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Transcriptional and Mutational Analysis of the Uptake Hydrogenase of the Filamentous Cyanobacterium *Anabaena variabilis* ATCC 29413

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A 10-kb DNA region of the cyanobacterium *Anabaena variabilis* ATCC 29413 containing the structural genes of the uptake hydrogenase (*hupSL*) was cloned and sequenced. In contrast to the *hupL* gene of *Anabaena* sp. strain PCC 7120, which is interrupted by a 10.5-kb DNA fragment in vegetative cells, there is no programmed rearrangement within the *hupL* gene during the heterocyst differentiation of *A. variabilis*. The *hupSL* genes were transcribed as a 2.7-kb operon and were induced only under nitrogen-fixing conditions, as shown by Northern blot experiments and reverse transcriptase PCR. Primer extension experiments with a fluorescence-labeled oligonucleotide primer confirmed these results and identified the 5′ start of the mRNA transcript 103 bp upstream of the ATG initiation codon. A consensus sequence in the promoter that is recognized by the fumarate nitrate reductase regulator (Fnr) could be detected. The *hupSL* operon in *A. variabilis* was interrupted by an interposon deletion (mutant strain AVM13). Under N_2-fixing conditions, the mutant strain exhibited significantly increased rates in H_2 accumulation and produced three times more hydrogen than the wild type. These results indicate that the uptake hydrogenase is catalytically active in the wild type and that the enzyme reoxidizes the H_2 developed by the nitrogenase. The Nif phenotype of the mutant strain showed a slight decrease of acetylene reduction compared to that of the wild type.

The only microorganisms with an O_2-producing photosynthesis that have a hydrogen metabolism are cyanobacteria and green algae (23, 25). In cyanobacteria, up to three enzymes can be involved in hydrogen metabolism: the nitrogenase which produces H_2 during nitrogen fixation (24), the membrane-bound hydrogenase which reoxidizes the H_2 (10), and the bidirectional hydrogenase catalyzing both oxidation of molecular hydrogen and reduction of protons (38). In cyanobacteria, the genetics of bidirectional hydrogenases are especially well characterized (3, 7). But, the H_2 production in filamentous cyanobacteria during the reduction of nitrogen to NH_3 is mainly catalyzed by the nitrogenase in the heterocysts. The *Anabaena* cells can oxidize the hydrogen with the uptake hydrogenase via the oxyhydrogen (Knallgas) reaction. It was suggested that the organism gets additional ATP while the Knallgas reaction can protect the O_2-sensitive nitrogenase by removing the oxygen in the heterocysts (30).

Hydrogenases have been described for a large number of microorganisms and studied intensively in diverse phylogenetic groups of bacteria (22, 34, 54). The uptake hydrogenases are membrane-bound enzymes which consist of two subunits with [Fe-S] clusters as prosthetic groups. The large subunit, HupL, carries additionally a Ni atom in the active center. In most of the bacterial families, the *hupSL* genes are clustered in an operon in which *hupS* is located upstream of the *hupL* gene (20, 51, 53). Recently, some hydrogenase sequences from filamentous cyanobacteria were published (10, 31, 38). The uptake hydrogenase in the heterocyst-forming organism *Anabaena* sp. strain PCC 7120 is interrupted by a 10.5-kb element. Under nitrogen-fixing conditions, this fragment is excised by a site-specific recombinase that is encoded inside the right border of the *hupL* element (10). This rearrangement is not found in the *hupSL* genes of *Nostoc* sp. strain PCC 73102 (31).

In the present study, we isolated and characterized the *hup* gene region in *Anabaena variabilis* ATCC 29413. In contrast to the best characterized filamentous cyanobacterium *Anabaena* sp. strain PCC 7120, the closely related strain *A. variabilis* has some interesting features. Heterocyst differentiation in *Anabaena* sp. strain PCC 7120 is accompanied by developmentally regulated genome rearrangements that affect *fdxN*, *nifD*, and *hupL* gene expression (9, 10). *A. variabilis* does not contain the *fdxN* element (6), and in this study we show that the *hupL* gene is also not rearranged in *A. variabilis*.

In order to analyze the function of the uptake hydrogenase in nitrogen and hydrogen metabolism, we constructed a *hupSL* deletion mutant by the insertion of an interposon in the *hupSL* operon. Physiological studies to compare the mutant phenotype with that of the wild type were carried out. The transcriptional regulation of the *hupSL* genes was investigated by Northern analysis and reverse transcriptase PCR. Further transcriptional investigations were made by determining the 5′ end of the mRNA by the primer extension technique and analyzing the promoter region.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids are listed in Table 1. Cells of the N_2-fixing cyanobacterium *A. variabilis* ATCC 29413 were grown either in BG11, $BG11_0$ (12), or $BG11_0$ medium supplemented with 5 mM NH_4Cl and 10 mM TES [*N*-tris (hydroxymethyl)methyl-2-aminoethane sulfonic acid] under continuous irradiance of 100 μmol m^{-2} s^{-1} and bubbled with air enriched with CO_2 to 1% (vol/vol) at 30°C. To induce heterocyst formation, the cultures were pelleted, washed twice with $BG11_0$, and grown in $BG11_0$ for 24 h.

For deletion mutagenesis, a wild-type strain of *A. variabilis* with a spontaneous mutation (FD strain) which also grows at 40°C was used. The mutant AVM13 was grown in BG11 medium supplemented with 50 μg of neomycin per ml. The growth conditions, media, and antibiotic concentrations for *Escherichia coli* strains were described elsewhere (47).

Nucleic acid isolation. Genomic DNA of the *A. variabilis* wild type and the mutant was isolated according to the method of Smoker and Barnum (42).

