Characterization and transcriptional analysis of \textit{hupSLW} in \textit{Gloeoeche} sp. ATCC 27152: an uptake hydrogenase from a unicellular cyanobacterium

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The structural genes (\textit{hupSL}) encoding an uptake hydrogenase in the unicellular cyanobacterium \textit{Gloeoeche} sp. ATCC 27152, a strain capable of aerobic N\textsubscript{2} fixation, were identified and sequenced. \textsuperscript{3}9-RACE experiments uncovered the presence of an additional ORF 184 bp downstream of \textit{hupL}, showing a high degree of sequence identity with a gene encoding an uptake-hydrogenase-specific endopeptidase (\textit{hupW}) in other cyanobacteria. In addition, the transcription start point was identified 238 bp upstream of the \textit{hupS} translational start. RT-PCR experiments revealed that \textit{hupW} is co-transcribed with the uptake hydrogenase structural genes in \textit{Gloeoeche} sp. ATCC 27152. In addition, Northern hybridizations clearly showed that \textit{hupSLW} are transcribed under nitrogen fixing conditions, but not in the presence of combined nitrogen. A putative NtcA binding site was identified in the promoter region upstream of \textit{hupS}, centred at \textasciitilde41-5 bp with respect to the transcription start point. Electrophoretic retardation of a labelled DNA fragment (harbouring the putative NtcA-binding motif) was significantly affected by an \textit{Escherichia coli} cell-free extract containing overexpressed NtcA, suggesting that NtcA is involved in the transcriptional regulation of \textit{hupSLW}.

\section*{INTRODUCTION}

Several species of bacteria and cyanobacteria are capable of N\textsubscript{2} fixation. During the N\textsubscript{2} fixation process H\textsubscript{2} is formed as a by-product. This nitrogenase-dependent H\textsubscript{2} production is often compromised by the presence of an uptake hydrogenase (encoded by \textit{hupSL}) that rapidly consumes the H\textsubscript{2} generated. In addition, a bi-directional enzyme (encoded by \textit{hoxEFUYH}) may be present which, depending on the growth conditions, may display the capacity of both producing and consuming H\textsubscript{2} (Lambert & Smith, 1981; Houchins, 1984; Schmitz \textit{et al.}, 2002). All cyanobacteria examined so far contain an uptake, a bi-directional or both the hydrogenases (Schmitz \textit{et al.}, 1995, 2002; Boison \textit{et al.}, 1996; Tamagnini \textit{et al.}, 2000, 2002; Sheremetieva \textit{et al.}, 2002; Schütz \textit{et al.}, 2004). Moreover, the uptake-type enzyme has been found in all nitrogen fixing cyanobacterial strains studied to date (Carrasco & Golden, 1995; Oxelfelt \textit{et al.}, 1998; Happe \textit{et al.}, 2000; Tamagnini \textit{et al.}, 2000, 2002; Schütz \textit{et al.}, 2004).

The first data on cyanobacterial \textit{hupSL} transcription appeared in 1995 (Carrasco & Golden, 1995). RT-PCR experiments on \textit{Anabaena}/\textit{Nostoc} sp. strain PCC 7120 demonstrated that \textit{hupL} transcription coincides with the formation of heterocysts. Subsequent studies, in other filamentous strains, have confirmed the induction of an \textit{hupL} transcript under nitrogen-fixing conditions only (Axelsson \textit{et al.}, 1999; Happe \textit{et al.}, 2000; Hansel \textit{et al.}, 2001). One exception is \textit{Anabaena variabilis} ATCC 29413, where a low level of \textit{hupL} expression has been detected in vegetative cells grown with the addition of ammonia (Boison \textit{et al.}, 2000). In all the cyanobacterial strains
examined so far, transcriptional studies have shown that the hupSL genes constitute a single transcript, containing no additional ORFs (Happe et al., 2000; Lindberg et al., 2000).

