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Characterization of the Genome Region Encoding an FdxH-Type Ferredoxin and a New 2[4Fe-4S] Ferredoxin from the Nonheterocystous, Nitrogen-Fixing Cyanobacterium *Plectonema boryanum* PCC 73110

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A genomic DNA region with four consecutive open reading frames, including an *fdxH*-type gene, has been sequenced and initially characterized for the nonheterocystous nitrogen-fixing cyanobacterium *Plectonema boryanum* PCC 73110. The *fdxH* gene encodes a [2Fe-2S]-type ferredoxin, 98 amino acids in length, with a deduced molecular mass of 10.9 kDa. Conserved residues include two characteristic lysines at positions 10 and 11, shown recently to be important for interaction with nitrogenase reductase (S. Schmitz, B. Schrautemeier, and H. Böhme, Mol. Gen. Genet. 240:455–460, 1993). The gene is transcribed only under anaerobic nitrogenase-inducing conditions, whereas the *Plectonema petF* gene, encoding a different (type 1) [2Fe-2S] ferredoxin, is only transcribed in cultures growing with combined nitrogen. The *fdxH* gene was expressed in *Escherichia coli* as a holoprotein. The purified protein was able to effectively donate electrons to cyanobacterial nitrogenase, whereas PetF from the same organism was not. The occurrence of FdxH in the nonheterocystous genus *Plectonema* demonstrates for the first time that FdxH-type ferredoxins are not exclusively expressed within heterocysts, as is true for cyanobacteria differentiating these cells for nitrogen fixation under aerobic growth conditions. Two open reading frames that precede *fdxH* have high similarity to those found at a corresponding location in *Anabaena* sp. strain PCC 7120. In the latter organism, they are transcribed only under nitrogen-fixing conditions, but the functions of their gene products remain unclear (D. Borthakur, M. Basche, W. J. Buikema, P. B. Borthakur, and R. Haselkorn, Mol. Gen. Genet. 221:227–234, 1990). An *fdxB*-type gene encoding a 2[4Fe-4S] ferredoxin not previously identified in cyanobacteria is located immediately downstream of *fdxH* in *P. boryanum*.

Ferredoxins of the [2Fe-2S] type are small acidic redox proteins found predominantly in plants, eukaryotic algae, and cyanobacteria. They function as electron carriers primarily at a low redox potential of about −400 mV (2, 30). In cyanobacteria, $NADP^+$ photoreduction and nitrogen fixation are quantitatively the most important processes requiring ferredoxin.

In heterocysts of cyanobacteria, a distinct [2Fe-2S]-type ferredoxin that is especially adapted to electron donation to nitrogenase and is spectroscopically and immunologically distinct from the type 1 [2Fe-2S] ferredoxin from vegetative cells was discovered (5, 37). The corresponding gene from *Anabaena* sp. strain PCC 7120, named *fdxH*, has been sequenced and shown to be coexpressed with the nitrogenase structural genes after combined nitrogen deprivation (3). Expression of the cloned gene as a holoprotein in *Escherichia coli* has been reported (4). Recently, the molecular structure of this protein was resolved at 1.7 Å (0.17 nm) (17). *fdxH* genes are also present in other heterocystous cyanobacteria, including *Calothrix* sp. strain PCC 7601 (38), *Fischerella* sp. strain PCC 7115 (39), and *Nostoc* sp. strain MAC RI (39). A comparison of the amino acid sequences derived from the two published *fdxH* sequences with those of other known [2Fe-2S] ferredoxins from cyanobacteria and plants revealed characteristic conserved regions not found in the latter group. Among these are two lysines at positions 10 and 11, which have now been shown to be important for a specific interaction with cyanobacterial nitrogenase reductase (34).

To learn more about this new type of ferredoxin (FdxH), we have begun studies including several approaches: (i) exchange of single or multiple amino acids by site-directed mutagenesis, leading to altered activity in systems reconstituting cell-free nitrogenase activity and eventually in other FdxH-dependent reactions, (ii) isolation of *fdxH* genes from other cyanobacteria as "natural mutants" to further confirm and identify distinctively conserved regions of their gene products and to assess their occurrence among cyanobacteria, and (iii) investigation of cellular localization, regulation of expression, and possible additional functions of FdxH ferredoxins.

