Mutants of the Cyanobacterium *Anabaena variabilis* Altered in Hydrogenase Activities

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Two mutants of the cyanobacterium *Anabaena variabilis* impaired in the utilization or formation of molecular hydrogen have been obtained by nitroso-guanidine mutagenesis. Cultures of both mutants did not show alterations in the growth characteristics or in the heterocysts frequency but evolved molecular hydrogen from nitrogenase with enhanced rates. Activity measurements in extracts showed that one mutant (PK84) did not perform $Na_2S_2O_4$-dependent H_2-formation and was, therefore, unable to express an active bidirectional hydrogenase. Both mutants (PK84, PK17R) were characterized by lower activity of phenazine-methosulphate-dependent H_2-uptake when extracts were assayed from younger cultures. In older cells, particularly when grown with nitrate in the medium, this H_2-uptake activity was, however, enhanced. Both mutants are likely affected in regulatory hydrogenase genes. The mutant PK84 offers perspectives for potential applications in solar energy conversion programs.

Introduction

Some cyanobacteria have the simplest nutrient requirements of all organisms in Nature. Species can grow photoautotrophically and meet their nitrogen demand by dinitrogen fixation. In this reaction, molecular hydrogen is released from the enzyme complex nitrogenase, simultaneously with the reduction of the dinitrogen molecule to ammonia (Bothe *et al.*, 1983). Cyanobacteria have often been discussed to offer perspectives for the commercial photoproduction of molecular hydrogen by using either liquid suspension cultures or immobilized cells (Böger, 1978; Gisby *et al.*, 1987; Kentemich *et al.*, 1990). In whole cells of nitrogen fixing cyanobacteria, H_2-evolution activities are usually very low compared to other metabolic activities, due to the fact that the H_2-evolved is immediately recycled by hydrogenases (Houchins, 1984; Papen *et al.*, 1986). Cyanobacteria possess at least two different hydrogenases which have recently been cloned and sequenced (Matveyev *et al.*, 1994; Carrasco *et al.*, 1995; Schmitz *et al.*, 1995). One of these enzymes, catalyzing H_2-uptake only, is integral component of the thylakoid membrane (Eisbrenner *et al.*, 1981). It is particularly active in heterocysts (Peterson and Wolk, 1978) but apparently also occurs in the unicellular *Anacystis nidulans* (Eisbrenner *et al.*, 1978; Peschek, 1979). The other hydrogenase, the so-called bidirectional or reversible enzyme, resides both in heterocysts and vegetative cells and its activity levels are enhanced by anaerobiosis (Houchins and Burris, 1981; Papen *et al.*, 1986). The isolated enzyme has a high affinity for H_2. Therefore it likely operates also in recycling molecular hydrogen in intact cells.

To examine maximal potentialities of H_2 production by intact cyanobacteria, mutants defective in H_2-utilizations would be desirable. The strategy for isolating such mutants has been described earlier for *Anabaena variabilis* ATCC 29413 (Mikheeva *et al.*, 1994). This communication characterizes two different mutants altered in hydrogen metabolism in assays both with intact cells and with extracts.

Abbreviations: MV, methyl viologen; NTG, N-methyl-N-nitroso-N-guanidine; PMS, phenazinemethosulphate.

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Materials and Methods

Strains and culture conditions

The wild-type strain of the heterocystous *Anabaena variabilis* ATCC 29413 and its mutants were

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grown in the nitrogen-free modified medium of Allen and Arnon (see Kentemich *et al.*, 1988), supplemented, when required, with either 20 mM $NaNO_3$ or 5 mM NH_4Cl. The concentration of agar at plate growth was 1.2%. Suspension cultures were grown under fluorescent light (50μE $m^{-2}s^{-1}$) at 28 °C. Growth rates in liquid media were determined spectrophotometrically by the absorbance at 540 nm. For the growth determinations of the wild type and the mutants, initial inocula were the same. Heterocyst frequency was determined in about 200 filaments for each probe by microscopic counting.

