Cloning, molecular analysis and insertional mutagenesis of the bidirectional hydrogenase genes from the cyanobacterium *Anacystis nidulans*

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Abstract **Among cyanobacteria, the heterocystous, N_2-fixing *Anabaena variabilis* and the unicellular *Anacystis nidulans* have recently been shown to possess an NAD^+-dependent, bidirectional hydrogenase. A 5.0 kb DNA segment of the *A. nidulans* genome is now identified to harbor the structural genes *hoxUYH* coding for three subunits of the bidirectional hydrogenase. The gene arrangement in *A. nidulans* and in *A. variabilis* is remarkably dissimilar. In *A. nidulans*, but not in *A. variabilis*, the four accessory genes *hoxW*, *hypA*, *hypB* and *hypF* could be identified downstream of *hoxH*. An insertional homozygous mutant in *hoxH* from *A. nidulans* was completely inactive in performing $Na_2S_2O_4$-dependent H_2 evolution but could utilize the gas with almost 50% of the activity of the wild type. These findings with the first defined hydrogenase mutant in any photosynthetic, O_2-evolving microorganism indicate that the unicellular cyanobacterium *A. nidulans* possesses both an uptake and a bidirectional hydrogenase. The physiological role(s) of the two hydrogenases in unicellular non-N_2-fixing cyanobacteria is not yet understood.**

Key words: Hydrogenase; Hydrogen metabolism; Cyanobacterial mutant; Nitrogen fixation; Cyanobacteria; *Anacystis nidulans*

1. Introduction

At least two different hydrogenases catalyzing the reaction $2\ H^+ + 2\ e^- = H_2$ have been demonstrated for the heterocystous, N_2-fixing cyanobacteria *A. variabilis* and *Anabaena* sp. PCC 7120 [1,2]. One of these enzymes, the so-called bidirectional or reversible hydrogenase, catalyzes both the $Na_2S_2O_4$- and methyl viologen-dependent H_2 evolution and the uptake of the gas with phenazine methosulfate (PMS) or methylene blue as electron acceptor. It occurs in both heterocysts and vegetative cells of *Anabaena* sp. [3,4] as well as in the unicellular *A. nidulans* [5]. Immunogold labeling experiments [5] as well as results with spheroplasts [6] indicated its location at the cytoplasmic membrane of *A. nidulans*. The gene set coding for the bidirectional hydrogenase from *A. variabilis* has been cloned and sequenced and surprisingly found to be related to the genes coding for an NAD^+-dependent hydrogenase from *Alcaligenes eutrophus* [7]. Such findings have recently been corroborated by the demonstration of an NADH-dependent H_2 evolution and an H_2-dependent NAD^+ reduction in extracts from *A. nidulans* [8] and by EPR data with the purified enzyme from *A. variabilis* [9].

The second enzyme is called uptake hydrogenase as it catalyzes only the uptake of the gas with PMS or methylene blue as electron acceptor. It is believed to be a component of the thylakoid membrane [10] only of heterocysts but not of vegetative cells in *Anabaena* sp. [11]. Its occurrence outside heterocystous, non-N_2-fixing cyanobacteria remained to be elucidated. Years ago, Peschek [12] put forward circumstantial physiological evidence that the unicellular *A. nidulans* possesses two hydrogenases.

The gene set coding for the bidirectional, NAD^+-dependent hydrogenase of *A. nidulans* will be described in the present communication. The data indicate that the gene arrangements in the unicellular *A. nidulans* and the heterocystous *A. variabilis* are largely different. A defined mutant unable to express this bidirectional hydrogenase has now been obtained by insertional mutagenesis. Enzyme determinations with this mutant indicate that an uptake hydrogenase is present in *A. nidulans*, thus in non-N_2-fixing cyanobacterial cells.