The maturation of hydrogenases is a complex process requiring a number of accessory proteins (Menon et al., 1993; Vignais & Toussaint, 1994; Maier & Triplett, 1996; Buhlke et al., 2001; Casalot & Rouset, 2001; Vignais et al., 2001; Blokesch et al., 2002; Paschos et al., 2002). One distinctive feature in the NiFe-hydrogenases maturation process is the endoproteolytic cleavage of a C-terminal peptide of the large subunit precursor, carried out by a specific C-terminal endopeptidase (Casalot & Rouset, 2001; Paschos et al., 2002). Until now, the available data on the maturation of cyanoacterial NiFe-hydrogenases are scarce. Recently, the presence and expression of endopeptidases specific for cyanobacterial hydrogenases was reported (Wünschers et al., 2003). These authors screened three completed cyanobacterial genome sequences [Anabaena/Nostoc sp. strain PCC 7120 (www.kazusa.or.jp/cyano/Anabaena), Nostoc punctiforme ATCC 29133/PCC 71302 (http://genome.jgi-psf.org/draft_microbes/nospu/nospu.home.html), and Synechocystis PCC 6803 (www.kazusa.or.jp/cyano/Synechocystis)] with the purpose of identifying genes putatively encoding C-terminal specific endopeptidases. In agreement with previous nomenclature they proposed the gene name hoxW (endopeptidase specific for the bi-directional hydrogenase) for the ORFs all0770 (Anabaena/Nostoc PCC 7120) and slr1876 (Synechocystis PCC 6803), whereas the ORFs slr1423 (Anabaena/Nostoc PCC 7120) and c509/r320 (Nostoc PCC 73102) were named hupW (endopeptidase specific for the uptake hydrogenase). These ORFs are not clustered with any known hydrogenase-related gene(s).

A strong correlation between nitrogen fixation and uptake hydrogenase activity has been demonstrated in filamentous cyanobacteria (Lambert & Smith 1981; Houchins, 1984; Wolk et al., 1994; Oxelfelt et al., 1995; Masukawa et al., 2002; Schütz et al., 2004). In cyanobacteria nitrogen control is mediated by a transcriptional regulator, NtcA, belonging to the CAP family (the catabolite gene activator or cAMP receptor protein) (Herrero et al., 2001). In response to ammonium withdrawal, NtcA binds to specific sites in the promoter region of regulated genes involved in nitrogen assimilation. The NtcA-activated promoter structure consists of a −10 box in the form TAN1T and an NtcA-binding site with the consensus sequence GTAN8TAC, usually located 20 to 23 nucleotides upstream of the −10 box, which appears to substitute for the −35 box (Luque et al., 1994; Muro-Pastor et al., 1999; Herrero et al., 2001). Other proposed consensus NtcA binding sites are TGTA9/10ACA, and TGTA9TACA (Ramasubramanian et al., 1994; Jiang et al., 2000; Wisén, 2003).

Up to now, only limited amounts of biochemical/physiological data are available concerning uptake hydrogenases in unicellular cyanobacteria (Lambert & Smith, 1981; Houchins, 1984; Schütz et al., 2004). Recently, in the unicellular cyanobacterium Gloeothec sp. strain ATCC 27152, the unequivocal presence of an uptake hydrogenase was reported, in contrast with the lack of hybridization signals when probes for hox genes were used (Schütz et al., 2004). However, a residual level of methyl-viologen-dependent H2 evolution could be detected, therefore the presence of a bi-directional hydrogenase in Gloeothec sp. ATCC 27152 cannot be excluded.

This study presents the first comprehensive molecular data on an uptake hydrogenase being present in a unicellular cyanobacterium, and provides new information on how oxygen-evolving photosynthesis and an essentially anaerobic process like hydrogen uptake can occur within a single cell. The structural genes (hupSL) encoding this enzyme in Gloeothec sp. ATCC 27152 were identified, sequenced and characterized. Moreover, a gene encoding a cyanobacterial hydrogenase specific endopeptidase – hupW – was found immediately downstream of hupL, and was shown to be co-transcribed with hupSL. The three genes are transcribed under nitrogen fixing conditions, but not in the presence of combined nitrogen. Evidence for the involvement of NtcA in the transcriptional regulation of hupSLW is also presented.