Isolation of mutants

Mutants altered in hydrogen metabolism were obtained by chemical mutagenesis of the wild type strain with N-methyl-N-nitroso-N-nitroguanidine (NTG). The exact procedure has been described earlier (Polukhina *et al.*, 1982). More than 1000 colonies could be depicted from agar plates (medium without combined nitrogen) which were smaller in size than the average. After growth of these colonies in suspension cultures, several showed higher H_2-formation activities when assayed under argon. Of these, the mutants PK84 and PK17R were chosen for the present study. The latter is a spontaneous morphological revertant of the mutant PK17 described earlier (Mikheeva *et al.*, 1994). Mutant PK17R forms blue-green colonies of normal size in contrast to the parental PK17 which shows more yellowish and smaller colonies in N-free medium on agar plates.

The mutants will be available from S. Shestakov, Moscow, upon request.

Enzyme assays

Nitrogen fixation activity was determined by the C_2H_2-reduction method as described (Bothe and Loos, 1972; Kentemich *et al.*, 1988). The quantity of C_2H_4 formed was measured in a gas chromatograph fitted with a flame ionization detector and a Carbosieve SII column (100/120 mesh/10 feet x 1/8 inches, for details see Kentemich *et al.*, 1988). H_2 was quantified in a gas chromatograph fitted with a thermal conductivity detector and molecular sieve column (5 Å, 45/60 mesh, 2 m x 1/8 inches). Protein in intact cells or in extracts was determined by a modification of the Lowry method as described by Herbert *et al.* (1971).

For preparing cell-free extracts, the cultures were harvested by centrifugation, washed with 0.02 M Tris-HCl-buffer pH 7.5, recentrifuged, resuspended and passed twice through a French Press at 1200 PSI (~ 137,000 kPa). The extract was centrifuged (20 min, 48000 x *g*), and the pellet was resuspended in 0.02 M Tris-buffer pH 7.5. The assays were performed in 7.0 ml Fernbach flasks covered with suba seals. The flasks were made anaerobic by repeatedly evacuating and flushing with argon. The reaction mixture in the Fernbach flasks contained in a final vol of 3 ml in mM: Tris[hydroxymethyl] aminomethan-HCl buffer pH 7.5 267, methyl viologen 0.8, $Na_2S_2O_4$ 12, supernatant 0.1–0.6 mg protein. The reaction was started by injecting the anaerobic solution through the suba seals of the flasks which were then incubated in a shaking water bath in the dark at 30 °C for 1–5 h. PMS dependent H_2-uptake was assayed also in the Fernbach flasks under argon, containing the same buffer and the same pellet concentration but 3 mM PMS. The reaction was started by injecting H_2 through the suba seals with a syringe (final concentration in the flasks 2.5%, v/v). The H_2-uptake determination by the electrode is based on the method developed by Hanus *et al.*(1980). The exact assay conditions in the 1.6 ml vessel have been described (Papen *et al.*, 1986). PMS (3 mM) served as electron acceptor also in these assays.

Results

The two mutants obtained by chemical mutagenesis were not impaired in their vitality. Growth rates (determined by the optical density 5 d after the inoculum) were the same in the wild type and in the PK84 and PK17R mutants grown either under N_2-fixing conditions or with nitrate in liquid medium (Table I). In filaments of the wild type and the mutants, heterocyst frequency was the same, being approximately twice as much under N_2-fixing conditions as compared to nitrate-grown cells (Table I). Likewise, C_2H_2-reduction activities did not show significant differences. In all three cultures, C_2H_4-formations were slightly higher when the cells were assayed in argon and not in air (Table I). The wild type culture showed no H_2-formation in air and marginal productions when

Table I: Growth, heterocyst frequency, C_2H_2-reduction, H_2-evolution and uptake activities of the wild-type and mutants from *Anabaena variabilis*.

