Arch Microbiol (1993) 160: 62–67

Archives of
Microbiology

Identification of the *nifJ* gene coding for pyruvate: ferredoxin oxidoreductase in dinitrogen-fixing cyanobacteria

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Received: 7 January, 1992/Accepted: 22 January 1993

Abstract. A 329 bp DNA segment from both *Anabaena variabilis* and *Anabaena* PCC 7119 was amplified using the polymerase chain reaction (PCR). The sequences from the two cyanobacteria showed strong similarities to the corresponding part of the *nifJ* gene from *Klebsiella pneumoniae* and *Enterobacter agglomerans*. The present findings underline earlier results of enzymatic studies that heterocystous cyanobacteria possess a pyruvate : ferredoxin (flavodoxin) oxidoreductase (PFO). The *nifJ* gene segment could not be detected in the non-dinitrogen-fixing, unicellular cyanobacterium *Anacystis nidulans* which is also in accord with previous findings from enzyme assays.

Key words: Pyruvate : ferredoxin oxidoreductase – *nifJ* Gene – Ferredoxin – Flavodoxin – Dinitrogen fixation – Heterocysts – Cyanobacteria

In the pyruvate clastic reaction (Wolfe and O'Kane 1953), this keto acid is cleaved to acetyl coenzyme A and CO_2, and the remaining two electrons are transferred to either ferredoxin or flavodoxin, depending on the organism. The enzyme catalyzing the reaction (PFO) occurs in *Clostridium* spp. (Mortenson et al. 1963; Uyeda and Rabinowitz 1971a) and other anaerobes (see Bothe et al. 1974), in facultative anaerobes of the Enterobacteriaceae, e.g. in *Klebsiella pneumoniae* (Nieva-Gomez et al. 1980; Shah et al. 1983), in *Escherichia coli* (Blaschkowski et al. 1982; Knappe 1987) and in *Enterobacter agglomerans* (Kreutzer et al. 1989) as well as in *Halobacterium* (Kerscher and Oesterhelt 1981a, b). The reaction ideally meets the requirement for N_2-fixation, since reduced ferredoxin or flavodoxin are the immediate carriers transferring electrons to nitrogenase, and since ATP can be formed from acetyl coenzyme A in reactions catalyzed by phosphotransacetylase and acetate kinase, e.g. in *Clostridium pasteurianum*. In *Klebsiella*, the gene coding for PFO (*nifJ*) is part of the cluster required for nitrogenase expression. The *nifJ* gene is apparently absent in other N_2-fixing bacteria like *Azotobacter* or *Rhizobium* (Bothe et al. 1983).

In cyanobacteria, reports about the occurrence of PFO have been controversial since a long time. Evidence for its presence in crude extracts was originally communicated by Leach and Carr (1971), and the enzyme was then characterized in detail both by the forward and reverse reaction (Bothe et al. 1974; Bothe and Nolteernsting 1975; Neuer and Bothe 1982; 1985). The enzyme was shown to be present in heterocysts (which are the site of nitrogenase in those cyanobacteria which differentiate these cells), to be oxygen-labile and subject to inhibition by the product acetyl coenzyme A (Neuer and Bothe 1982; 1985). In line with this, the enzyme activity was shown to be enhanced in extracts supplemented with oxaloacetate to convert acetyl coenzyme A to citrate (Böhme and Schrautemeier 1987). Other authors could not confirm the occurrence of PFO in cyanobacteria (Lockau et al. 1978; Privalle and Burris 1984; Schrautemeier et al. 1984). In reviews on N_2-fixation by cyanobacteria, the occurrence of PFO was disputed (Haselkorn 1978) or more recently even not mentioned (Fay 1992).

This communication describes the identification of the *nifJ* gene in the heterocystous *Anabaena variabilis* and *Anabaena* PCC 7119 and its absence in the unicellular, non N_2-fixing *Anacystis nidulans*.

Materials and methods

Strains and growth conditions

Anacystis nidulans (= Synechococcus leopoliensis) was strain 1402-1 of the Sammlung von Algenkulturen of the Pflanzenphysiologisches Institut, Universität Göttingen. *Anabaena variabilis* ATCC 29413 and *Anabaena* PCC 7119 were kindly made available to us by Professor C. P. Wolk, East Lansing and Dr. R. Rippka, Paris, respectively. The cyanobacteria were grown aerobically under continuous gassing with a mixture of air/CO_2 = 95/5 (by vol) with nitrate in the medium as described earlier (Neuer and Bothe 1982, 1985).