METHODS

Organism and growth conditions. Gloeothec sp. strain ATCC 27152 was cultured at 25°C in BG11 or BG11 for the Northern blot experiments (Rippka et al., 1979), under a 12 h light (10 μmol photons m−2 s−1)/12 h dark cycle regime.

Hydrogen uptake activity. In vivo hydrogen uptake was measured using a Hansatech DW1 O2/H2 electrode (Hansatech) according to the methods described previously (Oxelfelt et al., 1995).

Nucleic acid extraction and analysis. Genomic DNA was isolated from Gloeothec sp. ATCC 27152 cells by phenol/chloroform extraction as described elsewhere (Tamagnini et al., 1997). In order to obtain clean DNA (e.g. free from extracellular polysaccharides), additional washing steps were required: the cells were collected by centrifugation and resuspended in washing buffer [50 mM NaCl, 5 mM EDTA and 50 mM Tris/HCl pH 8.0 (Fiore et al., 2000)], followed by the addition of 0.5–1 g acid-washed 0.6 mm diameter glass beads and vortexing. The supernatant was collected and the washing step was repeated twice without the presence of glass beads. The DNA was then extracted following the protocol referred to above. After the precipitation step, the pellet was again resuspended in washing buffer and the DNA was precipitated. The DNA was recovered using a sterile micropipette tip, washed with 70% (v/v) ethanol, dried and dissolved in sterile water.

For RNA isolation, cells of Gloeothec sp. ATCC 27152 were harvested by centrifugation at 4°C, frozen in liquid nitrogen and left to thaw on ice. This freezing and thawing was repeated twice. Total RNA was then isolated following the protocol of Axelsson et al. (1999), with the exception that 20 U DNase I PFLCpure (Amersham Biosciences) was added during the hot phenol treatment. RNA used for the Northern hybridizations was isolated using TRIZOL reagent (Invitrogen), following the manufacturer’s instructions. In the homogenization step, 0.5 g acid-washed 0.2 mm diameter glass beads were added to the samples, and the disruption of the cells was accomplished using a
Mini-Beadbeater (Biospec Products). DNA and RNA were analysed by agarose gel electrophoresis using 1× TAE or TBE buffer (Sambrook et al., 1989).

PCR, DNA sequencing and sequence analysis. All oligonucleotides used in this study are listed in Table 1 (see also Fig. 1a). PCR amplifications were carried out in a Gene Amp PCR System 2400 (Perkin-Elmer) thermal cycler with Taq DNA polymerase (Amersham Biosciences) as previously described (Tamagnini et al., 1997).

DNA fragments were isolated from agarose gels using the QIAEX II gel extraction kit (Qiagen) or the NucleoSpin Extract kit (Macherey-Nagel), following the manufacturer’s instructions. Sequencing reactions were performed with an ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems), the thermal cycler mentioned above, and the ABI 377 DNA automated sequencer system (Applied Biosystems). Published sequences were retrieved from GenBank and computer-assisted sequence analyses were performed using CLUSTAL W (Thompson et al., 1994). Novel sequences associated with this study (Gloeoethece sp. ATCC 27152 hupSLW) are available in GenBank under the accession number AY260103.

Southern blot analysis. The probe used for Southern hybridization was obtained by PCR using genomic DNA from Gloeothece sp. ATCC 27152 and the primer pair GloS1A/HS1B [probe GhupS (Fig. 1a) Schütz et al. (2004)]. The identity of the probe was confirmed by sequencing.

