In aerobically grown cyanobacterial cells, the level of bidirectional hydrogenase activity, measured as methylviologen-dependent H2 evolution, usually is low. In heterocystous forms, a one to three order increase of the enzyme activity occurs under microaerobic growth conditions [11,12]. A similar induction is seen in the unicellular A. nidulans [13]. In contrast, in the unicellular strain Gloeocapsa alpicola CALU 743 the enzyme activity is not induced during microaerobic incubation, but increases considerably under light-limiting and nitrate-limiting growth conditions [14,15]. It has been suggested that the increase in activity involves additional protein synthesis due to the requirement of light as energy source in this process; the cyanobacterial hydrogenase activity usually increases about two-fold during a dark anaerobic incubation [12]. The present study is devoted to the identification of hox genes in G. alpicola; and to analysis of hoxY transcription in parallel with the increase of the hydrogenase activity in cells growing under nitrate-limiting conditions.
conditions. Furthermore, transcription of the same genes in non-N2-fixing cells of *A. variabilis* ATCC 29413 during microaerobic adaptation has been analyzed to compare the regulation of development of bidirectional hydrogenase in different forms of cyanobacteria.

2. Materials and methods

2.1. Strain and culture conditions

The unicellular non-N2-fixing cyanobacterium *G. alpicola* CALU 743 strain Fitzgerald 1052 (= *Synechocystis* sp. PCC 6308) was obtained from Alga Collection of St. Petersburg University (Russia). The cyanobacterium was grown in batch culture as described earlier [15], using BG11o medium with the addition of KNO₃ (2 or 10 mM).

For transcription analysis, cells were grown in medium containing 2 mM KNO₃. Three cultures were inoculated to the identical density, but with interval of several hours, and grew in the identical conditions. Growth parameters (optical density and protein content) and hydrogenase activity were measured in the samples of each culture at several time points. Growth curves have been done from the data of three cultivations.

The heterocystous cyanobacterium *A. variabilis* ATCC 29413 was grown as described earlier [16] using BG11o supplemented by 5 mM NH₄Cl, bubbled with a mix of air and CO₂ (3%). Cells taken early in exponential phase of growth were used for 24 h microaerobic adaptation, when air in gas phase was replaced by argon. Hydrogenase activity and protein content were measured after 0, 6, 12 and 24 h of adaptation.

2.2. Determination of optical density, protein content and the hydrogenase activity

Optical density of cultures was measured in a 2-mm cuvette at 750 nm. Protein concentration was determined according to the method by Lowry [17].

Hydrogenase activity was measured as evolution of H₂ by intact cells in the presence of methylviologen reduced by sodium dithionite, using gas chromatography [15]. Activity is expressed as μmol of H₂ evolved per hour per mg of protein.

All measurements were done in triplicate and mean values are presented.

2.3. DNA and RNA isolation

Genomic DNA was extracted with phenol/chloroform as described earlier [16].

For RNA isolation, cell of cyanobacteria were quickly separated by pre-cooled centrifugation and frozen in liquid nitrogen. Total RNA was extracted as described [18] using 60 U of RNase free DNase I (Amersham-Pharmacia Biotech) per 7–10 μg of RNA. The concentration of RNA before and after DNase treatment was determined by measuring the absorbance at 260 nm. The quality of RNA was checked using a 1% agarose gel.

2.4. PCR, TOPO-cloning and sequencing

PCR with genomic DNA of *G. alpicola* was carried out in the Mastercycler gradient PCR (Eppendorf). Reaction mixtures of 20 μl contained 2 μl DNA (3.6 ng/μl), each deoxynucleoside triphosphate at 0.2 mM, 1.5 U *Tag* buffer, 0.5 U *Tag* DNA polymerase (Amersham-Pharmacia Biotech) and 2 μM of each primer (Table 1). The reaction was performed for 40 cycles consisting of 94°C for 1 min, an annealing (gradient of temperature, 48–62°C) for 1 min and 72°C for 12 and 24 h of adaptation.