So far, *fdxH* genes have been found to be expressed only in heterocysts, the sites of nitrogen fixation in cyanobacteria differentiating these cells under aerobic growth conditions. The protein was therefore originally termed heterocyst ferredoxin. *Plectonema boryanum* PCC 73110 (hereafter referred to as *P. boryanium*) is a nitrogen-fixing cyanobacterium incapable of differentiating heterocysts. Nitrogen fixation occurs only under microaerobic conditions and therefore must be temporarily separated from photosynthesis in its natural habitat (28, 45).

In this paper, we report the isolation of an *fdxH*-type gene from *P. boryanum* PCC 73110. The gene encodes a [2Fe-2S]-type ferredoxin that is transcribed only under nitrogenase-inducing conditions. Its expression in *E. coli* is demonstrated, and functional studies with the resulting holoprotein confirm its role as a direct electron donor for nitrogenase. Open

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or source
Strains		
P. boryanum PCC 73110	Wild type	PCC[a]
A. variabilis ATCC 29413	Wild type	C. P. Wolk
E. coli MC1061	Propagation and expression of plasmids	7
Plasmids		
pUC18 and pUC19	Vectors for plasmid library, cloning of all resulting recombinant plasmids, and sequencing; Amp^r	47
pAn 321	0.64-kb *Eco*RI fragment containing the complete *fdxH* gene from *Anabaena* sp. strain PCC 7120	4
pAn 655.1	0.52-kb *Hpa*I-*Nhe*I fragment containing the complete *petF* gene from *Anabaena* sp. strain PCC 7120	33
pACE1	2.4-kb *Eco*RI chromosomal fragment from *P. boryanum* PCC 73110 with ORF1, ORF2, *fdxH*, and *fdxB*	This study
pEKA3	1.1-kb internal *Hin*dIII fragment of pACE1 containing ≥90% of *fdxH*	This study
pVAC1	1.9-kb *Hin*dIII fragment containing the *petF* gene from *P. boryanum* PCC 73110	This study

[a] PCC, Pasteur Culture Collection.

reading frames (ORFs) of interest flanking *fdxH* include ORF1 and ORF2, found similarly upstream of *fdxH* in *Anabaena* sp. strain PCC 7120 (6), and a gene encoding a 2[4Fe-4S] ferredoxin not described for cyanobacteria thus far.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are shown in Table 1.

Growth of cells and induction of nitrogenase activity. *P. boryanum* PCC 73110 was grown at 30°C in BG-11 medium with $NaNO_3$ as a nitrogen source (29). The cultures were illuminated with white fluorescent light at 100 microeinsteins m^{-2} s^{-2} and bubbled with air enriched with 1% (vol/vol) CO_2 (noninduced cells). For induction of nitrogenase activity, cells were transferred to 1-liter Erlenmeyer flasks, made and kept anaerobic by adding the photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea at a concentration of 5×10^{-6} M and bubbling with argon for 15 min. The serum-stoppered flasks were then incubated under low-light conditions (5 microeinsteins m^{-2} s^{-2}) with moderate shaking to keep the filaments suspended. At regular intervals, 1-ml samples were removed from the cultures to determine nitrogenase activity, measured as acetylene reduction in septum-closed 8-ml vials, made anaerobic with argon. The vials were incubated in light (200 microeinsteins m^{-2} s^{-2}) in a shaking water bath at 30°C. The ethylene formed was quantitated by use of an HP 5890 series II gas chromatograph equipped with a 2-m Porapak N 80/100 column and a flame ionization detector. After 3 to 5 h of induction (nitrogenase activity of between 10 and 60 μmol of acetylene reduced mg of chlorophyll a^{-1} h^{-1}), the cells were harvested for RNA preparation.

DNA isolation and amplification by the PCR. *Plectonema* genomic DNA was isolated as described previously (42). Degenerate *fdxH*-specific PCR primers for 5′-terminal and 3′-terminal parts of the gene were synthesized with a Gene Assembler Plus (Pharmacia) on the basis of the known derived amino acid sequences for FdxH from *Anabaena* sp. strain PCC 7120 and *Calothrix* sp. strain PCC 7601: 5′-TTGGATCCAT GGCN(A,T)(G,C)NTA(T,C)CA(A,G)GTN(A,C)G-3′ and 5′-TATCTAGACC(T,C)TTN(G,C)(A,T,C)CAT(T,C)TG(T,C) TC(A,G)TC-3′, respectively (N is all four nucleotides; restriction sites *Bam*HI and *Xba*I [underlined] were linked to the 5′ ends for convenient cloning of PCR products). PCR was performed with 100 ng of chromosomal DNA from *P. boryanum* PCC 73110, 200 pmol of each primer, 2 mM deoxynucleoside triphosphates, 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 25 mM KCl, 0.01% gelatin, and 2.5 U of *Thermus aquaticus* (*Taq*) DNA polymerase. After DNA denaturation for 3 min at 94°C, nucleotides, reaction buffer, primers, and enzyme were added. Except for an initial program with three preamplification cycles at an annealing temperature of 37°C and then 30 cycles at an annealing temperature of 46°C (nonstringent conditions), the following PCR program was routinely run: 30 cycles each of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. PCR products of desired sizes were excised from agarose gels, purified with a powdered glass suspension (Qiaex kit; Diagen, Düsseldorf, Germany), and reamplified as necessary.