2. Materials and methods

2.1. Culture, growth, preparation of extracts and activity measurements

All steps were performed as in the preceding publication [8]. *Anacystis nidulans* (= *Synechococcus leopoliensis* = *Synechococcus* sp. PCC 6301) was purchased from the Algensammlung des Pflanzenphysiologischen Instituts der Universität Göttingen, Germany (SAUG 1402-1). The wild type and the mutant were grown in BG-11 medium with nitrate under continuous gassing with a mixture of air/CO_2 = 95/5 (by volume), with continuous illumination (approximately 5000 lux at the surface of the growth tubes) and at a temperature of 30°C. Cells (200–400 ml) were centrifuged ($4000 \times g$, 10 min, 15°C), and the pellet was suspended in 20 ml Tris-HCl buffer (pH 7.5) and broken twice in a French press at 137 000 kPa. The extract was centrifuged ($48\,000 \times g$, 20 min, 15°C), and the supernatant was assayed for activities. $Na_2S_2O_4$- and methyl viologen-dependent H_2 formation tests were performed in 7.0 ml Fernbach flasks, and the amount of the gas evolved was determined by gas chromatography as described previously [8]. Similarly, the determination of the phenazine methosulfate-dependent uptake of the gas by the H_2 electrode has been described in the preceding publication [8]. All manipulations with the extracts were performed under argon to ensure anaerobiosis as far as possible.

2.2. Construction and isolation of the insertional mutant

The *hoxH* gene coding for the large subunit of the hydrogenase dimer [7] was inactivated by inserting the cassette with the kanamycin (Km) resistance conferring gene from pUC4K [13] into the *Bam*HI site of a 5 kb *Eco*RI/*Hin*dIII segment cloned into pUC18, encoding part of the *hox* and *hyp* cluster (see Fig. 1). Orientation of the cassette in the construct was determined by restriction analysis and hybridization. The protocol for the transformation of *A. nidulans* was adopted from [14]. Cells of a 4 day old culture were harvested by centrifugation ($5000 \times g$, 15 min), resuspended in 1 ml Bg-11 medium, and transformed with the construct (termed pKmB16) containing Km resistance, the 5 kb segment with the inactivated *hoxH* and the other

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genes listed in Fig. 1 either by natural transformation or by electroporation. Natural transformation was performed with $3–6 \times 10^8$ cells/ml, which were incubated in the light for 2 h with 2.7 μg/ml DNA. In the case of electrotransformation, the mixture of cells ($1.5–3 \times 10^9$/ml) and DNA (18 μg/ml) was treated with 12.5 kV/cm for 5 ms. After natural transformation or electroporation, the cells were transferred to BG-11–agar plates and incubated at 30°C in the light. Transformants were selected after 14 days on a Km gradient, obtained by adding 1.75 mg Km in 0.5 ml to one point of the plate after 2 days. The recombinants segregated during 10 passages on BG-11–agar plates by increasing the Km concentrations from 10 to 50 μg/ml. Segregation and identity of the mutants were checked by restriction and by Southern blot analysis of genomic DNA with the *hoxH* probe of *Anabaena* sp. PCC 7119 (see Fig. 2) as well as the probe for the Km resistance gene.

2.3. Others

Hybridizations, labeling and sequencing followed standard protocols described in detail previously [7]. The genomic DNA bank of *A. nidulans* in λ-GEM11 was constructed as described for the *A. variabilis* bank [7]. Protein was determined by the Lowry method, and all chemicals came from Boehringer, Mannheim.

The DNA sequences of the hydrogenase *hoxU, Y* and *H* genes and of the accessory genes *hoxW, hypA* and *hypB* have been deposited to the EMBL/GenBank/DDBJ databases (accession no. X97797).