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Synthesis of oligonucleotides

The oligonucleotides were synthesized by a Pharmacia LKB Gene Assembler Plus (Pharmacia LKB, Uppsala, Sweden). After removal from the column they were purified by separation on a 1.5 ml NAP™-10 column (Pharmacia LKB).

Polymerase chain reaction

The assays contained in a final volume of 50 µl: Taq-polymerase (Boehringer Mannheim, FRG), 2U; 10× Taq-polymerase buffer (Boehringer), 5 µl; deoxynucleotides, 10 nmol of each; oligomers 50 pmol of each; cyanobacterial DNA, 20–50 ng. The reaction was performed in 30 cycles using the following conditions: 30 s denaturation at 92 °C, 30 s annealing at 36, 46, 68 or 72 °C and 60 s polymerization at 72 °C.

DNA sequencing

The amplified PCR-products were cloned into pCR™II (TA Cloning™, Invitrogen, San Diego, Calif. USA). *XbaI/Hin*dIII fragments containing the entire PCR-segments were subsequently cloned into M13mp18/M13mp19. The sequences of the inserts of the M13-derivatives were determined on both strands by the dideoxy mediated chain-termination technique using the TAQuence™ Sequencing Kit (United States Biochemical Corporation, Cleveland, Ohio, USA).

Labeling

A purified *Eco*RI-fragment of the pCR™II vector, containing the entire subcloned PCR-segment, was labeled with 20–50 µCi [^{32}P]-dCTP by the Random Primed DNA Labeling Kit (Boehringer).

DNA isolation, restriction, blotting and hybridization

The DNA of the cyanobacteria was isolated as described by Adams (1988). For each lane of the Southern blots, 10 µg of DNA were digested by 20 U (2 h) of *Cla*I, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Ssp*I or *Xba*I or by a combination of two of these enzymes as given in Fig. 4. The DNA was separated on a 0.7% (w/v) agarose gel and blotted onto nitrocellulose filters (United States Biochemical Corporation). After 16 h of transfer, the DNA was immobilized by UV-irradiation (254 nm, 2 min) and incubating at 60 °C for 2 h. The Southern blots were prehybridized by a buffer containing 50% formamide, 5×SSC, 0.5% SDS, 5× Denhardt's solution and 100 µg/ml denaturated herring sperm DNA. After 2–3 h of prehybridization at 42 °C, the filters were transferred into the hybridization buffer, which differed from the prehybridization buffer in the concentration of herring sperm DNA (20 µg/ml) and the Denhardt's solution (1×) and additionally 100 ng of the ^{32}P-labeled denaturated DNA-probe. Hybridization was performed for 20 h decreasing the temperature from 42 to 30 °C stepwise. The filters were washed twice in 5× SSC, 0.5% SDS at 30 °C, dried at room temperature and exposed to an X-ray film (Hyperfilm™-β_{max}, Amersham) for 24–72 h.

Transformation, plasmid preparation, restriction analysis and ligation were performed by conventional techniques (Sambrook et al. 1989).

Results

This investigation initially aimed at identifying the genes coding for hydrogenases in cyanobacteria. The strategy employed computer analysis of published DNA sequences from diverse organisms to identify conserved parts within the hydrogenase genes. This should provide informations necessary for the synthesis of oligonucleotide primers to be used for gene amplification by PCR. For unknown reasons, this strategy did not result in much success for cyanobacterial hydrogenases as yet, even when primers of a single region were used to amplify flanking sequences by inverse PCR (Ochman et al. 1990). Within the smaller subunit (about 30 kDa) of hydrogenases from *Azotobacter chroococcum*, *A. vinelandii*, *Bradyrhizobium japonicum*, *Rhodobacter capsulatus*, *Desulfovibrio baculatus* and *D. vulgaris*, sequences from the EMBL data bank show strong similarities in two short regions separated from each other by 19–20 amino acids. These conserved sequences contain three cysteine residues as possible Fe- and/or Ni-binding sites. This region corresponding to the amino acid positions 266–304 in the sequence of the hydrogenase from *B. japonicum* (Sayavedra-Soto et al. 1988) was chosen to synthesize the two oligodeoxynucleotide primers:

(a) 5′ GTT GTA GGT GGT AGG ACC CTT GCA ACC 3′
T T T T A

(b) 5′ TGC ATC GGT TGC TCT GAA GAT GGT TTC TGG 3′
T T T C C

taking into account the codon usage of cyanobacteria (Tandeau de Marsac and Houmard 1987). Either nondigested or partially *Taq*I-digested DNA from *Anabaena variabilis* served as template to amplify fragments with

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        <------- primer (a) ------>
                                    <----- primer nifJ1 ------>
A.var.  GTTGTAGGTTGTAGGACCTTTGCAACCATAGATGGCAATGAGGCTGTTGCCC  52

A.var.  AAGTCGTCTATCAAATCAATGAAGTTATTGCTATTTATCCGATTACACCTTC 104
A.7119                                                    C

A.var.  TTCACCGATGGCGGAATGGTCAGATGCTTGGGCGAGTGAAGGTAAACCAAAT 156
A.7119             A  G   G C          T              C

A.var.  ATTTGGGGTACTGTACCGACGGTAGTACAAATGCAGAGTGAAGGAGGAGTAG 208
A.7119                          G                    G

A.var.  CGGGTGCAGTACATGGGGCGTTGCAAACAGGGTCATTAACAACGACATTTAC 260
A.7119        C                                   A

            <----- primer nifJ2 ------>          <----------
A.var.  CGCATCCCAGGGATTATTGCTGATGATCCCTAATATGTACAAGGTTGCAAGG 312
A.7119

        primer (a) ----->
A.var.  GTCCAACCACCTACAAC 329
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Fig. 1. Nucleotide sequence of the PCR amplified partial *nifJ* sequences from *Anabaena variabilis* ATCC 29413 and *Anabaena* PCC 7119. Only the nucleic acids different to the sequence of *A. variabilis* (*A. var.*) are shown for A PCC 7119 (A. 7119). The positions of the primers used to amplify the segments are given above the sequence

these two primers. By this, an about 300 bp segment was obtained at the low annealing temperature of 36 °C. Sequencing showed that this segment had an exact length of 329 bp and ended with primer (a) at both sides. The DNA sequence of the segment (Fig. 1) showed no similarity to any hydrogenase gene but a strong one to the *nifJ* gene (61% identical bases with the *Klebsiella pneumoniae* gene and 63% identity with that from *Enterobacter agglomerans* in the amplified part of 275 bp within this segment).

The knowledge of the sequence allowed to synthesize internal primers (designated as *nifJ*1 and *nifJ*2 in Fig. 1) by which a fragment of 267 bp could consistently be amplified by PCR at all employed annealing temperatures from 46 °C (very strong signal) to 68 °C (strong signal) up to 72 °C (weak signal). The same primers could be used to amplify a segment of the same length even at high stringencies up to 68 °C when DNA from *Anabaena* PCC 7119, but not from *Anacystis nidulans*, was taken as template (Fig. 2). The segment from A. 7119 showed 95% DNA sequence homology to that from *A. variabilis* with a mismatch of 11 bases. The CLUSTAL V multiple sequence alignment program (Higgins and Sharp 1988) indicated one single conservative amino acid exchange only in this 213 bp segment from the two cyanobacteria (Fig. 3). The G/C-contents of the sequences from *A. variabilis* and *A.* 7119 were 48 and 49%, respectively, and thus in the upper range for DNA from heterocystous cyanobacteria (Herdman et al. 1979).

The 329 bp *nifJ* segment from *A. variabilis* was cloned into M13 and used for DNA hybridizations with DNA isolated from *A. variabilis*, *A.* 7119 and *Anacystis nidulans* which had been digested by one of the restriction enzymes *Eco*RI, *Hin*dIII, *Xba*I, *Eco*RV, *Hin*cII, *Cla*I and *Ssp*I or by a combination of two of them (Fig. 4). In the Southern blots, positive signals were obtained with all of the digested DNA samples from *A.* 7119 (Fig. 4a) and *A. variabilis* (not shown), whereas no distinct ones were seen in the case of *Anacystis nidulans* DNA (Fig. 4b). A physical map for the DNA region containing the *nifJ* segment was constructed from the restriction pattern (Fig. 4c), and this knowledge should allow to clone and sequence the whole gene if desired.