Hybridization and gene isolation techniques. Genomic DNA for Southern hybridization or library construction was digested with *Eco*RI or *Hin*dIII, and the resulting fragments were separated on an 0.8% agarose gel. For gene isolation, a fragment size-selected plasmid library was constructed as follows. Chromosomal restriction fragments of the desired sizes were excised and purified (Qiaex kit; see above). The fragments were ligated into linearized pUC18. *E. coli* MC1061 was transformed with the ligation products and plated on Luria-Bertani agar (31). After colonies were transferred to nylon filters (Hybond N; Amersham Buchler, Braunschweig, Germany), colony hybridization (at 65°C) was performed according to the DIG applications manual (Boehringer GmbH, Mannheim, Germany), except that the DNA was fixed onto the membranes by UV light. Southern hybridization was performed the same way, with homologous or heterologous probes under stringent (65°C) or nonstringent (usually 40°C) conditions. Two heterologous hybridization probes were used: the 0.64-kb *Eco*RI fragment from pAn 321 (4), containing the entire *fdxH* gene from *Anabaena* sp. strain PCC 7120, and the 0.52-kb *Hin*dIII-*Hpa*I fragment (pAn 665.1, derived from pAn 665 (4), carrying the complete *petF* gene from the same organism (1). The 240-bp PCR product amplified from *Plectonema* genomic DNA and heterologous hybridization probes were labeled with digoxigenin-11-dUTP by the random priming technique (10). Hybridizing probe DNA was detected with an anti-digoxigenin antibody coupled to alkaline phosphatase. Probe labeling and color detection were performed with a nonradioactive DNA labeling and detection kit (Boehringer) according to the instructions of the manufacturer.

Other nucleic acid techniques and DNA sequence analysis. Standard methods were used for *E. coli* plasmid isolation and transformation by calcium chloride treatment (31). Alterna-

tively, electroporation with a Bio-Rad Gene Pulser was performed by use of 0.2-cm cuvettes with the following settings: 2,500 V, 200 Ω, and 25 μF. Southern transfer was accomplished by use of an LKB 2016 Vacugene vacuum blotting system (Pharmacia). When chromosomal digests were transferred, the DNA was depurinated by incubating the gels for 20 min with 0.25 N HCl before denaturing the samples for 30 min with 0.5 M NaOH–1.5 M NaCl. Transfer to Hybond N nylon membranes was performed with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate), with subsequent UV fixation. DNA sequences were determined from pUC18 and pUC19 plasmids by use of a T7 sequencing kit (Pharmacia) and pUC forward and reverse sequencing primers. Synthetic oligonucleotides were used to close gaps of a part of the sequence not accessible to subcloning. The EMBL and GenBank data libraries were searched by use of the FASTA search program (27).

RNA isolation and Northern (RNA) analysis. Total RNA from nitrogenase-induced and noninduced cultures was prepared essentially as described by Golden et al. (14). RNA (20 μg per lane) and molecular size standards were glyoxylated and separated on a 1% agarose gel for 4 h at 50 V with 0.1 M Na_2HPO_4 (pH 7.0) as the electrophoresis buffer. RNA was transferred to a nylon membrane (GeneScreen Plus; Dupont, New England Nuclear Research Products) with 10× SSC by the capillary blot technique. The following fragments were used as probes: first, the terminal 354-bp *Hin*cII-*Hin*dIII fragment from pEKA3, containing the *Plectonema fdxH* gene, except for 15 bp of the 3′-terminal coding region, and second, a 292-bp *Mae*I fragment starting at the fifth coding nucleotide and ending at the last coding nucleotide of the *petF* gene from *P. boryanum* PCC 73110, isolated in our laboratory (8). Probe DNA fragments (50 to 100 ng) were excised from agarose gels, purified as described above, and labeled with [α-^{32}P]dATP by use of the random primed DNA labeling kit (Boehringer). Excess label was removed with Sephadex G-50 in a Pasteur pipette equilibrated with TE buffer (31). Prehybridization was performed for 2 h with 25 ml of a solution containing 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 1% sodium dodecyl sulfate, and 0.25 mg of salmon sperm DNA per ml. Hybridization was performed at 65°C overnight with 10 ml of the same solution plus 10% dextran sulfate and a denatured DNA probe.