3. Results

3.1. Characterization of the structural genes of the bidirectional hydrogenase

As described in a preceding publication, an 1.35 kb DNA segment from several cyanobacteria could be amplified by PCR with specific primers, and sequencing of this segment indicated its strong identity to part of the large subunit (*hoxH* gene) of an NAD^+-reducing hydrogenase [7]. The digoxigenin-labeled DNA segment from *Anabaena* sp. PCC 7119 served as probe to screen for positive clones in a λ-gene bank of *A. nidulans*. Two different hybridizing clones strongly overlapped from which a 5 kb *Eco*RI/*Hin*dIII segment and the adjacent 1.4 kb *Hin*dIII/*Sst*I segment were subcloned into Bluescript, pUC18 and M13mp18/M13mp19. A 5.0 kb fragment could also be identified in *Eco*RI/*Hin*dIII digests of genomic DNA from *A. nidulans* with the 1.35 kb probe in Southern blotting experiments. Further restriction analysis and hybridization with this probe verified the restriction pattern of the analyzed segment on the *A. nidulans* chromosome. Southern analysis indicated that one *hoxH* copy only was observed in the *A. nidulans* genome under the high stringency employed.

The 5 kb DNA segment was completely sequenced on both strands. The average GC content of 57% was typical for *A. nidulans*. The segment contained the complete *hoxH* gene with a sequence identity of 64% (on an amino acid basis) to the corresponding gene product from *A. variabilis*.

In the previous investigation [7], the bidirectional hydrogenase from *A. nidulans* had been purified and digests had been sequenced. The determined amino acid sequence of a digest of the large subunit (*hoxH* gene product) was identical with that deduced from the DNA sequence in the case of *A. nidulans* and was strongly homologous to that deduced from the *A. variabilis* DNA composition, as shown in the table below.

(K) (I) (S) V F L D D Q G N A E	amino acid sequence of the *A. nidulans* protein[1]
K I S V F L D D Q G N A E	deduced amino acid sequence from *A. nidulans*
K I S I Y L D D T G Q V S	deduced amino acid sequence from *A. variabilis*

In addition to *hoxH*, the 5.0 kb segment contained upstream of *hoxH* the hydrogenase genes *hoxY* (coding for the smaller subunit of the hydrogenase dimer, sequence identity to the *A. variabilis* gene product 61% on an amino acid basis) and *hoxU* (coding for the smaller subunit of the diaphorase part, sequence identity to *A. variabilis* = 63%). However, *hoxF* (coding for the larger subunit of the diaphorase part) could not be detected either as a direct sequence on the 5 kb segment or by heterologous probing with the *A. variabilis* gene on a 13 kb λ-clone containing the 5 kb segment. The *hoxF* gene could, however, be identified on genomic DNA cleaved with several restriction enzymes, indicating that *hoxF* is not located directly upstream of *hoxU*. In this region, separated from *hoxU* by some 160 bp, part of an ORF is located on the opposite strand, possibly encoding a NifS-like protein. In the case of *A. variabilis*, two open reading frames are located within the gene cluster (ORF3 between *hoxY* and *hoxH* and ORF8 between *hoxU* and *hoxY*; see Fig. 1 and [7]). ORF3 could not be detected on genomic DNA from *A. nidulans* by heterologous probing even at the low hybridization temperature of 56°C. In addition, sequences homologous to the *A. variabilis* ORF3 and ORF8 (the latter downstream of *hoxU*) were not present on the 5 kb segment of *A. nidulans*.

The deduced amino acid sequences of the *hoxU, hoxY* and *hoxH* genes all have the same sequence characteristics described for the homologous genes from *A. variabilis* [7], in particular the conserved cysteine motifs for binding potential Fe-S clusters and for the Ni-binding sites. The HoxU protein (the small subunit of the diaphorase part of the enzyme) possesses 238 amino acids with 16 cysteines (for comparison 238 amino acids with 15 Cys in HoxU of *A. variabilis* [7]). A putative [4Fe4S] cluster liganded by the motif $Cx_{33}Cx_2Cx_2C$ unusually present in this protein from *A. nidulans* (this study) and *A. variabilis* [7] has been discussed to serve as a link in distributing the electrons from H_2 to either NAD^+ or respiration [15].

The *hoxY* gene product represents the smaller subunit of the hydrogenase dimer and consists of 184 amino acids with 6 cysteines (181 amino acids and 5 cysteines in the case of *A. variabilis*). The reading frames between *hoxU* and *hoxY* overlap in *A. nidulans* and the tandem repeats in the intergenic region present between the two genes of *A. variabilis* [7] are missing in the case of *A. nidulans*. The gene seems to start with an ATG codon, since the Val triplet GTG and the stretch observed in the *A. variabilis* gene are absent in *hoxY* of *A. nidulans*. The protein contains only one Fe-S cluster-binding site and a leader sequence at the N-terminus is apparently absent as in the protein from *A. variabilis* and *A. eutrophus*.

