Cloning and sequencing of the genes encoding the subunits of bidirectional hydrogenase of *Anabaena variabilis* IAM M58

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**Introduction**

Photobiological H₂ production by cyanobacteria is expected to become an environmentally acceptable future alternative to fossil fuel resources, because heterocystous N₂-fixing cyanobacteria have the unique ability of sustained H₂ production simultaneously with photosynthetic O₂ evolution without anaerobic treatments of the cells. In addition, they are able to produce H₂ using sunlight and water with the simplest nutritional requirements.

Previously, we reported the rates of H₂ production of 12 heterocystous N₂-fixing cyanobacterial strains and *Anabaena variabilis* IAM M58 being most active in H₂ production. In this study, we have sequenced *hoxFUY* genes, a partial *hoxH* gene and two unidentified ORFs in the genome of *A. variabilis* IAM M58 by a PCR-based strategy. The sequences of the *hox* genes showed high homologies with those of other cyanobacteria.

**Materials and methods**

*Anabaena variabilis* IAM M58 was obtained from IAM culture collection, University of Tokyo. The cyanobacteria were grown in BG11 medium with continuously stirring and bubbled with air at 26 °C, under continuous illumination with white fluorescent light at 80 μE m⁻² s⁻¹ PAR. *Escherichia coli* strain JM109 was grown in LB medium at 37 °C. The concentrations of antibiotics were 100 μg/ml for ampicillin.

Genomic DNA was extracted from the cyanobacterial cells by the glass beads method. PCR amplification was carried out using a PCR Thermal Cycler PERSONAL2 (Takara) and Ex-Taq polymerase (Takara). Oligonucleotides used as PCR primers were designed on the basis of highly conserved sequences identified by aligning the *hox* DNA sequences from *A. nidulans* PCC 6301 (Boison et al. 1996, 1998), *Synechocystis* PCC 6803 (Kaneko et al. 1996), *A. variabilis* ATCC 29413 (Schmitz et al. 1995) and *Anabaena* PCC 7120 (Table 1). The *Anabaena* PCC 7120 *hox* genes have been identified in ongoing genome project of *Anabaena* PCC 7120 (http://www.kazusa.or.jp/ctn/cyan/anaabena/). The amplified PCR products were purified by using Quantum Prep Freeze ‘N Squeeze DNA Extraction Spin Columns (Bio-Rad) after electrophoresis on 1% agarose gels and then cloned into pGEM-T.
Easy Vector (Promega). Nucleotide sequencing was performed by the dideoxy-chain termination method using SequiTherm EXCEL 11 DNA Sequencing Kit-LC and fluorescent dye-labeled M13 primer (Epicentre Technologies). The DNA sequences of the hydrogenase \( hoxF \), \( hoxU \) and \( hoxY \) genes and ORFs have been deposited to the DDBJ/EMBL/GenBank databases (accession no. AB057405).

**Results and Discussion**

Nine PCR fragments generated by using primer pairs shown in Table 1 turned out to be parts of \( hox \) genes. By arranging these fragments, complete sequences of \( hoxF \), \( hoxU \), \( hoxY \) gene and two ORFs and partial sequence of \( hoxH \) gene were determined. The organization of \( hox \) genes of \( A. \ variabilis \) IAM M58 shown in Figure 1 is the same as those of *Anabaena* PCC 7120, *A. variabilis* ATCC 29413, *Synechocystis* PCC 6803 and *A. nidulans* PCC 6301 except that the genes are interrupted by different ORFs at different positions. An ORF (ORF8) located between \( hoxU \) and \( hoxY \) is also identified in the same position as in *A. variabilis* ATCC 29413 and *Anabaena* PCC 7120, but an unidentified ORF between \( hoxF \) and \( hoxU \), that gave no significant homologies in the available databases, is missing in any of the other strains. In *Anabaena* PCC 7120, an additional ORF (\( hoxE \)) is located upstream of \( hoxF \), but we could not identify it by PCR amplifications with several primers that were based on \( hoxE \) sequence of *Anabaena* PCC 7120.