Expression in *E. coli*, protein purification, and native PAGE. *E. coli* MC1061 harboring pACE1, pAn 321, pAn 665.1, or pVAC1 was grown in 2× YT medium (31) with 40 μM $FeSO_4$ in the presence of ampicillin (100 μg/ml). Growth, harvesting, cell disruption, and purification by anion-exchange chromatography were performed as described previously (4). For high-resolution separation of the recombinant ferredoxins, native polyacrylamide gel electrophoresis (PAGE) was used with a low bisacrylamide/acrylamide ratio (34).

Enzyme assays. Reconstitution of cyanobacterial nitrogenase activity with purified recombinant ferredoxins was performed with anaerobically prepared heterocyst homogenates from *Anabaena variabilis* ATCC 29413 (40). The modified assay mixture contained, in a final volume of 200 μl, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.5), 2.5 mM glucose 6-phosphate, 0.06 mM $NADP^+$, 0.2 mg of bovine serum albumin, and 10 μl of heterocyst homogenate, corresponding to 5 μg of chlorophyll *a*. An ATP-generating system was included and consisted of 5 mM ATP, 5 mM $MgCl_2$, 15 mM creatine phosphate, and 20 μg of creatine kinase. Ferredoxins were added as described in Results and Discussion.

Nucleotide sequence accession number. The nucleotide sequence reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number X71865.

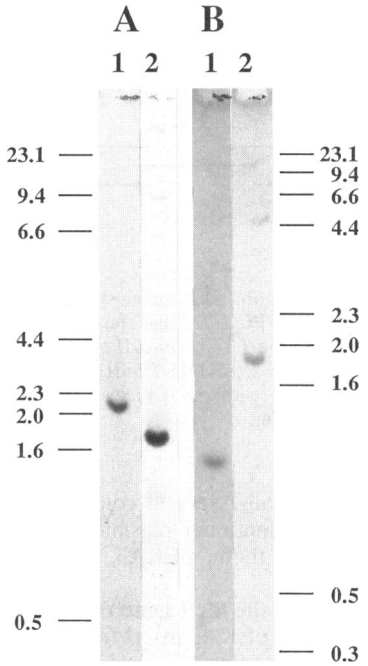

FIG. 1. Southern hybridization of total DNA from *P. boryanum* PCC 73110. Genomic DNA was restricted with *Eco*RI (A) and *Hin*dIII (B), electrophoresed, and blotted. Blots were hybridized with *fdxH* (lanes 1) and *petF* (lanes 2) gene fragments from *Anabaena* sp. strain PCC 7120 as described in Materials and Methods. Molecular size standards (in kilobase pairs) are shown on the left (A) and right (B).

RESULTS AND DISCUSSION

Gene isolation. To detect genes coding for different [2Fe-2S] ferredoxin types in *P. boryanum* PCC 73110, the *fdxH* and *petF* genes of *Anabaena* sp. strain PCC 7120 were used as heterologous hybridization probes. Southern hybridization with these two probes under nonstringent conditions identified fragments clearly different in size: the *fdxH* probe detected 2.4-kb *Eco*RI and 1.1-kb *Hin*dIII fragments, whereas 1.6-kb *Eco*RI and 1.9-kb *Hin*dIII fragments hybridized with the *petF* probe (Fig. 1). These results suggested the presence of an *fdxH*-type gene distinct from a *petF*-type gene.

Two degenerate *fdxH*-specific oligonucleotides were synthesized on the basis of the consensus amino acid sequence of the two *fdxH* genes known so far. One included the initiator codon plus six residues (MA[S,T]YQVR), and the other comprised residues 68 to 74 (DEQM[G,S]KG; see Fig. 6). This primer pair proved suitable for the isolation of several *fdxH* genes from other cyanobacterial species (39). When a band of the expected size (240 bp) appeared among others on agarose gels after one or more PCR amplification rounds, it was excised, purified, and reamplified until a single 240-bp product was left (≥1 μg of DNA). The purified product was then used as a homologous hybridization probe without further cloning. It hybridized to the same fragment sizes on a Southern blot as did the heterologous *fdxH* probe in Fig. 1, suggesting successful amplification of part of an *fdxH*-type gene.