Culture	Growth rate		Heterocyst frequency		C_2H_4-formation		H_2-evolution		H_2-uptake
	N-free	$NaNO_3$	N-free	$NaNO_3$	Air	Argon	Air	Argon	Air
Wild type	0.70	0.76	6.2	3.3	0.51	0.68	0.00	0.02	0.26
PK84	0.78	0.80	6.3	3.5	0.58	0.76	0.22	0.65	<0.02
PK17R	0.75	0.81	6.2	2.8	0.56	0.75	0.20	0.75	<0.02

The determinations were made as follows:
a) growth rate: optical density at 540 nm after 5 d of cultivation in a medium either free of combined nitrogen or with 20 mM $NaNO_3$;
b) heterocyst frequency: % heterocysts counted among the cells of the filaments;
c) C_2H_2-reduction: μmol C_2H_4 formed/(h x mg protein), incubation for 1 h in the light;
d) H_2-evolution: μmol H_2 evolved/(h x mg protein), 5 h incubation in the light;
e) H_2-uptake: μmol H_2 consumed/(h x mg protein), 5–20 h incubation in the light in the presence of 3 mM PMS and 5% H_2.
The activities were determined in 5 d old cultures.

assayed under argon. Intact cells of both mutants, however, produced significantly higher amounts of H_2, the rates almost equalling the C_2H_4-formation activities (Table I). Measurements of the H_2-uptake capabilities (performed with the cells in the presence of 3 mM PMS) already indicated that the mutants were impared in H_2-uptake, since the wild type only could consume the gas efficiently (Table I).

H_2-production activity in intact cells of the mutants was maximal in cultures of the exponential growth phase (Table II). The activity declined with the age of the cultures, and cells in the stationary growth phase had only 25–30% of the maximal activity. Heterocyst frequency did not grossly change with the age of the cells. In cultures of the wild-type and the mutants, the addition of nitrate to the medium suppressed heterocyst frequency and drastically decreased H_2-production rates, confirming earlier findings that the gas produced stems from nitrogenase (Papen *et al.*, 1986).

Table II: H_2-formation by the wild type and the mutants of intact *Anabaena variabilis*: Dependence of the activity on the age of the culture.

Culture	Time of cultivation in days		
	3	7	21
Wild type	3.5	6.1	0.9
PK84	214.6	89.1	50.8
PK17/R	133.9	159.2	56.0

Rates are given in nmol H_2-formed/(h x mg protein). The cultures were grown under N_2-fixing conditions in the light at 28 °C.

The bidirectional hydrogenase was assayed by the $Na_2S_2O_4$-dependent H_2-formation in cell-extracts. Rates were linear for at least 0.6 mg protein/3 ml assay vol in the vessels and 3 h incubation time with extracts from the wild-type and from PK17R (not documented). In the wild-type, the activity was present both in NO_3^-- or NH_4^+-grown and N_2-fixing cells, indicating that this enzyme is not confined to heterocysts (Table III). After a centrifugation of the extract the enzyme level was much higher in the supernatant than in the pellet, also confirming earlier observations that this enzyme is soluble or can readily be solubilized from membranes (Kentemich *et al.*, 1989, 1991). The mutant PK84 has almost completely lost the $Na_2S_2O_4$-dependent H_2 formation activity whereas the rate was virtually not altered in the PK17R culture.

Phenazine methosulfate (PMS)-dependent H_2-uptake was determined by gas chromatography. The activity proceeded linearly for at least 4 h and 1.2 mg protein/3 ml assay vol in the vessel (not documented). Whereas extracts from wild-type cells effectively utilized the gas with approximately threefold higher activity than in H_2-formation, this uptake was significantly reduced in young cultures (up to 7 d old) of the two mutants (Fig. 1). Older cultures of PK17R and also NO_3^--grown cells of both mutants showed high H_2-utilization levels, even exceeding the rates observed

Table III: Activity of the bidirectional hydrogenase in cell-extracts from the wild-type and mutants of *Anabaena variabilis*.