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TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant characteristic(s)	Reference(s) or source
Strains		
E. coli		
MC1061	*araD139* Δ(*ara-leu*)*7696* Δ(*lac*)*L74 galU galK hsdR2*(r_K^- m_K^+) *mcrB1*	11
HB101	F^- *supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	18
A. variabilis		
FD	Temperature-resistant wild type	14
AVM13	*A. variabilis hupSL*::*npt*	This study
Plasmids		
pUC18, pUC19	Cloning vectors, Ap^r, *mcs*	44, 55
pBluescript SK(−)	Cloning vector, Ap^r, *mcs*	40
pSUP202	Mobilizable plasmid, Ap^r, Cm^r, Tc^r	41
pRL443	Conjugal plasmid, Ap^r, Tc^r, Km^s, derivative of RP4	19
pRL623	Helper plasmid, Mob_{ColK}, M.*Ava*I, M.*Eco*4711, M.*Eco*T221, Ap^r, Cm^r	19
pRL648	Source of C.K3 cassette encoding Nm^r	18
pBMB19	Plasmid carrying the *bom* site in pUC18, Ap^r	45
pKS1	4.8-kb *Hin*dIII fragment carrying *A. variabilis hupSL* cloned in pBluescript	This study
pKS4I/II	Nm^r gene replacing 2.1-kb *Xba*I fragment of pKS1	This study
pKS13	0.7-kb *Xba*I-*Hin*dIII fragment, subclone of pKS1 with 3′ end of *hupL*	This study
pKS14	0.9-kb *Xba*I-*Hin*cII fragment, subclone of pKS1 with part of *hupS*	This study
pKS15	0.3-kb *Hin*cII-*Xba*I fragment, subclone of pKS1 with 5′ end of *hupS*	This study
pKS17	0.5-kb fragment containing part of *hupL* amplified by inverse PCR	This study
pKS18	4.8-kb *Xba*I fragment carrying *A. variabilis hupL* downstream region	This study
pAVM13	pSUP202 containing a 3.7-kb *Hin*dIII fragment carrying *hupSL*::Nm^r	This study
pIF1	0.8-kb *Hin*dIII-*Asp*700 fragment carrying part of *hupL* of *Anabaena* sp. strain PCC 7120	This study
pTS1	0.6-kb *Asp*700-*Hin*dIII fragment carrying part of *hupL* of *Anabaena* sp. strain PCC 7120	This study

[a] Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nm, neomycin; Tc, tetracycline.

Plasmid DNA was obtained by standard techniques (36). Total cellular RNA was isolated from 200-ml cultures grown in different media (BG11, $BG11_0$, and NH_4^+). The cells were disrupted with glass beads (450 microns), followed by organic extraction and ethanol precipitation. To remove contaminating DNA and proteins, a cesium-chloride gradient ultracentrifugation purification step (4) was done. The concentration of RNA was determined by measuring the absorbance at 260 nm.

Hybridization. For Southern blot hybridization analysis, chromosomal DNA of *A. variabilis* and the exconjugants, as well as the plasmid DNA from the clones of the partial libraries, was isolated and cut with appropriate restriction enzymes. Following separation by electrophoresis in 1% agarose gels, the DNA was transferred to Hybond N nylon membranes (Amersham Pharmacia Biotech) by the capillary method described by Sambrook et al. (36). Prehybridization, overnight hybridization, and washing steps were carried out at 58 to 62°C. Labeling of probes and detection of hybridization signals were performed with the DIG Labeling and Detection kit (Roche Diagnostics, Boehringer Mannheim).

Northern blot experiments were carried out by separating total RNA of the different cultures in 1% denaturing formaldehyde agarose gels and transferring to Hybond N nylon. pKS14 was labeled with digoxigenin-UTP by in vitro transcription with the T7-RNA-polymerase (Roche Diagnostics, Boehringer Mannheim). RNA-RNA hybridization was performed overnight at 65°C in DIG Easy Hyb solution.

Screening of partial gene banks and sequencing. Genomic DNA of *A. variabilis* was digested by different restriction endonucleases. After separation on a 1% agarose gel, *Hin*dIII and *Xba*I fragments between 4.5 and 5.0 kb were eluted out of the gel pieces. The extracted gel fragments were ligated to *Hin*dIII- and *Xba*I-linearized and -dephosphorylated pBluescript SK(−) vectors and transformed into competent *E. coli* MC1061 cells by the $CaCl_2$ method (13; modified by reference 36). A 0.8-kb *Hin*dIII-*Asp*700 fragment (pIF1) and a 0.6-kb *Asp*700-*Hin*dIII fragment (pTS1), obtained from the plasmid pAM1311 (10), were digoxigenin-labeled and used for Southern blot experiments with the clones of the partial libraries.

The plasmids pKS1 and pKS18 were treated with exonuclease III and S1 nuclease of an Erase-a-Base kit (Promega). Following ligation and transformation, the deletion clones were analyzed by restriction. Sequencing was then performed by the dideoxy chain termination method (37).

PCR and primer extension analysis. The 5′ end of the uptake hydrogenase mRNA was determined by a primer extension protocol (29) with fluorescence-labeled oligonucleotides and an automated DNA sequencer (2). The synthetic cy5-labeled oligonucleotide HupS7 (5′-Cy5-CGCATACTGTCGGTTCTTCGG C-3′) was a 22-mer complementary to the bases 85 to 64 downstream of the translation start codon ATG of the *hupS* gene.