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**Table 1. Oligonucleotide primers used in the present study**

For the specific positions within the hupSLW sequence see Fig. 1(a).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5′→3′</th>
<th>References</th>
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<td>This study</td>
</tr>
<tr>
<td>S1 (2)</td>
<td>AACGTACTCGGCTGAGAGG</td>
<td>Tamagnini et al. (2000)</td>
</tr>
<tr>
<td>Srev (3)</td>
<td>CACCCTCTGGAGAGTACGTT</td>
<td>This study</td>
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<tr>
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<td>GCTAGGAGAACATCTGCAAC</td>
<td>Schütz et al. (2004)</td>
</tr>
<tr>
<td>GloS1B (5)</td>
<td>GTTGAAGATGTGCTCTCAAC</td>
<td>This study</td>
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<tr>
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**Fig. 1.** (a) Physical map of hupSLW in Gloeothece sp. ATCC 27152. The transcriptional start point is indicated by +1, and is located 238 bp upstream from the hupS start codon. The numbered grey arrowheads represent the oligonucleotide primers used in this study (see also Table 1). GhupS indicates the homologous probe used in Southern hybridization for the identification of the HindIII fragment (clone GhSL1, dashed line not to scale). Black vertical arrows indicate the restriction sites. The sequence harbouring the hupSLW (3891 kb) is available from GenBank under accession no. AY260103. (b) Nucleotide sequence of the promoter region upstream of hupS in Gloeothece sp. ATCC 27152. A putative NtcA binding site is boxed. The transcription start point is indicated by +1 and a putative −10 consensus sequence is underlined. The start codon of hupS is shown in bold and the deduced N-terminal amino acid sequence is given below.
Radioactive and non-radioactive Southern hybridizations were carried out at 57 °C, following previously described protocols (Tamagnini et al., 1997; Schütz et al., 2004).

Construction of a partial genomic library of Gloeothece sp. ATCC 27152 and identification of hupSL. Genomic DNA was digested by the restriction endonuclease HindIII and separated on a 1 % (w/v) agarose gel. A region between 3-5 and 4-5 kb was cut out, and the DNA was extracted from the gel as described above. Ligation into the vector pGEM 3Zf(+) (Promega), transformation and screening (using the probe GhpSp; see also Fig. 1a) were performed as described previously (Oxelfelt et al., 1998). Positive clones were detected using a Typhoon 8600 Variable Mode imager (Amersham Biosciences).

To identify the 3' end of hupL in Gloeothece sp. ATCC 27152, 3'-RACE was performed essentially following the manufacturer’s protocol (FirstChoice RLM-Race kit; Ambion). As in the 5'-RACE, 2 μl of the reverse transcription (RT) reaction was used in the outer PCR and that the extension was prolonged to 1 min in both the outer and inner PCR amplifications. The gene-specific antisense primers from the 5’ end of hupS of Gloeothece sp. ATCC 27152 used were: S1rev, GloS1B and GloSR1B.

To locate the transcription start point, 5'-RACE experiments were carried out, using a commercially available kit (FirstChoice RLM-Race kit; Ambion). The instructions of the manufacturer were followed, except that a double volume (2 μl) of the reverse transcription (RT) reaction was used in the outer PCR and that the extension was prolonged to 1 min in both the outer and inner PCR amplifications. The gene-specific antisense primers from the 5’ end of hupS of Gloeothece sp. ATCC 27152 used were: GloH4A and GloH4A2.

PCR products were cloned, using the pGEM-T Easy vector system (Promega), into XL-1 Blue supercompetent cells (Stratagene). Plasmid PCR products were cloned, using the pGEM-T Easy vector system were: GloH4A and GloH4A2.

RESULTS AND DISCUSSION

Identification and characterization of hupSL

The presence of an uptake hydrogenase in Gloeothece sp. ATCC 27152 was recently demonstrated by Schütz et al., 2004. These authors showed that Gloeothece sp. ATCC 27152 is able to fix nitrogen both under aerobic and anaerobic conditions, yet no nitrogenase-mediated H2 evolution was observed, most likely due to consumption by an active-uptake hydrogenase. Subsequently, these results were confirmed using additional homologous probes (generated using oligonucleotide primers designed against conserved regions within other cyanobacteria) in Southern hybridizations, and by measuring hydrogen uptake activity using a H2 electrode (this work, data not shown). Moreover, to obtain a contiguous sequence Gloeothece sp. ATCC 27152-specific primers were designed, and the respective PCR products sequenced. This revealed the majority of the hupSL sequence, disclosing the presence of a 259 bp intergenic region. Concomitantly, a partial genomic library was constructed, and a 4 kb HindIII fragment (clone GhSL1) was isolated and sequenced (see Fig. 1a). To obtain the remaining 3'-end of hupL, 3'-RACE reactions were carried out, and a number of cDNA clones of different lengths were obtained (most likely due to the fact that the mRNA polyadenylation in prokaryotes appears to be a relatively indiscriminate process, able to occur at all mRNA’s 3'-ends, and does not require a specific consensus sequence as in eukaryotes; see Sarkar, 1997; Rauhut & Klug, 1999; Wagner, 2000). Sequencing revealed that all these clones consisted of the 3'-end of hupL. Multiple sequence alignments were carried out to obtain the sequence
hupSLW genes in Gloeothecae sp. ATCC 27152 are co-transcribed