Culture	H_2-formation Activity under N_2-fixing conditions			in $NaNO_3$-medium		
	Extract	Super-natant	Pellet	Extract	Super-natant	Pellet
Wild type	19.4	25.5	5.7	7.0	16.0	3.3
PK 84	1.1	1.9	0.2	0.4	0.6	0.3
PK 17 R	23.8	25.3	11.6	5.4	11.5	2.2

Rates are given in nmol H_2 evolved/(h x mg protein). Cultures grown for 5 d were assayed for dithionite and MV-dependent H_2-formation activity.

with wild type cultures of the same age (Table IV). Extracts from NH_4^+-grown cells could not perform this reaction (not documented).

The determination of the H_2-uptake by gas chromatography requires an incubation of at least 2–4 h to detect significant differences in the H_2-uptake due to the low sensitivity of the method and/or the relatively low activity levels in cyanobacterial cells. It was, therefore, tried to verify the unexpected increase of the activity levels in older cultures by measuring H_2-utilization by an independent method. The H_2-electrode allows to monitor H_2-utilization continuously and within a short incubation period. The graphs (Fig. 2) confirm that older cultures of the mutant PK17R utilized the gas even more rapidly than the wild type. In contrast, the activity of the N_2-grown PK84 was lower than that of the wild type. Thus the electrode measurements independently confirm the data of Table IV and that the results on H_2-uptake are not due to leaky suba seals or any other technical insufficiency during the determination of H_2-uptake by the gaschromatographic procedure.

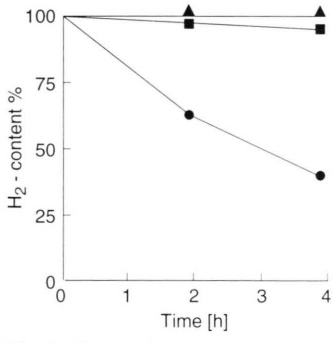

Fig. 1. Phenazinemethosulphate dependent H_2-uptake in cell-extracts of the wild-type and mutants of *Anabaena variabilis*. Cultures were grown under N_2-fixing conditions for 7 d. The initial concentration of H_2 was 2.5% in the assay vessels and is set to 100% in the fig. In the assays, the protein content was 1.5 mg/3 ml reaction mixture and the concentration of PMS was 3 mM. The rate was 580 nmol H_2 consumed/(h x mg protein) for the wild type. The experiment was performed three times with the same results.

● ——— ● Wild type;
■ ——— ■ PK17R;
▲ ——— ▲ PK84.

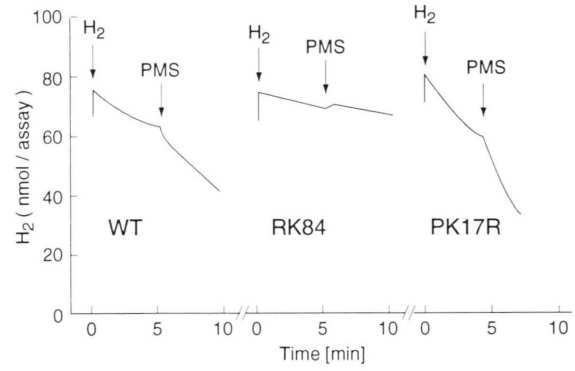

Fig. 2. The consumption of H_2 by intact cells of the wild type and the two mutants of *Anabaena variabilis* determined by the H_2 electrode. The figures represent the original recorder tracings. 3 weeks old cultures grown under N_2-fixing conditions were directly assayed without concentrating by centrifugation. Activity calculated from the tracings: 70 nmol/(h x mg protein), for the wild type.

Discussion

Under all assay conditions, H_2-formation activity was highest in N_2-fixing *A. variabilis*, decreased in parallel with heterocyst frequency in nitrate-

Table IV: PMS-dependent H_2-uptake activity in extracts from the wild type and mutants of *Anabaena variabilis*.