HoxH (large subunit of the hydrogenase dimer, 476 amino acids, 10 cysteines, in *A. variabilis* 487 amino acids and 8

[1] Sequence of part of the HoxH subunit=47 kDa band in SDS gels of purified bidirectional hydrogenase [7].

Table 1
The bidirectional hydrogenase of *A. nidulans*: comparison of the products of the structural genes *hoxU*, *hoxY* and *hoxH* and of the accessory genes *hoxW*, *hypA*, and *hypB* with homologous proteins involved in H_2 metabolism of other bacteria

Organism	Gene product	Identical amino acids to the *A. nidulans* gene products					
		HoxU	HoxY	HoxH	HoxW	HypA	HypB
A. variabilis	HoxU	63					
	HoxY		61		?	?	?
	HoxH			64			
A. eutrophus	HoxU	34					
	HoxY		35				
	HoxH			43			
	HoxM				26		
	HoxW				23		
	$HypA_1$					30	
	$HypB_1$						47
A. vinelandii	HoxK	–	23				
	HoxG			26			
	HoxM				21		
	HypA					34	
	HypB						48
R. capsulatus	HupS	–	20				
	HupL			27			
	HupD				24		
	HypA					32	
	HypB						46
B. japonicum	HupS	–	19				
	HupL			26			
	HupD				27		
	HypA					27	
	HypB						43
E. coli	HyaA	–	20				
	HyaB			24			
	HybA		16				
	HybC			27			
	HycG		20				
	HycE			18			
	HyaD				28		
	HybD				23		
	HycI				22		
	HypA					29	
	HypB						43
M. mazei	VhtG	–	23				
	VhtA			28			
	VhtD				25	?	?

The data represent the percentage of identical amino acids between the two proteins referred to the *Anacystis* protein length. Data refer to the following accession numbers of EMBL/GenBank/DDBJ database or SWISS PROT (marked by asteriks) database: *A. variabilis* HoxFUYH (X79285), *Alcaligenes eutrophus* HoxUYH, HoxM, HoxW, $HypA_1B_1$ (P22318*/P22319*/P22320*, P31909*, X92988+M55230, X70183), *A. vinelandii* HoxKGM, HypAB (P21950*/P21949*/M80522, X63650), *R. capsulatus* HupSLD, HypAB (P15283*/P15284*/Q03004*, X61007), *B. japonicum* HupSLD, HypAB (P12635*/P12636*/S39401, L24513), *E. coli* HyaAB, HybAC, HycGE, HyaD, HybD, HycI, HypAB (M34825/P19927*, U09177, P16433*/P16431*, P19930*, P37182*, X17506, U29579/P24190*), *M. mazei* VhtGAD (X83112/X91851).

cysteines) possesses characteristic features similar to *A. variabilis* HoxH [7]. The gene seems to start with an ATG codon located 10 bp after the stop codon of *hoxY*. Near the carboxy terminus, HoxH possesses a strongly conserved motif ending with a histidine. The formation of the mature protein and the incorporation of Ni into it might require the cleavage of a stretch of 26 amino acids behind this His similar as in the proteins from *Methanococcus voltae* [16], *E. coli* (hydrogenase 3) [17], and *A. eutrophus* [18]. An N-terminal leader sequence is also not observed in HoxH of *A. nidulans*.