Primer extension mixtures included 10 to 20 μg of RNA of the different preparations, 10 pmol of the HupS7 oligonucleotide, 50 U of RNasin Ribonuclease Inhibitor, 1 mM concentrations of deoxynucleoside triphosphates, and 5 U of avian myeloblastosis virus reverse transcriptase. The mixtures were incubated at 42°C for 1 h. The dideoxy sequencing reaction using a clone containing the 5′ region of the *hupS* gene (pKS15) and the same HupS7 primer was performed according to the instructions of the cy5-Auto Read Sequencing kit (Amersham Pharmacia Biotech). The primer extension product and the sequence ladder were loaded together onto a gel of the A.L.F. Express DNA Sequencer, and the transcription start point was determined by comparing their retention times.

Inverse PCR as described by Pang and Knecht (32) was used to determine the flanking sequences of the 3′ end of the *hupL* gene. Two primers which were complementary to bases 1230 to 1310 (HupL2 [5′-CGCTTGGCGATATAACT TGA-3′]) and identical to bases 1337 to 1362 (HupL3 [5′-GTCACTGGATAG ATATCGAAGGTGGC-3′]) of the *hupL* gene were selected. These primers, facing outwards from the known DNA sequence, were used to carry out PCR with genomic DNA from *A. variabilis*.

Constructions of plasmids and conjugative plasmid transfer. Different cargoplasmids were constructed and then transferred from *E. coli* HB101 to *A. variabilis*. A 2.15-kb *Xba*I fragment containing the *hupS* gene and part of the *hupL* gene was removed from the plasmid pKS1 by replacing it with a 1.1-kb neomycin resistance cassette from C.K3, yielding pKS4. C.K3 contains the *npt* gene from Tn*5* with a promoter from the *psbA* gene of *Amaranthus hybridus* (18). pAVM13 was constructed by inserting the resulting 3.77-kb *Hin*dIII fragment from pKS4 in pSUP202 (41).

The resulting cargoplasmid pAVM13 was introduced from *E. coli* cells into the *A. variabilis* FD strain via triparental mating (using the conjugal plasmid pRL443 and the helper plasmid pRL623 for mobilization). The conjugative plasmid transfer was performed according to the method of Elhai et al. (19). Instead of the normal wild-type strain, a spontaneous mutant (FD strain) which grows at 40°C was used for triparental mating. Exconjugants were selected by plating on BG11 plates containing 50 μg of neomycin per ml. Nm^r exconjugants were picked and grown in liquid medium, one added with neomycin and one added with ampicillin, to select the Amp^s exconjugants which have made the marker rescue via double crossover. One ampicillin-sensitive mutant chosen for further studies was designated AVM13. Insertion of the neomycin resistance cassette and complete segregation in AVM13 was shown by Southern hybridization.

Determination of nitrogenase and hydrogenase activity. In vivo nitrogenase activity was measured by acetylene reduction assay (17) using a Hewlett-Packard gas chromatograph Model 5890 Series II with a 6-ft Porapak N80/100 column for acetylene-ethylene separation and a flame ionization detector. The specific nitrogenase activity was expressed as follows: nanomoles of ethylene (C_2H_4)/micrograms of chlorophyll $\alpha \times$ h. The developed H_2 was quantified in a gas chromatograph (Hewlett-Packard Model 5890 Series II) equipped with a thermal conductivity detector and a molecular sieve column and expressed as follows: nanomoles of H_2/nanograms of chlorophyll $\alpha \times$ h. The hydrogen uptake was

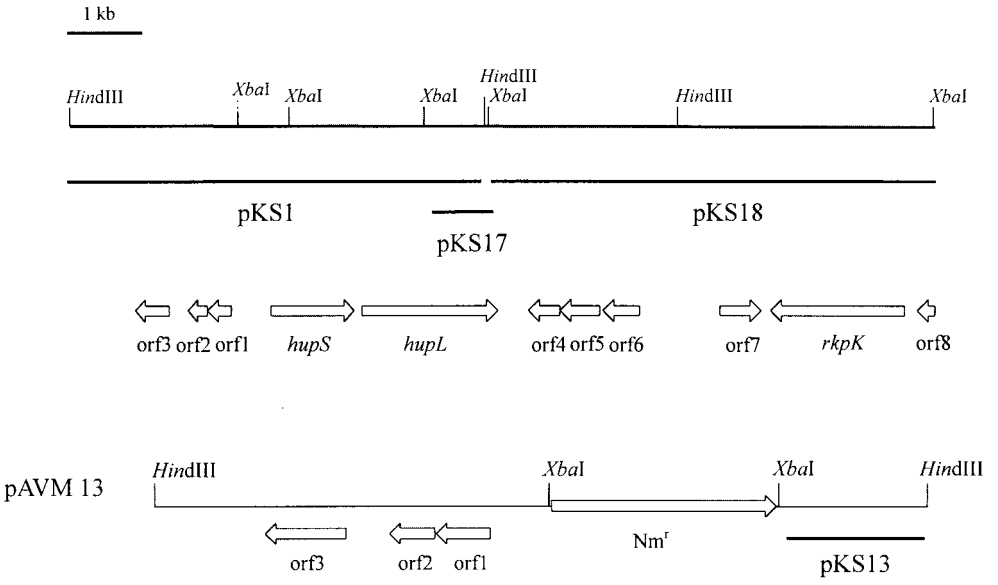

FIG. 1. Physical and genetical maps of the 10-kb *hupSL* gene region of *A. variabilis*. The physical maps are for the enzymes *Hin*dIII and *Xba*I. The region is divided into three subclones, marked with horizontal lines. The organization of the genes is indicated by arrows under the restriction map; ORF8 has been only partially sequenced. *hupSL* code for the two subunits of the uptake hydrogenase; *rkpK* probably encodes the UDP-glucose dehydrogenase. The gene arrangement of the cargoplasmid pAVM13 with the integrated neomycin resistance cassette (Nm^r) is shown. Note that pAVM13 is drawn on a different scale.

measured during incubation at 25°C by the decrease of a known amount of H_2 (2 ml of 0.3% H_2–99.7% argon) added to the probe.