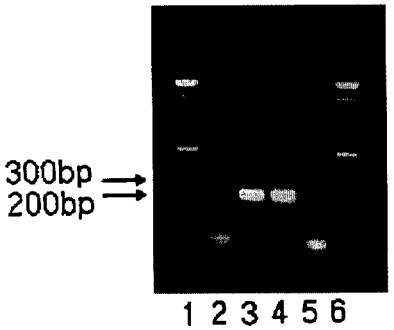

Fig. 2. Agarose gel electrophoresis of products from PCR experiments with genomic DNA of *Anabaena variabilis* ATCC 29413, A. PCC 7119 and *Anacystis nidulans* 1402-1 using primer *nifJ*1 and *nifJ*2. *Lane 3* = *A. variabilis*, *lane 4* = A. PCC 7119, *lane 5* = *Anacystis nidulans*. The sizes of the amplified fragments determined to be 267 bp by DNA sequencing were in agreement with the sizes estimated from the molecular weight markers (*lanes 1 and 6*, 100 bp-ladder obtained from Life Technologies, Eggenstein, Germany) A PCR assay without genomic DNA but including the primers was used as a control (*lane 2*). The PCR experiments shown were performed at an annealing temperature of 46 °C as described under 'Materials and methods'. Segments were separated by 1.5% agarose gel electrophoresis

Discussion

Although a fragment of only 329 bp and thus approximately only 1/10 of the complete gene encoding PFO with 1171 amino acids in *Klebsiella pneumoniae* (Cannon et al. 1988) has been sequenced, the strong similarities to the sequences of the genes from *K. pneumoniae* and *Enterobacter agglomerans* (Kreutzer et al. 1989) clearly indicate that the segment originates from the *nifJ* gene of *Anabaena variabilis* and *A.* PCC 7119. Kreutzer et al. (1989) sequenced a slightly larger segment of the gene and came to the same conclusion in the case of *E. agglomerans*. The oligonucleotide primers synthesized for PCR were originally chosen to correspond to the presumed Fe-binding sites of the smaller subunit of hydrogenase. Similarly, PFO is an iron protein containing 2 (4 Fe–4 S) clusters in the prosthetic group (Kerscher and Oesterhelt 1981b; Uyeda and Rabinowitz 1971b). The segment sequenced from the two *Anabaena* strains presumably contains the conserved functional Fe–S-cluster binding sites within the enzyme. Thus the current genetic findings strongly

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A.var.          IDGNEAVAQVVYQINEVIAIYPITPSSPMAEWSDAWASEGKPN
A.7119                VVYQINEVIAIYPITPSSPMAEWADAWASEGKPN
K.pneum.    MSGKMKTMDGNAAAAWISYAFTEVAAIYPITPSTPMAENVDEWAAQGKKN
E.agg.      MPGKMKTMDGNTAAAYVSYAFTDVTAIYPITPSTPMAESVDEWAAQGKKN
                      . * . .* ********.****  * **..** *

A.var.      IWGTVPTVVQMQSEGGVAGAVHGALQTGSLTTTFTASQGLLLMIPNMY
A.7119      IWGTVPTVVQMQSEGGVAGAVHGALQTGSLTTTFTA
K.pneum.    LFGQPVRLMEMQSEAGAAGAVHGALQAGALTTTYTASQGLLLMIPNMYKI
E.agg.      LFGQTVKIMEMQSESGAAGAIHGALQAGALATTYTASQGLLLMTPNMYKI
            . *    ...****.* ***.*****.*.*.**.**
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Fig. 3. Multiple amino acid sequence alignment of NifJ segments from different microorganisms. *Anabaena variabilis* ATCC 29413 = *V. var.*, *Anabaena* PCC 7119 = A. 7119, *Klebsiella pneumoniae* = *K. pneum.*, *Enterobacter agglomerans* 333 = *E. agg.* The sequences of *K. pneumoniae* (Cannon et al. 1988) and *E. agglomerans* (Kreutzer et al. 1989) start at amino acid position 1. The sequence alignment was performed using of the program CLUSTAL V (Higgins and Sharp 1988). Identical residues were marked by an asterisk, conservative amino acid exchanges by a point below the sequence