A combined physical and genetic map of the cyanobacterium *Synechocystis* sp. PCC 6803 has recently been constructed by contigue mapping using pulse field gel electrophoresis for separating restricted fragments of genomic DNA and a cosmid library [19]. Screening of this library, supplied by Dr. V. Shestopalov (Moscow), with the 1.35 kb *hoxH* probe from *Anabaena* sp. PCC 7119 revealed several positive clones, some of which also containing the genes coding for the β and ε subunits of F_0F_1 ATPsynthase (*atpBE*), others carrying genes encoding the PSI subunits PsaFJ. The distance between the *atpBE* and *psaFJ* gene markers is approximately 30–40 kb (V. Shestopalov, personal communication). These data made it possible to localize *hoxH* on the MluA fragment at position 3200 kb of the *Synechocystis* chromosome [19]. Referring to the mapping of *atpBE* and *psaFJ* reported by [20], *hoxH* must be located in the vicinity of *ndhL* at position 48–48.5′ of the

chromosome. Specific probes for *hoxU, Y* and *H* resulted in a hybridization signal at 5.6 kb with genomic DNA from *Synechocystis* restricted with *Nco*I/*Hin*dIII, indicating that these three structural genes coding for the bidirectional hydrogenase belong to the same gene cluster also in *Synechocystis*. In this cyanobacterium the hydrogenase accessory gene *hypF* is located at position 69′ of the chromosome with no other structural or accessory genes in the vicinity [21], thus indicating different organizations of the hydrogenase gene cluster in *Synechocystis* and *Anacystis nidulans* (see below).

3.2. Characterization of accessory genes

In *A. variabilis*, transcription terminates immediately downstream of *hoxH*, since two open reading frames with no apparent hydrogenase function (ORF5, ORF6) are located on the opposite strand [7]. The DNA sequence from *A. nidulans* does not show any similarity to these ORFs in that region. In contrast, as in *A. eutrophus* [22], sequences for the accessory genes *hoxW, hypA, hypB* and *hypF* begin immediately downstream of *hoxH* on the same strand (Fig. 1, Table 1). A sequence for a protein with no obvious function (ORF2) resides between *hoxW* and *hypA* in *A. eutrophus* [18]. Such a protein is not encoded in the same region on the *A. nidulans* genome.

HoxW (152 amino acids) might start with an ATG codon 9 bp after the stop codon for *hoxH*. No termination signal for transcription is apparent between the two genes. HoxW might be a protease involved in the maturation of HoxH by releasing a stretch of 26 amino acids at the carboxy terminus behind the histidine just mentioned as in *E. coli* or *A. eutrophus*. The amino acid identity is only 20–25% with the hydrogenase proteases from other organisms (Table 1). Such a low value is not surprising, since these proteases appear to cleave stretches

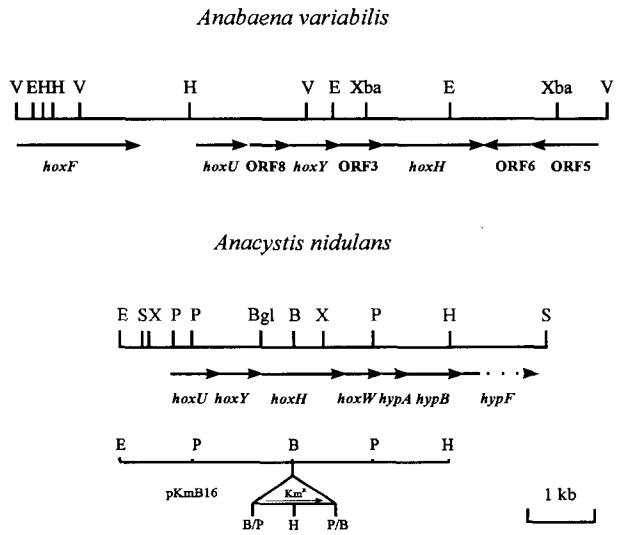

Fig. 1. The arrangement of genes coding for the bidirectional hydrogenase in *A. variabilis* and *A. nidulans* in comparison. The arrangement of the genes is shown by arrows under the restriction map. *HypF* has only been partially sequenced as yet (indicated by the straight line, dotted line represents the expected size, as in *A. eutrophus*). The construct pKmB16 (consisting of the 5 kb *Eco*RI/*Hin*dIII segment and the Km resistance conferring gene from pUC4K ligated into the *Bam*HI site in the middle of *hox*H) is indicated below the map for *A. nidulans*. Restriction enzymes used: E = *Eco*RI, X = *Xho*I, S = *Sst*I, P = *Pst*I, Bgl = *Bgl*II, B = *Bam*HI, H = *Hin*dIII, Xba = *Xba*I.