![Fig.1. Determination of hox genes of Anabaena variabilis IAM M58 by PCR. Positions of primers used for PCR amplification and the amplified fragments A-I are indicated above the gene cluster. Oligonucleotides used in the PCRs are shown in Table1.](image)

<table>
<thead>
<tr>
<th>PCR product</th>
<th>Sense primer Name</th>
<th>Sequence 5'--&gt;3'</th>
<th>Antisense primer Name</th>
<th>Sequence 5'--&gt;3'</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A ( hoxEs2 )</td>
<td>GGTGCTTGTGGGATATGCTCC</td>
<td>( hoxFa1 )</td>
<td>GCATAGGCAGCGATCGCCATT</td>
<td>804</td>
<td></td>
</tr>
<tr>
<td>B ( hoxEs2 )</td>
<td>GGTGGCGGATGGGATATGCTCC</td>
<td>( hoxFa3 )</td>
<td>TTGAGGTGCAATAGGCGCAT</td>
<td>1729</td>
<td></td>
</tr>
<tr>
<td>C ( hoxFs5 )</td>
<td>GTCATCTGAAAGCCGATGAA</td>
<td>( hoxFa3 )</td>
<td>TTGAGGTGCAATAGGCGCAT</td>
<td>1032</td>
<td></td>
</tr>
<tr>
<td>D ( hoxFs3 )</td>
<td>GTCATCGCTAATCCCGATGTTT</td>
<td>( hoxUa1 )</td>
<td>TCTAAATGGCGACAGTGGGAAG</td>
<td>1180</td>
<td></td>
</tr>
<tr>
<td>E ( hoxFs4 )</td>
<td>CAACACATGATGTAATGATTTT</td>
<td>( hoxYa1 )</td>
<td>AAGGACATATGACAGGCGAACA</td>
<td>1607</td>
<td></td>
</tr>
<tr>
<td>F ( hoxUs1 )</td>
<td>ATTCCGACACCTGCGATGTTT</td>
<td>( hoxYa1 )</td>
<td>AAGGACATATGACAGGCGAACA</td>
<td>1259</td>
<td></td>
</tr>
<tr>
<td>G ( hoxUs2 )</td>
<td>ATCGCGCCAACTCCCGATGTTT</td>
<td>( hoxYa2 )</td>
<td>TTAGCCAAATTGATAAGTTTCC</td>
<td>1178</td>
<td></td>
</tr>
<tr>
<td>H ( hoxYs3 )</td>
<td>TGGCGCTAATGGAACCCGAGCAT</td>
<td>( hoxHa3 )</td>
<td>AGGGCTGCGGATCGCATTCTT</td>
<td>3.0 kb*</td>
<td></td>
</tr>
<tr>
<td>I CMhoxH-s</td>
<td>ATTTGTGGTATTTGTCCGGTGAGTC</td>
<td>CMhoxH-a</td>
<td>GAACAACTTAAACAGGGGTCAAAGC</td>
<td>1187</td>
<td></td>
</tr>
</tbody>
</table>

*, fragment size of the PCR product I was calculated from electrophoresis.

The deduced amino acid sequences of the \( hoxF \), \( hoxU \), \( hoxY \) and \( hoxH \) genes showed 81, 89, 79 and 86% identities with the corresponding homologs of *A. variabilis* ATCC 29413, respectively. The products of the \( hoxF \), \( hoxU \) and \( hoxY \) genes all share the characteristics that are conserved in the homologous gene products from other cyanobacteria, in particular, putative FeS-cluster binding motifs and NAD-, FMN-, Ni-binding regions (Fig. 3).
The bidirectional hydrogenase is suggested to be a heterotetrameric enzyme consisting of a hydrogenase part (HoxYH) and a diaphorase part (HoxFU). The diaphorase part is proposed to have dual roles in both the bidirectional hydrogenase and respiratory complex I because of significant sequence similarities between the NADH dehydrogenase part of complex I from *E. coli* and the diaphorase part of the NAD⁺-reducing hydrogenase. However, *Nostoc* PCC 73102, which lacks not only *hoxYH* but also *hoxEFU*, respires at rates similar to those of other cyanobacteria and the respiration was unaffected by knocking out the diaphorase genes in *Synechocystis* PCC 6803 and *A. nidulans*. Though the physiological role of the bidirectional hydrogenase is still controversial, it has been so far assigned roles in H₂ uptake as a means of collecting additional reducing power during growth in anaerobic environments due to its capability of either consumption or production of hydrogen and to its low $K_m$ for H₂. If so, disruption of *hox* genes may improve H₂ production.

**References**


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**Fig. 2.** Organization in the bidirectional hydrogenase genes from different cyanobacteria.
Fig. 3. Alignment of (A) HoxF, (B) HoxU, (C) HoxY and (D) HoxH. The deduced amino acid sequences from (3) *A. variabilis* IAM M58 are compared with those from (1) *Anabaena* PCC 7120, (2) *A. variabilis* ATCC 29413, (4) *Synechocystis* PCC 6803 and (5) *A. nidulans* PCC 6301. Conserved cysteines are highlighted. NAD, FMN and putative FeS-cluster binding motifs are underlined and their consensus sequences are indicated.