RT-PCR experiments, using total RNA extracted from Gloeothecae sp. ATCC 27152 cells grown under nitrogen-fixing conditions, were performed for the transcriptional analysis of the hupS, hupL and hupW genes. The culture was harvested 5 h into the dark phase, hydrogen uptake activity was confirmed using a hydrogen electrode (data not shown), and RNA was extracted. An RT reaction using GhpWIR as the hupW-specific antisense primer was performed, and the resulting cDNA was used as template in PCR amplifications for the detection of hupS–hupL and hupL–hupW co-transcription. The results showed that the three genes (hupSLW) are indeed transcribed together (Fig. 3). The 3′-RACE results described above already indicated that hupW could be transcribed along with hupL. In the cyanobacterial strains studied so far, the hupSL genes constitute a single transcript with no additional ORFs (Happe et al., 2000; Lindberg et al., 2000). Moreover, the transcription of hupW in Anabaenae/Nostoc PCC 7120 and N. punctiforme ATCC 29133/PCC 73102 has been shown

Fig. 2. Deduced amino acid sequence alignment of the putative hydrogenase-specific protease of Gloeothecae sp. ATCC 27152 (HupW G27152) and the corresponding protein in Anabaenae/Nostoc sp. PCC 7120 (HupW N73102) and N. punctiforme ATCC 73102 (HupW N73102). hupW genes were co-transcribed with hupL in the strains studied.
to occur independently of that of hupSL (Wünschiers et al., 2003). Thus, this is the first time that the gene (hupW) encoding an uptake-hydrogenase-specific endopeptidase has been reported to be co-transcribed with the uptake hydrogenase structural genes (hupSL) in cyanobacteria.

Transcription start point identification and characterization of the hupS promoter region

5’-RACE permitted the identification of the transcription start point (tsp), 238 bp upstream from the hupS start codon (Fig. 1). In Gloeothece sp. ATCC 27152, a putative −10 box (TAATGT) is located six nucleotides upstream of the tsp, matching well with the consensus σ70-like −10 box sequence (TAN3T) found in other cyanobacteria (Luque et al., 1994; Herrero et al., 2001). A putative NtcA binding site (GTAAACCAAGATTAC) was also identified 22 nucleotides further upstream of the −10 region. This sequence contains the highly conserved palindromic NtcA-binding region signature GTAN8TAC (Luque et al., 1994; Herrero et al., 2001), and is flanked by T-rich sequences both upstream and downstream.