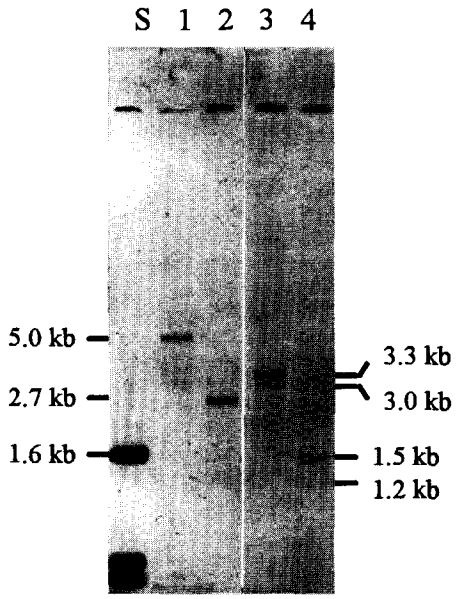

Fig. 2. Demonstration of the insertion of the Km resistance cassette into the hydrogenase gene *hoxH* and of the complete segregation in the mutant obtained. Genomic DNA of *A. nidulans* and of the mutant B16 was restricted with *Hin*dIII/*Eco*RI or *Pst*I. Hybridization with the 1.35 kb *hoxH* probe from *Anabaena* sp. PCC 7119 at 68°C resulted in two bands of the predicted size in each case (see text). lane S: 1 kb ladder, the 1.6 kb hybridizing band is marked; lane 1: DNA of the wild type restricted with *Hin*dIII/*Eco*RI; lane 2: DNA of the wild type restricted with *Pst*I; lane 3: DNA of the mutant restricted with *Hin*dIII/*Eco*RI; lane 4: DNA of the mutant restricted with *Pst*I.

specifically from distinct hydrogenases. The motif $GxGNx_4DD/EGxG$, common in proteases cleaving the hydrogenase large subunit [18], occurs in HoxW of *A. nidulans* also.

HypA (112 amino acids) may start with a Met encoded by GTG, which implies that the genes *hoxW* and *hypA* overlap. An intergenic stretch between the two genes seen in *A. eutrophus* [18] is missing in the *A. nidulans* sequence. The motif $Cx_2Cx_{12-13}Cx_2C$, found in all HypA proteins investigated so far supposedly functioning as a metal-binding domain [23], occurs also in HypA of *A nidulans*, however, with the distance between the two Cx_2C being only 10 amino acids. The sequence identities to the other homologous proteins are about 30%. Work with deletion mutants has to show whether HypA modulates the activities of hydrogenases in *A. nidulans* as in *E. coli* [24].

The following gene *hypB* has been extensively studied in other organisms. HypB is a Ni-binding protein which can take up to 18 Ni atoms per dimer in *Bradyrhizobium* [25]. It might be involved in Ni donation to the HoxH apoprotein [25], and this reaction is dependent on GTP hydrolysis [25–27]. The *A. nidulans hypB* gene (protein 259 amino acids) has a start codon for Met immediately behind the stop codon for *hypA*. HypB from *A. nidulans* possesses the putative GTP-binding regions G1–4 seen in the GTPase superfamily [28], which are for *A. nidulans*: G1: ALNLLSSPGSGKT, G2: DAQRLQAT, G3: PAAFDLGEE, and G4: LVLITKVD (in bold conserved amino acids, as in *E. coli* [27], compare also [28]). In almost all organisms investigated so far, the protein has a stretch extremely rich in histidine residues at the N-terminus which might be involved in Ni binding [25]. Two

Table 2
The uptake and evolution of H_2 in mutant B16 and the wild type of *A. nidulans*

	$Na_2S_2O_4$/MV-dependent H_2 evolution	PMS-dependent H_2 uptake
Wild type	430	65
Mutant B16	0	30

MV = methyl viologen, PMS = phenazine methosulfate. Rates are given in nmol/(mg protein × h). The determinations were done with cells grown aerobically at 30°C. H_2 uptake was performed with intact cells supplemented with PMS in the H_2 electrode. $Na_2S_2O_4$/methyl viologen-dependent H_2 formation was determined by gas chromatography, assaying crude extracts obtained by breaking the cells in a French press and by spinning down undesired unbroken cells and debris.