Nucleotide sequence accession number. The nucleotide sequence data reported in this study is available from the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Y13216.

RESULTS

Isolation, characterization, and sequence comparison of the *hupSL* region in *A. variabilis* ATCC 29413. To identify the *hup* gene region in *A. variabilis* ATCC 29413, the *hupSL* genes were cloned by partial genomic library screening using different fragments within the *hupL* gene of *Anabaena* sp. strain PCC 7120 as probes in Southern hybridization experiments (see Materials and Methods). First, a *Hin*dIII (pKS1) clone with a 4,824-bp insert was isolated. This nucleotide sequence has two open reading frames (ORFs) that show over 90% homology to the *hupSL* genes of *Anabaena* sp. strain PCC 7120. As a result, no stop codon was found at the 3′ end of the *hupL* gene, indicating that *hupL* was only partially encompassed by the insert of pKS1.

A second partial gene bank was constructed to obtain the remaining part of *hupL*. After screening with a DNA probe (pTS1) containing the downstream region of the *Anabaena* sp. strain PCC 7120 *hupL* gene, one positive clone (pKS18) which contained the 120 bp of the 3′ end of the *A. variabilis hupL* gene was obtained. The missing 30 bp of the *hupL* gene were then identified by inverse PCR (32) using two inverted oligonucleotide primers which were designed from within the *hupL* sequence of the *Hin*dIII fragment. Both strands of the inserts, which contain the complete sequence of the *hupSL* gene region, were determined (data not shown). Together, the nucleotide sequence revealed 10 complete ORFs and one partial ORF (Fig. 1). Comparisons of their deduced amino acid and DNA sequences with known sequences in databases were performed (1, 21).

Two ORFs encode the small and large subunits of the uptake hydrogenase (*hupSL*). The proteins consist of 321 and 531 amino acid residues with calculated molecular masses of 35.1 and 61.9 kDa, respectively. Comparisons of the deduced amino acid sequences of *hupS* and *hupL* gene products with the HupS and HupL proteins of the filamentous cyanobacteria *Anabaena* sp. strain PCC 7120 and *Nostoc* sp. strain 73102 revealed very high similarities. Both subunits in *A. variabilis* showed more than 95% identity with the homologous region from *Anabaena* sp. strain PCC 7120. This is not surprising for these closely related organisms since 90% and higher similarities are also known for the *nifHDK* cluster (95% nucleotide identity) (6) and the *ndh* genes (93% nucleotide identity; T. Happe, personal communication). The intergenic region between *hupS* and *hupL* in *A. variabilis* consists of 76 bp and is only 60% homologous to the intergenic DNA sequence (54 bp) of *Anabaena* sp. strain PCC 7120. Unlike the *hupL* gene of *Anabaena* sp. strain PCC 7120, the *hupSL* operon in vegetative cells of *A. variabilis* is not interrupted by a 10.5-kb element. In *Anabaena* sp. strain PCC 7120, this DNA fragment is excised during heterocyst differentiation. Southern hybridizations with different DNA probes support the sequence data of a contiguous *hupSL* operon in *A. variabilis*.

The three ORFs upstream of the *hupSL* genes (Fig. 1) encode products that showed no similarity to any known protein. They probably do not belong to the *hupSL* operon because they are transcribed in the opposite direction. Also, no homologies were found for ORF4, ORF5, and ORF6. Interestingly, one (ORF7, 159 amino acids encoded) showed about 44% amino acid identity to a regulator protein of *Enterococcus faecalis*. One ORF (453 amino acids) 2 kb downstream of the *hupL* gene, showed homology to a UDP-glucose dehydrogenase of *Synechocystis* sp. strain PCC 6803 (46% identity) and *Sinorhizobium meliloti* (43% identity). In *S. meliloti*, the dehydrogenase catalyzes the reaction from UDP-glucose to UDP-glucuronic acid and is involved in the synthesis of capsular polysaccharides. The partial ORF at the 3′ end of the *Xba*I clone demonstrated homology to the C-terminal end of a UDP-glucose dehydratase from *Synechocystis* sp. strain 6803 (48% identity).

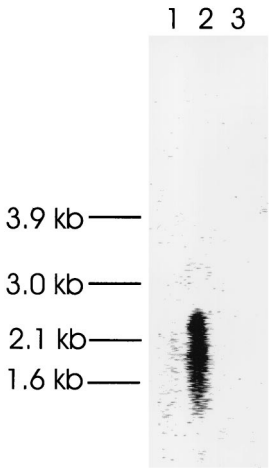

FIG. 2. RNA gel blot analysis of the *hupSL* transcript. Hybridization of the *hupS* gene containing a 950-bp *Hin*cII fragment to total RNA (10 μg each) isolated from cells grown with NO_3^- (1), NH_4^+ (3) in the medium and under nitrogen-fixing conditions (2). The locations of the RNA size markers are shown.

Transcriptional analysis. Northern blot analysis was performed to examine the expression of the *hupSL* operon in N_2-fixing and non-N_2-fixing cultures. The Northern blot was hybridized with a digoxigenin-labeled probe (pKS14) from within the *hupS* gene. The transcript was only detected in RNA isolated from N_2-fixing cultures (Fig. 2). Using an RNA marker, the size of the *hupSL* operon was determined to be 2.7 kb. Transcripts were completely missing in cells grown in medium supplemented with either ammonia or nitrate. The results of the Northern analysis were in good agreement with the results made by RT-PCR using total RNA from BG11 and $BG11_0$ cultures (data not shown). These results indicate that the *A. variabilis hupSL* operon is either nitrogen regulated or induced by hydrogen. In the cyanobacterium *Nostoc* sp. strain PCC 73102, the *hupL* transcript is also induced during heterocyst differentiation (5).