Culture	H_2-uptake rate			
	7 d old culture		21 d old culture	
	under N_2-fixing conditions	in NO_3^- medium	under N_2-fixing conditions	in NO_3^--medium
Wild type	580	103	84	75
PK84	10	92	19	256
PK17R	24	456	382	356

Rates are given in nmol H_2 formed /(h x mg protein).
The protein content in the assay (3 ml vol) was 1.2 mg. Concentration of PMS = 3 mM, incubation time = 3 h. The cells assayed had been grown for 7 and 21 d, respectively, in light and air.

grown cultures and was completely abolished when ammonia served as N-source for growth. The present investigation thus confirmed the earlier findings that virtually all H_2-production in intact *A. variabilis* as in other heterocystous forms comes from nitrogenase (Papen *et al.*, 1986). Hydrogenases in intact cyanobacteria can evolve the gas with at best marginal activities (Hallenbeck *et al.*, 1981). It had also been stated earlier that net H_2-formation of intact cells can be enhanced by blocking hydrogenase dependent H_2-uptake by using the inhibitors CO and C_2H_2 (Bothe *et al.*, 1977). In such assays, the concentration of inhibitors was critical to block hydrogenase only and not to affect other metabolic traits within the cells. It had been stated (Hallenbeck *et al.*, 1981; Papen *et al.*, 1986) that maximal H_2-production activity can probably be obtained only by the use of mutants defective in H_2-utilizations.

In the present study, the uptake and evolution of H_2 were differentially affected in the two mutants which corroborates the statement that two different hydrogenases occur in cyanobacteria. Whereas mutant PK84 is inactive in performing $Na_2S_2O_4$-dependent H_2-formation and has thus lost the capability for expressing a functional bidirectional hydrogenase, PK17R has this activity unimpaired. Both mutants show lower H_2-utilizations with PMS as electron acceptor in younger cultures. However, this activity is enhanced at later growth stages in the medium with nitrate even in the case of mutant PK84 which has low activity under N_2-fixing conditions. Mutant PK17R grown with N_2 as the N-source shows significantly higher PMS-dependent H_2-uptake in the 21 d old culture than the corresponding wild type and PK84 cells (Table IV).

The enhancements of activities in older cultures is difficult to be explained, but was measured by two experimentally different approaches. Both mutants are unlikely defective in any of the structural hydrogenase genes but are probably affected in the regulation of their expression. Otherwise the restoration of the activity beyond the level seen in the wild-type strain is not conceivable.

As regard to the nature of these mutations, complementation analysis may permit to reveal regulatory genes controlling the expression of the uptake and the bidirectional hydrogenase or both. A suitable complementation system by transformation has been developed for some cyanobacteria (Houmard, 1994), but is unfortunately not yet available for *A. variabilis*.

This investigation unambiguously showed by the use of hydrogenase mutants that these enzymes are not essential for the cyanobacteria growing vividly which had earlier been inferred from studies with wild-type strains (Papen *et al.*, 1986). As alterations in the uptake or formation activities do not provide a modified phenotype, characterization of hydrogenase mutants requires enzymic measurements. It remains to be shown which physiological role is to be assigned to the hydrogenases in cyanobacteria as also in green algae (Kessler, 1974). The possession of two hydrogenases may become favourable when the energy demand for the cell is high, e. g. under light-limiting growth conditions. The enzymes may have had a functional role in the early earth history when the atmosphere had reducing conditions, and the genes may have been preserved as ancient relicts.

The mutant PK84 offers some perspectives in solar energy conversion programs as it is impaired in the bidirectional hydrogenase activity and as the

rate of H_2-uptake is also reduced in this mutant grown under N_2-fixing conditions. Applications of such mutants will be useful to define the maximal H_2-production rates by cyanobacteria for biotechnological solar energy exploitation. Such studies are prerequisital to decide whether systems based on microorganisms can compete with the photovoltaic or any other established energy generating system.

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