His only are found in this part of the protein in *E. coli* [26] whereas this region is missing in *A. nidulans*. In this cyanobacterium, however, four histidines are located immediately downstream of the N-terminus. All these sequence and motif similarities indicate that HypB might act as a Ni-donating GTPase also in *A. nidulans*.

The *A. nidulans* gene downstream of *hypB* has only been partly sequenced as yet. The 310 bp sequenced reveal similarities to *hypF*. The protein encoded by *hypF* is believed to be involved in the H_2 stimulation of hydrogenase expression. In many organisms with the exception of *A. eutrophus* [18] HypF possesses a Cys arrangement typical of a zinc-finger protein which is possibly present on the part not yet sequenced. As mentioned above, *hypF* is not located in the vicinity of other hydrogenase genes in *Synechocystis* 6803 [21], indicating either a different organization of the hydrogenase accessory genes in this cyanobacterium or the presence of a second copy of this gene.

In *Anacystis*, the accessory genes *hoxW, hypA, hypB* and *hypF* appear to form a contiguous transcriptional unit with partially overlapping genes and no obvious termination signals, which has to be verified by Northern blot analysis.

3.3. A mutant in hoxH of A. nidulans and its hydrogenase activities

As described in Section 2, a Km resistance conferring cassette was inserted into the 5.0 kb DNA segment carrying the *hoxUYH* genes of *A. nidulans*. Transformation of *A. nidulans* with this construct was achieved either with naturally competent cells or by electroporation. The frequency of natural transformation via homologous recombination ranged from 10^{-6} to 10^{-7} per recipient cell. In the case of electroporation, the frequency of transformation was about 10-fold higher. This appears to be the first clear-cut demonstration for genetic transformation of this *A. nidulans* strain which was subject to extensive investigations in the past. The insertion of the cassette into the *hoxH* gene of one mutant, termed B16, is documented in Fig. 2. The cassette contains a *Hin*dIII site. Therefore, hybridization of the inserted genomic DNA from *A. nidulans* restricted by *Eco*RI/*Hin*dIII with the 1.35 kb probe carrying the *hoxH* gene resulted in two bands (lane 3 in Fig. 2) which in total had the predicted size of 6.3 kb (5.0 kb for the cyanobacterial DNA segment plus 1.3 kb for the cassette). In addition, both the cassette and the 5.0 kb segment have a *Pst*I site at each end. Hybridization of genomic DNA carrying the cassette and restricted with *Pst*I with the 1.35 kb *hoxH* probe (Fig. 2, lane 4) also resulted in two bands of the right size of 2.7 kb in total. Thus the insertional mutant B16 obtained was completely segregated and homozygous.

The *hoxH* mutant B16 was assayed for $Na_2S_2O_4$- and methyl viologen-dependent H_2 evolution (determined by gas chromatography) and for PMS-dependent uptake of the gas (in the H_2 electrode). In contrast to the wild type which had both activities, $Na_2S_2O_4$- and methyl viologen-dependent H_2 formation was absolutely nil in the mutant (Table 2). The mutant, however, performed PMS-dependent H_2 uptake with a specific rate a little lower than 50% of that of the wild type (Table 2). This differential effect on the forward and reverse activities clearly indicates that the unicellular *A. nidulans* must possess both an uptake and a bidirectional hydrogenase. The growth rate of the mutant was unimpaired.