The 5′ end of the *hupSL* mRNA was determined by means of the primer extension technique with a fluorescence-labeled oligonucleotide primer and an automated DNA sequencer. Performing the extension reaction with RNA isolated under N_2-limiting conditions, a major extension product was detected. By comparing the retention time of this product with the signal in the sequence chromatogram (Fig. 3), the first transcribed nucleotide, a thymidine, was located 103 nucleotides upstream of the translational start codon ATG of the *hupS* gene (Fig. 4). No primer extension product was found with RNA isolated from BG11 cultures (Fig. 3). This provides further evidence that the *hupSL* operon is expressed only under N_2-fixing conditions. Fifteen base pairs upstream of the transcription initiation, a sequence that resembles a −10 consensus region (TAAACT) of the *hup* operon in *E. coli* (26) was found. Half of a sequence motif identical to the consensus Fnr-binding sequence 144 bp upstream of the transcription start site (Fig. 4) was also found. In addition, a fivefold direct repeat of the sequence TA/GACAAC upstream of the ATG start codon was obvious to identify.

Construction of a *hupSL* mutant and physiological characterization. To investigate the biological properties of the *hupSL* genes, a mutant strain (AVM13) in which the complete *hupS* gene and the 5′ end of the *hupL* gene were deleted was constructed. A 2.15-kb *Xba*I fragment from within the plasmid pKS1 (4.8-kb *Hin*dIII fragment) was replaced by the selectable marker gene *npt* (Fig. 1).

Total DNA from two homocygote recombinant clones (13_1 and 13_2) was prepared, and marker rescue and complete segregation was confirmed by Southern hybridization using a 715-bp fragment from within the *hupL* gene as a probe (pKS13) (Fig. 1 and 5). The probe hybridized with wild-type DNA at approximately 4.8 kb, corresponding to the original

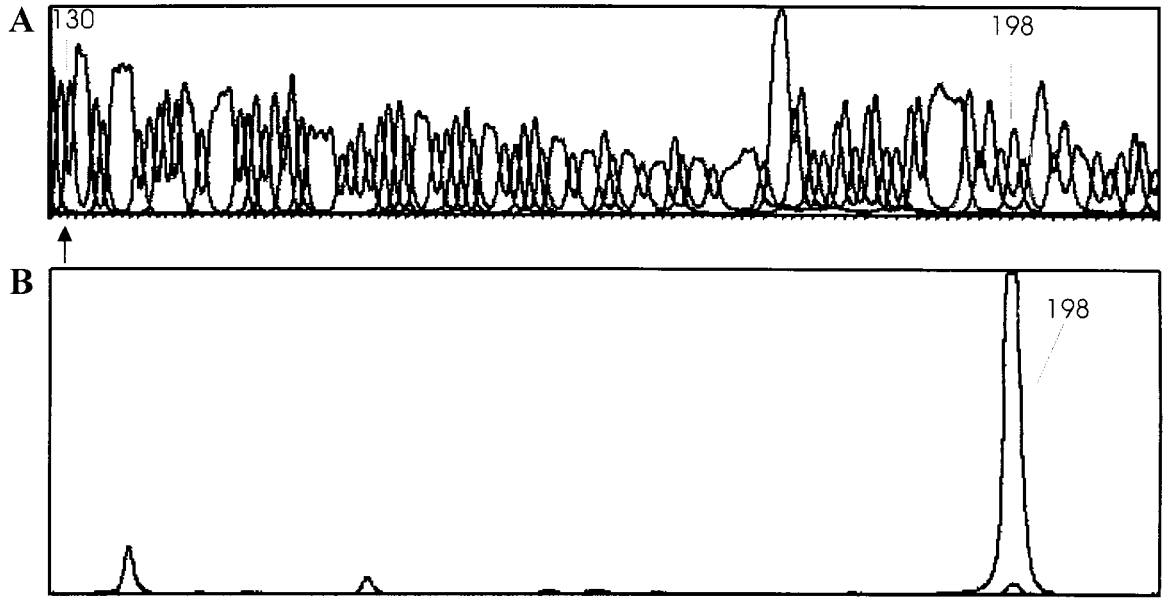

FIG. 3. Localization of the transcription start point of the *hupSL* genes. (A) Nucleotide sequence of the pKS1 clone containing the promoter region of the *hupSL* operon. Arrow indicates the start codon in the genome sequence. (B) Primer extension products obtained with RNA isolated from BG11 and $BG11_0$ cultures as the template and the oligonucleotide HupS7 as the primer. The retention times of the start codon and of the major extension product are shown. All signals result from the $BG11_0$ RNA except for the smaller peak at retention time 198.