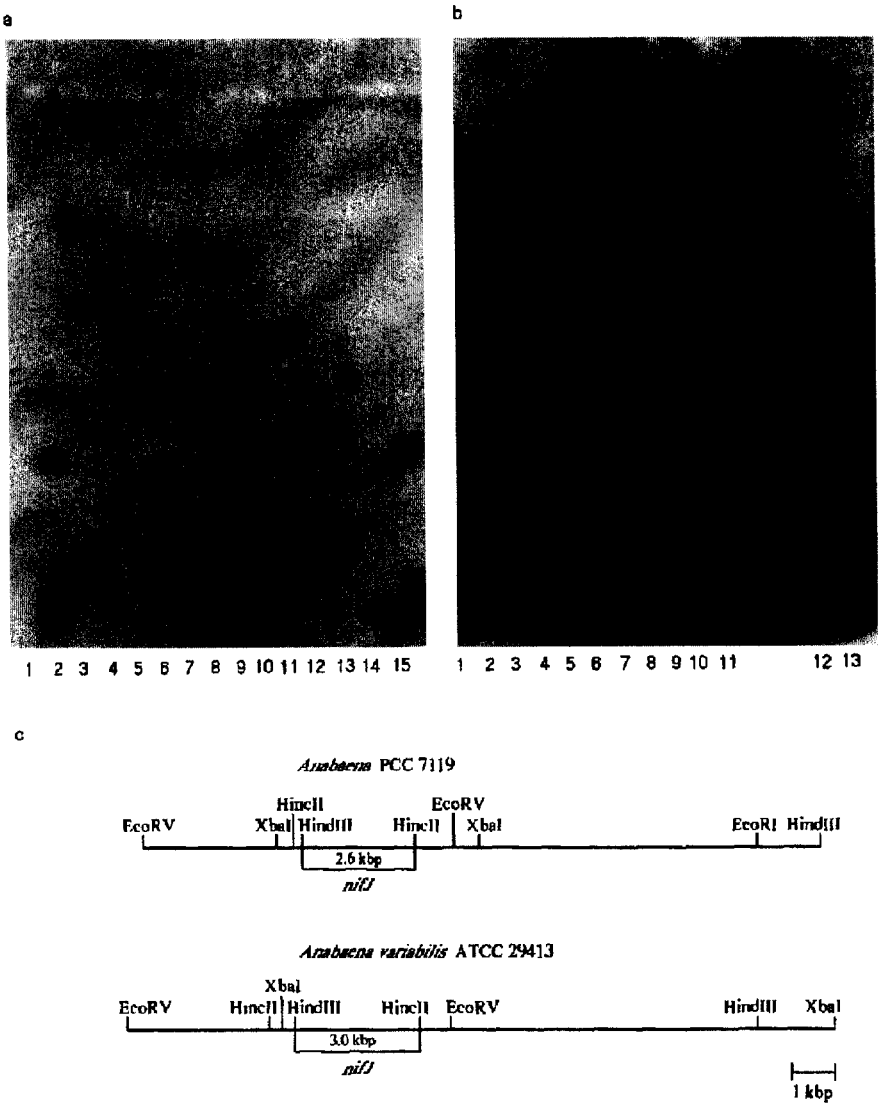

Fig. 4. Southern blots of digested genomic DNA of *Anabaena* PCC 7119 (**a**) and *Anacystis nidulans* 1402-1 (**b**) hybridized with the cloned ^{32}P-labeled 329 bp PCR segment of *Anabaena variabilis* ATCC 29413. The signals in the lanes correspond to hybridizations with DNA digested by the following restriction enzymes: (**a**) *Eco*RI = 2, *Hin*dIII = 3, *Xba*I = 4, *Eco*RV = 5, *Hin*cII = 6, *Eco*RI/*Hin*dIII = 7, *Eco*RI/*Xba*I = 8, *Eco*RI/*Eco*RV = 9, *Hin*dIII/*Xba*I = 10, *Hin*cII/*Eco*RI = 11, *Hin*cII/*Hin*dIII = 12, *Hin*cII/*Xba*I = 13 (**b**) *Eco*RI = 2, *Hin*dIII = 3, *Xba*I = 4, *Eco*RV = 5, *Eco*RI/*Hin*dIII = 6, *Eco*RI/*Xba*I = 7, *Eco*RI/*Eco*RV = 8, *Hin*dIII/*Xba*I = 9, *Hin*dIII/*Eco*RV = 10, *Eco*RV/*Xba*I = 11. *Lanes 1, 15* (**a**) and *lanes 1, 13* (**b**) are the molecular weight marker (1 kbp ladder, Life Technologies, Eggenstein, Germany). *Lane 14* (**a**) and 12 (**b**) are controls with the cloned 329 bp PCR segment in pCR™II digested by *Eco*RI. Details are described in 'Materials and methods'. The maps of the restriction sites in the *Anabaena* PCC 7119 and *Anabaena variabilis* ATCC 29413 DNA regions adjacent to the locus identified by the 329 bp *nifJ* probe of *Anabaena variabilis* are given in **c**

indicate the occurrence of PFO in heterocystous cyanobacteria which had earlier been demonstrated biochemically (Leach and Carr 1971; Bothe et al. 1974; Neuer and Bothe 1982; 1985).