Table 1

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Specific organism</th>
<th>Gene</th>
<th>Sense primer sequence 5′→3′</th>
<th>Antisense primer sequence 5′→3′</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Av1</td>
<td><em>A. variabilis</em></td>
<td>hoxY</td>
<td>ggtgagttctggctgctatgt</td>
<td>gttcgacctgtgaa</td>
<td>[2,16]</td>
</tr>
<tr>
<td>Av3</td>
<td><em>A. variabilis</em></td>
<td>hoxH</td>
<td>cctctcctattattacctaatct</td>
<td>cctctcctattattacctaatct</td>
<td>[2,16]</td>
</tr>
<tr>
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<td><em>A. variabilis</em></td>
<td>hoxH</td>
<td>cccctctctattattacctaatct</td>
<td>cccctctctattattacctaatct</td>
<td>[2,16]</td>
</tr>
<tr>
<td>HG</td>
<td><em>G. alpicola</em></td>
<td>hoxH</td>
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<td>cccctctctattattacctaatct</td>
<td>This work</td>
</tr>
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<td>YS</td>
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<td>hoxY</td>
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<td>tgcgtggtggctgctgctaggg</td>
<td>This work</td>
</tr>
<tr>
<td>ES</td>
<td><em>Synechocystis</em> sp. PCC 6803</td>
<td>hoxE</td>
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<td>ttttgctactggaagagagga</td>
<td>This work</td>
</tr>
<tr>
<td>FS</td>
<td><em>Synechocystis</em> sp. PCC 6803</td>
<td>hoxF</td>
<td>tgaatagggcccagagagga</td>
<td>tgaatagggcccagagagga</td>
<td>This work</td>
</tr>
<tr>
<td>US</td>
<td><em>Synechocystis</em> sp. PCC 6803</td>
<td>hoxU</td>
<td>gttgtgtgcaggtggaagaggt</td>
<td>gttgtgtgcaggtggaagaggt</td>
<td>This work</td>
</tr>
</tbody>
</table>
amino acid sequences and compared with hox sequences from other microorganisms (BLAST, GenBank).

2.5. Southern blot hybridization

Genomic DNA, digested by HincII (Pharmacia), was used for Southern blot hybridizations. DNA was separated electrophoretically in a 1% agarose gel and, after denaturation and neutralization, transferred overnight to a nylon membrane (see [16]). Southern blot hybridization was performed at 60°C. Cloned and sequenced DNA fragments generated by PCR with genomic DNA of G. alpica and hox specific primer pairs (Table 1) were used as probes. The Digoxigenin DNA Labeling and Detection kit (Boeringer, Mannheim, Germany) was used for labeling and hybridization.

2.6. Reverse transcription (RT)-PCR

RT was performed as described [18], with some modifications. An 11-μl RT mixture contained 0.6 or 0.8 μg of total RNA (G. alpica and A. variabilis, respectively) and 2.0 μl of 2.0 μM antisense primers. 2.5 μl of RT reactions were used for PCR. Each PCR (25 μl) contained cDNA (2.5 μl), 0.2 μM of primers, 0.2 mM of dNTP mix, 1× Tag buffer and 2.5 U of Tag polymerase. Reaction was carried out for 30 cycles. In case of G. alpica, they consisted of 94°C for 30 s, an annealing 61°C for 30 s and 72°C for 45 s. In case of A. variabilis, they consisted of 94°C for 10 s, an annealing 55°C for 1 min and 72°C for 1 min. A final extension was performed at 72°C for 7 min. Negative controls included 75 or 100 ng (for G. alpica and A. variabilis, respectively) of the template RNA without cDNA (for each RNA sample) or no template at all.

Genomic DNA of G. alpica and A. variabilis was used as a positive control.

3. Results

3.1. Identification and partial sequencing of hox genes

PCR with genomic DNA from G. alpica as a template and primer pair Av3 (Table 1) resulted in amplification of a DNA fragment of 984 bp, which was cloned and sequenced. The comparison of the sequence obtained with corresponding sequences from other microorganisms showed its high similarity with cyanobacterial hoxH, especially with Synechocystis sp. PCC 6803 (Table 2). Southern blot hybridizations of genomic DNA against the cloned PCR product as a probe showed that there is a single copy of hoxH gene in the genome of G. alpica as well as confirmed its origin (Fig. 1). The presence of other
structural hox genes was demonstrated, using PCR with genomic DNA of *G. alpicola* and the specific primer pairs (Table 1) constructed on base of annotated hox cluster sequence from *Synechocystis* sp. PCC 6803 [4]. Parts of hoxE (499 bp), hoxF (1004 bp), hoxU (702 bp) and hoxY (535 bp) were cloned, sequenced and confirmed by hybridization. When comparing obtained deduced amino acid sequences from *G. alpicola*, all of them are almost identical to corresponding sequences in *Synechocystis* sp. PCC 6803 (Table 2).