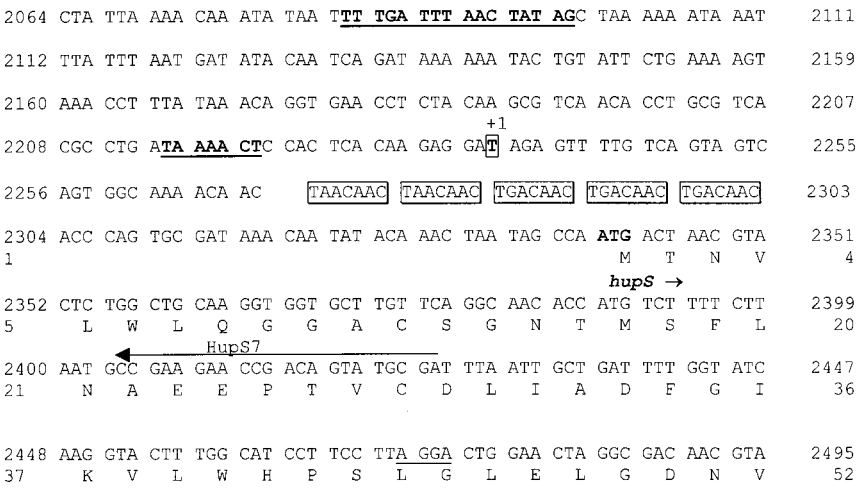

FIG. 4. Nucleotide sequence of the *hupSL* promoter region. The start codon of *hupS* is in bold; the N-terminal end of the amino acid sequence is written under the nucleotide sequence. Shine-Dalgarno homologies are underlined. The transcription start point is given by +1. The repetitive elements in the promoter sequence are boxed. The primer HupS7, used for primer extension, is also shown. The putative −10 consensus sequence and the Fnr-binding site are underlined and in bold.

*Hin*dIII fragment. On the other hand, there is only one signal at 3.8 kb in the lane containing genomic DNA of the mutant 13_1. This result indicates that the genome of this clone is completely segregated while the genome of 13_2 is not because two signals at 4.8 and 3.8 kb occurred on the blot. The mutant 13_1 was designated AVM13 and was chosen for further investigations.

The physiological effects of the mutation in strain AVM13 were investigated by comparing the diazotrophic growth under aerobic conditions and the hydrogen metabolism (the in vivo nitrogenase and hydrogenase activity) of the mutant and the wild-type strains. Because the uptake hydrogenase is only active in heterocysts during nitrogen fixation, the deletion of the *hup* genes should have effects on a culture grown in medium without combined nitrogen. The *A. variabilis* wild type and mutant strain AVM13 were grown in media with combined nitrogen (BG11) or without combined N_2 ($BG11_0$). Following the growth, the chlorophyll α content of the cultures was measured. In BG11, AVM13 cells have growth rates similar to that of the wild type (data not shown). During the first 80 h, the diazotrophic growth of both strains was nearly the same; however, after 80 h and until the end of the measurements, the mutant strain grew worse than did the wild type (Fig. 6A). Since the deletion of the *hupSL* genes in mutant AVM13 cells affected the growth under N_2-fixing conditions, the effect of the mutation on the nitrogenase, the key enzyme of nitrogen fixation which is also involved in hydrogen metabolism, was examined. The maximum of the nitrogenase activity could be determined about 24 h after induction of the cells. Both cultures had nearly the same nitrogenase activity after 24 h, but in the following days, much higher values of acetylene reduction for the wild type were measured (Fig. 6B).

Concerning the H_2 evolution, this paper demonstrates that the mutant AVM13 and the wild type produced equal rates of hydrogen in the first 16 h after induction with $BG11_0$ medium. At this time, the *hupSL* genes encoding the uptake hydrogenase were not expressed (data not shown). Contrastingly, after the expression of the *hupSL* operon, the H_2 evolution of the mutant strain AVM13 increased dramatically in the next 20 h, while for the wild type no change in the rates of H_2 evolution could be seen (Fig. 6C). Similar results were obtained for another mutant, in which the neomycin resistance cassette was cloned in the opposite direction to the *hupSL* operon. Under nitrogen-fixing conditions, the mutant developed very high rates of H_2 (68 nmol H_2/μg Chl α × h) because the produced hydrogen could not be oxidized. The wild-type cells, however, consumed even more hydrogen than the nitrogenase produced (−36 nanomoles of H_2/micrograms of chlorophyll α × h). The activity of the bidirectional NAD^+-hydrogenase assayed under non-N_2-fixing conditions was very low for both the wild type and the mutant.

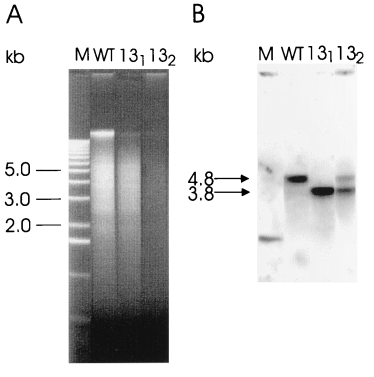

FIG. 5. Southern analysis of the *hupSL* operon. (A) Total DNA (5 μg) from *A. variabilis* and the two mutant strains 13_1 and 13_2 was restricted with *Hin*dIII. (B) pKS13 containing part of the *hupL* gene was used as a probe. The arrows indicate the hybridization signals of the wild type (4.8 kb) and the mutants (3.8 kb).

DISCUSSION

The similarity of the uptake hydrogenase from *Anabaena* sp. strain PCC 7120 and *A. variabilis* allowed for the isolation of a 4.8-kb *Hin*dIII fragment and of a 4.8-kb *Xba*I fragment of *A. variabilis* via hybridization of partial genomic libraries using the *Anabaena* sp. strain PCC 7120 *hupL* gene as a probe. The high degree of similarity between the *hupSL* operon of *A. variabilis* and other filamentous cyanobacteria (*Anabaena* sp. strain PCC 7120, 95%; *Nostoc* sp. strain PCC 73102, 90%) may reflect more stringent requirements for the conservation of amino acids in the uptake hydrogenase. The noncoding region