In the unicellular non-N_2-fixing *Anacystis*, a similar segment could not be amplified by PCR with the same primers, and the Southern blots with the *A. variabilis* probe were also negative. These molecular biological results suggesting that *Anacystis* apparently does not possess PFO corroborate earlier enzymatic and physiological findings (Bothe and Nolteernsting 1975). *Anacystis* was shown to cleave pyruvate by a pyruvate dehydrogenase complex. In addition, organisms possessing PFO (among them the N_2-fixing *Anabaena cylindrica*) were shown to contain no or an extremely low amount of lipoic acid, whereas the level of lipoic acid in *Anacystis* was sufficient to account for the occurrence of the pyruvate dehydrogenase complex. Thus, cyanobacteria can be distinguished by their modes to cleave pyruvate. PFO has been demonstrated only in prokaryotes up till now. Higher plants presumably evolved from a cyanobacterial ancestor possessing the pyruvate dehydrogenase complex like *Anacystis*.

PFO typically occurs in anaerobes or is synthesized during anaerobiosis in enteric bacteria. The occurrence of this enzyme in heterocysts (Neuer and Bothe 1985) is not an exception to this rule, since the inside of these cells is anaerobic or semianaerobic due to the thick cell wall with special lipooligosaccharides forming a barrier against the diffusion of oxygen (Walsby 1985). In cell-free preparations, PFO utilizes either ferredoxin or flavodoxin as electron acceptor with more or less the same efficiency (Bothe 1969; 1977). In *Anacystis* (Trebst and Bothe 1966) and in vegetative cells of *Anabaena* PCC 7119 (Fillat et al. 1991), flavodoxin is synthesized under iron-deficiency in the medium. Heterocysts of *A.* 7119 produce flavodoxin constitutively. *Anabaena* ATCC 29211 lacks the potential to synthesize flavodoxin (Sandmann et al. 1990). *Anabaena variabilis*, on the other hand, was reported to express a special ferredoxin (encoded by the *fdxH* gene) in heterocysts under N_2-fixing conditions (Böhme and Haselkorn 1989). Claims were forwarded that this ferredoxin is particularly active in N_2-fixation while being similarly efficient as the ferredoxin from vegetative cells in photosynthetic $NADP^+$-reduction (Schrautemeier and Böhme 1987).

This would be somewhat surprising since the smaller subunit of nitrogenase (nitrogenase reductase) is strongly conserved in all organisms and generally accepts electrons from many kinds of low-potential carriers unspecifically (Bothe et al. 1983). There is no obvious reason why cyanobacteria, depending on the strain, utilize either ferredoxin or flavodoxin in N_2-fixation.

Likewise, the physiological role of PFO is not clear as yet. The pyruvate clastic reaction is apparently not the only one providing reductant to nitrogenase (Bothe and Neuer 1988). It is generally agreed that the hexose monophosphate pathway is particularly active in degrading glucose-6-P to NADPH which reduces ferredoxin or flavodoxin in a reaction catalyzed by NADPH:ferredoxin oxidoreductase in heterocysts (Bothe 1970; Apte et al. 1978). In addition, electrons for the reduction of ferredoxin or flavodoxin can be generated via photosystem I from NADH and a dehydrogenase (Houchins and Hind 1982) or from H_2 and uptake hydrogenase (Eisbrenner and Bothe 1979). The flow of electrons from the different donors (pyruvate, NADH, H_2, NADPH) needs to be regulated. A special role of the thioredoxin system in the regulation of the hexosemonophosphate pathway and the generation of reductant for nitrogenase (Rowell et al. 1988) is now unlikely due to the recent findings that heterocysts and vegetative cells produce essentially the same level of this protein (Dai et al. 1992). Mutational inactivation of the *nifJ* gene might give insights into the role of PFO in the electron transfer to nitrogenase and its regulation.

Acknowledgement. This work was kindly supported by the BMFT in the program "Biologische Wasserstoffgewinnung" (grant no. 0319342A).

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