hupSLW transcription under different growth conditions

Northern blot experiments were performed with total RNA extracted from Gloeothece sp. ATCC 27152 cells grown under nitrogen-fixing or non-nitrogen fixing conditions, and 12 h light/12 h dark cycles. Samples were collected at four different time-points from both growth conditions (Fig. 4). The hybridizations were carried out using a hupS-specific probe. The results clearly show that the transcript(s) is present when Gloeothece sp. ATCC 27152 cells are grown under nitrogen-fixing conditions, but totally absent under non-nitrogen fixing conditions (NaNO3, Fig. 4). In addition, there is an evident light/dark regulation, with the highest transcript levels detected during the light cycle. This is interesting since Gloeothece sp. ATCC 27152 has been shown to fix nitrogen mainly in the dark (Reade et al., 1999), and consequently displays a higher hydrogen-uptake activity during the dark cycle (confirmed in this study using a hydrogen electrode, data not shown). The appearance of the transcript(s) prior to a detectable hydrogen uptake activity may be due to the fact that the uptake hydrogenase requires a complex maturation process. In contrast to heterocystous strains (Anabaena/Nostoc PCC 7120 and Nostoc PCC 73102), in which hupW is transcribed independently from hupSL (Wünschiers et al., 2003), hupSLW in Gloeothece sp. ATCC 27152 appear to be co-transcribed. This difference, together with the fact that there is a temporal separation between photosynthesis and nitrogen fixation/hydrogen-uptake activity in Gloeothece sp. ATCC
27152, may result in an extended period between transcription and activity. Furthermore, the Northern hybridizations revealed the presence of at least three different transcripts [or possibly a combination of transcript(s) and degradation products]. The largest transcript being approximately 3800 nt, and therefore probably corresponding to hupSLW, a smaller transcript of about 2000 nt (possibly a degradation product), and a third transcript of about 1200 nt possibly corresponding to hupS alone. These results corroborate the RT-PCR data (co-transcription of hupSLW), but also suggest the possibility of multiple transcripts.

Binding of NtcA to the promoter region of the hupS gene

The potential NtcA binding site, identified in the Gloeothece sp. ATCC 27152 hupS promoter region, is centred at -41·5 bp with respect to the transcription start point. In order to confirm binding of NtcA to the promoter region, gel mobility shift DNA-binding assays were performed. Cell-free extracts were prepared from E. coli BL21(DE3) (pREP4, pCSAM70), where the overexpression of NtcA had been induced by 1 mM IPTG (Muro-Pastor et al., 1999). The mobility shift assays were carried out with a 366 bp, 32P-labelled DNA fragment covering the promoter region of hupS (-101 to +265). Fig. 5 shows that electrophoretic retardation of the labelled DNA fragment was significantly effected by the E. coli cell-free extract containing the overexpressed NtcA, whereas no retardation could be detected when a non-related E. coli cell-free extract (not carrying a cloned ntcA gene) was used. Band retardation of the 32P-labelled fragment was successfully outcompeted by the identical unlabelled DNA fragment (Fig. 5, lane 6). These results suggest that NtcA binds specifically to the identified putative site (GTAACCAAGATTAC) in the promoter region of hupS of Gloeothece sp. ATCC 27152, and thus may be involved in the transcriptional regulation of hupSLW.

A variety of sequence motifs involved in the binding of NtcA have been reported (Jiang et al., 2000; Herrero et al., 2001; Lindberg, 2003). TGT(N9/10)ACA has been suggested as a consensus recognition site in Anabaena/Nostoc PCC 7120 (Ramasubramanian et al., 1994), whereas the motif GTAN8TAC was described as being the NtcA binding site in a number of other cyanobacterial strains (Luque et al., 1994; Herrero et al., 2001). In a study examining the binding of NtcA to a library consisting of DNA fragments with 13 random nucleotides followed by ACA, an eight-base palindromic sequence (TGTAN8TACA) was found to be the optimal binding site (Jiang et al., 2000). The NtcA motif upstream of hupS in Gloeothece sp. ATCC 27152 matches well with the GTAN8TAC sequence signature, while the corresponding motif identified in N. punctiforme (Lindberg, 2003) is more similar to the consensus sequence from Anabaena/Nostoc PCC 7120. N. punctiforme is a heterocystous cyanobacterium and thus able to carry out nitrogen fixation concomitantly with photosynthesis (Whitton & Potts, 2000). The hupSL NtcA binding site in N. punctiforme falls into the same group of binding sites found in promoter regions of several genes regulated during heterocyst differentiation (Lindberg, 2003). Being a unicellular cyanobacterium, Gloeothece sp. ATCC 27152 fixes N2 almost exclusively during the dark, effecting a temporal separation between photosynthesis and N2 fixation (Reade et al., 1999). Therefore it is possible that the NtcA binding motif upstream of hupS in Gloeothece sp. ATCC 27152 has a different signature compared to N. punctiforme, due to the lack of cells specialized in N2 fixation.

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