### 3.2. Analysis of hoxH and hoxY transcription

For the transcription analysis of hoxH in *G. alpicola* cells, the specific primer pair HG was constructed (Table 1). Gradient PCR with genomic DNA resulted in an amplification of a single product of 450 bp. Obtained DNA fragment was cloned and sequenced to confirm its identity. For the transcriptional analysis of hoxY, primer pair YS was used. Analyses of hoxH and hoxY transcript abundance in cells of *G. alpicola* growing under nitrate limiting conditions were performed by RT-PCR. Each reaction resulted in one primary DNA fragment of expected size (Fig. 2B). The relative intensity of DNA fragments corresponding to the relative amount of transcripts was approximately the same for all samples examined, even though the hydrogenase activity in them increased significantly (Fig. 2A).

Analysis of the abundance of hoxH and hoxY transcripts in cells of *A. variabilis* was performed using Av4 and Av1 primer pair, respectively (Table 1). The RT-PCR resulted in single DNA fragments of expected size (Fig. 3B). The most abundant transcripts occurred in samples corresponding to 12 and 24 h after start of adaptation; the level of transcripts was lower at time zero, when the cells showed almost no hydrogenase activity, and at 6 h, when the hydrogenase activity just started to increase (Fig. 3A).

### 4. Discussion

The presence of hox homologues in the genome of unicellular non-N₂-fixing cyanobacterium *G. alpicola* indicates that this strain possesses a typical cyanobacterial NAD(P)⁺-reducing bidirectional hydrogenase. However, in contrast to heterocystous cyanobacteria [11,12], the activity of this enzyme in the cells of *G. alpicola* is not affected by removal of O₂ from the gas phase of a growing culture (microaerobic growth), but increases in response to, for example, the depletion of combined nitrogen in the medium [14,15]. In agreement, a nitrogen-limited culture of *Synechocystis* sp. PCC 6803 was used to obtain high amount of active bidirectional hydrogenase for the purification and further biochemical analyses of the enzyme [6]. In non-N₂-fixing cyanobacteria nitrogen starvation leads to the inactivation of PSII and consequently to

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**Table 2**

Comparison of the deduced amino acid sequences of parts of hox genes from *G. alpicola* with the corresponding sequences from other cyanobacteria

<table>
<thead>
<tr>
<th>Cyanobacteria</th>
<th>hoxE (%)</th>
<th>hoxF (%)</th>
<th>hoxU (%)</th>
<th>hoxY (%)</th>
<th>hoxH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Synechocystis</em> sp. PCC 6803</td>
<td>100</td>
<td>100</td>
<td>99</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>57</td>
<td>69</td>
<td>70</td>
<td>54</td>
<td>72</td>
</tr>
<tr>
<td><em>Prochlorotrix hollandica</em></td>
<td>N/A</td>
<td>N/A</td>
<td>77</td>
<td>72</td>
<td>76</td>
</tr>
<tr>
<td><em>A. variabilis</em> ATCC 29413</td>
<td>N/A</td>
<td>76</td>
<td>62</td>
<td>60</td>
<td>66</td>
</tr>
<tr>
<td><em>Anabaena</em> sp. PCC 7120</td>
<td>66</td>
<td>75</td>
<td>62</td>
<td>60</td>
<td>66</td>
</tr>
</tbody>
</table>

N/A – no data in GenBank.
a decrease of photosynthetic O$_2$ evolution, while respiration remains unaltered [20] leading to an anaerobic intracellular environment [15]. Generally, it might be concluded that the cyanobacterial bidirectional hydrogenase is regulated by intracellular O$_2$ pressure, which can be reduced in distinct ways for different cyanobacterial strains.

The fact that the maximal enzyme activity requires light may reflect an energy requirement for a new protein synthesis. Indeed, an increase of hoxH and hoxY transcription at low level of O$_2$ takes place in _A. variabilis_. Similar effect was clearly demonstrated in N$_2$-fixing cyanobacterium _Nostoc muscorum_ [21]. At the same time we found that in _A. variabilis_ the activity during microaerobic adaptation while the amount of hydrogenase protein increased only twice [22]. Most probably, the positive regulatory effect of an anaerobic condition on the cyanobacterial bidirectional hydrogenase gene takes place both on the transcription level and post-transcriptional stages. However, in _G. alpicola_ the enhancement in the hydrogenase gene activity does not correspond to a similar increase in the level of hoxH and hoxY transcripts. The regulation of the bidirectional hydrogenase in unicellular cyanobacteria such as _G. alpicola_ requires further studies.

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**References**