4. Discussion

The arrangement of the genes coding for the bidirectional NAD^+-dependent hydrogenase is surprisingly different in the heterocystous, N_2-fixing *A. variabilis* [7] and in the unicellular *A. nidulans* (this communication). In the latter organism, the structural genes, *hoxUYH*, are contiguous, and the fourth gene, *hoxF*, resides somewhere apart on the *A. nidulans* genome. The cluster *hoxUYH* together with the accessory genes *hoxWhypABF* may form a transcriptional unit which has to be proven by Northern analysis. In *A. variabilis* [7], these accessory genes are not localized downstream of *hoxH*. The two open reading frames ORF8 and ORF3 between the structural genes *hoxFUYH* in *A. variabilis* [7] are unusual for hydrogenase gene clusters. The function, if any, of the proteins encoded by these ORFs in the expression or regulation of this bidirectional hydrogenase is likely not correlated with heterocyst differentiation and/or nitrogenase synthesis, since the enzyme also occurs in vegetative cells [29]. Both expression and activity of the bidirectional hydrogenase in cyanobacteria are subject to complex and not yet fully understood regulation [1,2,30]. Its activity levels in both heterocysts and vegetative cells of *A. variabilis* and in the unicellular *A. nidulans* are enhanced by incubating the cultures under anaerobic conditions and even further by adding molecular H_2 to the anaerobic headspace of the cultures [1,2].

The different organization of the hydrogenase genes in *A. variabilis* and *A. nidulans* suggests a different transcriptional regulation in the two cyanobacteria. The present article reports the first ever description of accessory hydrogenase genes in a cyanobacterium. Their organization in *A. nidulans* is similar to those coding for the NAD^+-dependent hydrogenase in *A. eutrophus*, and further genes coding for auxiliary proteins in hydrogenase synthesis and expression might exist also in *A. nidulans*. The present work with the mutant proved that *A. nidulans* must possess an uptake hydrogenase in addition to the bidirectional enzyme. The function of uptake hydrogenase in heterocysts of *A. variabilis* is coupled with nitrogenase activity [11]. Since the uptake hydrogenase in *A. nidulans* must be functionally different, its activity and expression may also be regulated in a different way. Little is known about the expression and also about the molecular biology of the uptake hydrogenase in cyanobacteria. The presumptive genes coding for the small and large subunits of the uptake hydrogenase (*hupSL*) from *Anabaena* sp. PCC 7120 have been cloned and sequenced and the large subunit has been shown to undergo gene rearrangement in parallel with heterocyst differentiation

and prior to expression [31,32]. Recent sequence comparisons indicate that these genes sequenced could alternatively represent the *hupUV* genes (J. Golden, personal communication).

The isolate described here is the first defined mutant of a structural hydrogenase gene in a photosynthetic, O_2-evolving microorganism. Both the uptake and the bidirectional hydrogenase have a low K_m for H_2 and might operate in recycling the gas in vivo. The uptake hydrogenase feeds in electrons to the respiratory chain at or close to the plastoquinone site in cyanobacteria [4], and thermodynamics do not permit a substantial H_2 evolution ($\varepsilon_o' = -420$ mV for the $H_2/2\ H^+$ couple) from NADH ($\varepsilon_o' = -320$ mV for $NADH/NAD^+$) catalyzed by the bidirectional hydrogenase. Indeed, the highest H_2 formations ever reported for *A. nidulans* are marginal [33]. It is not surprising that the growth rate of the mutant is not affected by the inactivation of *hoxH*. In the mutant, uptake hydrogenase may utilize H_2 in place of the bidirectional enzyme, therefore the phenotype may not be changed. However, in a previous study, a regulatory mutant of *A. variabilis* obtained by nitrosoguanidine treatment was found to be deficient in the expression of both the uptake and bidirectional hydrogenase, and growth was also unaffected in this mutant [34]. Thus the function(s) of these enzymes in photoautotrophically growing cyanobacteria like in green algae is an enigma. It could be argued that these enzymes played an essential role in the early history of the earth. However, it would then be surprising that cyanobacteria have retained the complex gene sets coding for two different non-essential hydrogenases for more than 3×10^9 years of their existence.

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