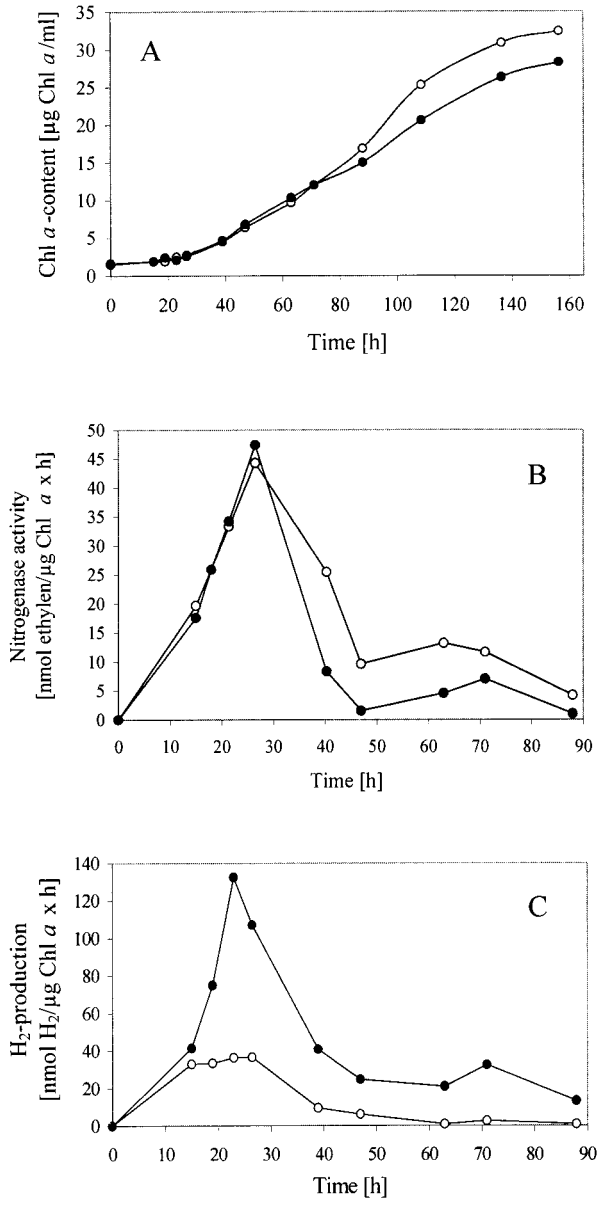

FIG. 6. Bacterial growth (A), nitrogenase activity (B), and hydrogen production (C) of the *A. variabilis* wild type and AVM13 mutant strain under nitrogen-fixing conditions. After growing in BG11 medium containing combined nitrogen, the cells of the wild type and the mutant were washed with N-free medium $BG11_0$ and adjusted to the same cell density (measured by the chlorophyll α content). Open circles, *A. variabilis* wild type; closed circles, AVM13 (*A. variabilis hupSL* interposon mutant).

between *hupS* and *hupL*, however, shows only slight similarities between *A. variabilis* and *Anabaena* sp. strain PCC 7120 and no similarities between *A. variabilis* and *Nostoc* sp. strain PCC 73102. This may hint not only at the physiological importance of the uptake hydrogenase but also at the phylogenetic divergence during evolution in filamentous cyanobacteria.

Protein sequence alignment of the HupS and HupL subunits was done with several Ni-Fe hydrogenases from different bacterial groups by using the ClustalW program (48; data not shown). The large subunit of Ni-Fe hydrogenases contains the Ni atom in the active site. As in the other organisms, one putative Ni-binding site (R-X-C-G-X-C) is located at the N-terminal site of the HupL protein in *A. variabilis*. The second conserved Ni-binding site (D-P-C-X-X-C) is found at the C-terminal end. The small subunit of *A. variabilis* contains 11 Cys residues. Nine of them correspond well to nine Cys residues of the known X-ray structure of the hydrogenase from *Desulfovibrio gigas* (52). HupS of *D. gigas* has two [4Fe-4S] clusters and one [3Fe-3S] cluster as prosthetic groups. The typical features of the small subunit of the dimeric Ni-Fe hydrogenase are the presence of a signal peptide at the N terminus and a motif located at the C terminus for anchoring the uptake hydrogenase to the membrane (51). Both features could not be found for the HupS protein of *A. variabilis* and of *Nostoc* sp. strain PCC 73102 (31). As Oxelfelt et al. pointed out, the role of the motifs is not yet clear and some examples of uptake hydrogenases which lack these features exist.

The cloning and sequencing of hydrogenase genes cluster led to the discovery of a number of accessory genes just up- and downstream of the two structural genes (51). In *Alcaligenes eutrophus*, up to 20 ORFs encoding proteins that are essential for the formation of the active hydrogenase have been characterized (20). Therefore, we sequenced 3 kb upstream and 4 kb downstream of the *hupSL* operon. Eight ORFs were discovered, but none of them showed similarities to other known accessory genes. Two kilobases downstream of the *hupL* gene, two ORFs which might encode for proteins of glucose metabolism could be detected. This means that the accessory genes of the uptake hydrogenase must be located elsewhere in the bacterial genome.

During late stages of heterocyst differentiation, three DNA rearrangements were found in *Anabaena* sp. strain PCC 7120 (10, 24). In vegetative cells, the genes *nifD*, *fdxN*, and *hupL* are interrupted by insertions of DNA elements. After excision by site-specific recombinases, the intact transcripts can be expressed in heterocysts. The *nif1* region of *A. variabilis*, however, possesses only the 11-kb element in the *nifD* gene and no interruption in the *fdxN* gene. The data in this study revealed the presence of a contiguous *hupSL* operon in *A. variabilis*. These results are supported by the fact that the specific recombinase *xisC* of the *hupL* rearrangement of *Anabaena* sp. strain PCC 7120 could not be detected in Southern blot analysis with genomic DNA of *A. variabilis* (5). Beside the *nif1* region, *A. variabilis* has an alternative Mo-dependent nitrogenase gene cluster (*nif2*) which is transcribed only under anaerobic conditions (46). Low-stringency Southern hybridization indicated that the *hupL* gene is a single-copy gene and that no similar ORFs for *hupSL* genes exist. Since the *hupSL* transcript is induced only under nitrogen-fixing conditions, *A. variabilis* has to regulate the uptake hydrogenase in a different way. In Northern analysis, we could show that the *hupSL* genes are probably transcribed as a dicistronic operon of a 2.7-kb size. In lithoautotrophic bacteria, most of the known genes for the uptake hydrogenase are clustered in a polycistronic operon, often with a third gene encoding HupC (51). The *hupSL* transcript could only be detected in cells grown 24 h on an N-free medium. The *nifDHK* genes of *A. variabilis* are induced earlier (12 h) during heterocyst differentiation (data not shown). This observation suggests that the *hupSL* genes are transcribed during the late stages of heterocyst differentiation when the nitrogenase is already active. It has been reported that the levels of uptake hydrogenase activity in *Anabaena* sp. strain PCC 7120 are up to five times higher in cultures grown under H_2-N_2-CO_2 conditions (25), so the endogenous H_2 produced by the nitrogenase may act as an inducer of the hydrogenase synthesis.

In cyanobacteria, promoter DNA elements that displayed a conserved element at −10 from the transcription initiation site could be found (15), which conforms to the *E. coli* −10 pro-

FIG. 7. Nucleotide sequences of the consensus Fnr-binding site, the Fnr-binding site within the *napF* promoter of *E. coli*, and the promoter of the *hupSL* operon of *A. variabilis*. Three nucleotides out of 10 are different between the consensus sequence of the Fnr-binding site in *E. coli*, the *napF* (periplasmic nitrate reductase) promoter, and the *hupSL* promoter of *A. variabilis*.

moter consensus sequence (TATAAT). The controlling promoters of genes encoding chimeric hydrogenases of hydrogen-oxidizing bacteria have also been characterized (8, 35, 50). In most of the cases, sequence elements resembling a −24/−12 consensus sequence of σ^{54}-dependent promoters are located just upstream of the transcription start (39). Neither a TAT AAT motif nor a −24/−12 consensus sequence could be detected upstream of the *hupSL* operon of *A. variabilis*. Interestingly, the location of a fumarate nitrate reductase regulator (Fnr)-binding motif and a −10 consensus motif, which was found during the regulation of the *hyp* operon in *E. coli* (26), was confirmed. Comparing the putative Fnr-binding site of the upstream region of the *hupSL* operon with the promoter of the *hypBCDE* transcript, the same succession of bases could be demonstrated; only the distance of the motifs to the transcription site is different.

DNase footprinting experiments and in vitro transcription confirm the unusual localization of the Fnr-binding site in the *napF* control region in *E. coli* (16). As shown in Fig. 7, four bases of the *napF* promoter sequence are modified from the Fnr consensus sequence, but only one base is exchanged in the promoter region of *hupSL* as compared with the *napF* promoter. During anaerobic growth, the Fnr protein induces the expression of several operons in *E. coli* (43). In *A. variabilis*, the induction of the *hupSL* operon occurs within the heterocysts. Heterocysts are terminally differentiated cells whose interiors become anaerobic. This suggests a similar regulation of the *hupSL* operon compared with that of *E. coli*.

The fivefold repeats (TA/GACAAC) downstream of the transcription site are another interesting structure which represent a new type of short tandemly repeated heptamers. Cyanobacterial genomes have a variety of such tandemly repeated sequences with unknown function, but most of them are specific to the heterocystous strains (24). It has been reported that these sequences might be a target of specific DNA-binding proteins for chromosome condensation (27). Further promoter analyses are needed to understand the transcription of the *hupSL* operon.

We also studied the induction of in vivo hydrogen uptake and nitrogenase activities under N-limiting conditions in the wild type and the *hupSL* mutant of *A. variabilis*. There are two hints that, in the light, the measured hydrogen originates from the nitrogenase activity. Firstly, hydrogen photoproduction of *A. variabilis* cultures did not occur under NO_3^--saturated conditions in which the nitrogenase genes are not expressed. In BG11 medium, a slight H_2 production was measured only in the dark, which indicates a low catalytic activity of the bidirectional hydrogenase. Secondly, the curve diagram of the H_2 evolution corresponds well with the one of acetylene reduction. After 24 h of growth under nitrogen-fixing conditions, the maximal H_2 production and nitrogenase activity could be observed. Similar effects have been described earlier (28, 49). Though a lot of biochemical and genetical studies have been done, the in vivo function of the uptake hydrogenase is poorly understood at present. In this study, the physiological data clearly show that the uptake hydrogenase reoxidizes the produced hydrogen at high rates in the wild type. Three to five times more hydrogen is produced by the *hupSL* mutant, dependent on the growth situations. It was shown that the electrons of the oxidized H_2 are fed into the respiratory chain, proceeding to an oxyhydrogen reaction coupled to oxidative phosphorylation (33). The electron transport from H_2 to O_2 supports ATP synthesis and thereby supplies part of the energy required by the nitrogenase and also protects the nitrogenase by lowering intracellular O_2 levels. The *hupSL* mutant fixed nitrogen after 35 h at lower rates than the wild type. However, the growth curves were almost the same, suggesting that the uptake hydrogenase is not absolutely essential under diazotrophic culture conditions. Obviously, the reduced rates of fixed nitrogen have only a slight effect on the growth of the mutant strain.

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