A size-selected plasmid library with chromosomal *Hin*dIII fragments about 1.1 kb in size was constructed, and colony hybridization with the 240-bp PCR fragment was performed

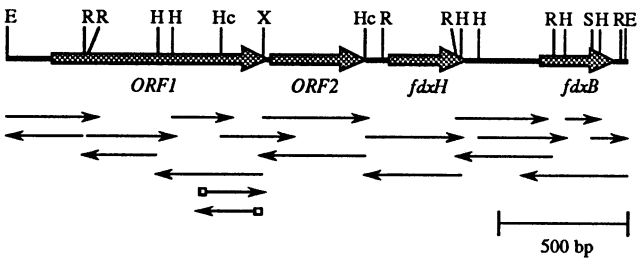

FIG. 2. Physical map of and sequencing strategy for the *fdxH* gene region from *P. boryanum* PCC 73110 (The 2.4-kb *Eco*RI fragment from pACE1 containing the 1.1-kb *Hin*dIII fragment from pEKA3 is shown.) Restriction sites: E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; R, *Rsa*I; S, *Sau*3AI; X, *Xba*I. Arrows with open boxes indicate region-specific primers used for sequencing.

under stringent conditions. Several colonies gave strong signals, and most of them contained plasmids with identical insert sizes (1.1 kb). One of them, pEKA3, was used for further analysis.

Sequence analysis of the *fdxH* gene region. Initial sequencing from one end of the pEKA3 insert fragment detected most of a reading frame encoding an *fdxH*-type ferredoxin gene. Colony hybridization of a size-selected plasmid library with the PCR probe was used to isolate the 2.4-kb *Eco*RI fragment (pACE1) shown to hybridize to the heterologous *fdxH* gene (Fig. 1). This fragment was sequenced completely, and 15 nucleotides encoding the C-terminal 5 amino acids of the *fdxH*-type gene were shown to lie outside pEKA3. In addition, the fragment was found to contain three more ORFs (Fig. 2 and 3).

As shown in Fig. 4A and B, the two ORFs preceding *fdxH* show a high degree of identical or similar amino acids when compared with ORF1 and ORF2 found upstream of *fdxH* in *Anabaena* sp. strain PCC 7120 in a very similar organization (6); 71% identity and 80% similarity are found for ORF1, and 64% identity and 83% similarity are found for ORF2. ORF1, as reported previously (6), lacks a ribosome binding site 5′ of the gene. However, a sequence alignment with the corresponding *Plectonema* DNA region reveals high identity values reaching to 45 bases upstream from the previously assumed ATG initiator codon (Fig. 5). Here, properly spaced ribosome binding sites are followed by putative GTG initiator codons in both organisms. Accordingly, alignment of the derived 15 amino acids in this N-terminal region reveals 80% identity and 93% similarity (Fig. 4A).

Sequence comparisons of the same region from three other heterocyst-forming cyanobacteria, *Fischerella* sp. strain PCC 7115, *Nostoc* sp. strain MAC RI, and *A. variabilis* ATCC 29413 (35), reveal the same putative GTG initiator codon for ORF1 in the *fdxH* gene region, including classical upstream ribosome binding sites. Moreover, in *A. variabilis* ATCC 29413, an ATG initiator codon is lacking at the previously assumed coding start site for ORF1. These data strongly suggest that GTG is used as an initiator codon for ORF1. Evidence for GTG initiation in cyanobacteria has been reported elsewhere, e.g., for the *psbC* gene of *Synechococcus* sp. strain PCC 7942 (15) and for a gene (*lpcB*) encoding a red light-induced phycobilisome linker protein in *Fremyella diplosiphon* UTEX 841 (confirmed by protein sequencing) (24). In addition, GTG initiation for one gene (encoding ADP-glucose pyrophosphorylase) in two different cyanobacteria has also been observed and was confirmed by protein sequencing (9, 19). Possible regulatory implications at the posttranscriptional level related to GTG initiation remain to be established (for a review, see reference 13).

As in *Anabaena* sp. strain PCC 7120, the ATG initiator codon for ORF2 in *P. boryanum* PCC 73110 follows a few base pairs downstream from the 3′ end of ORF1. However, the termination codon for ORF2 is 30 bp further downstream in *P. boryanum* PCC 73110 (and also in *A. variabilis* ATCC 29413) than in *Anabaena* sp. strain PCC 7120 (6). Again, high identity values on the DNA and derived amino acid levels (including a termination codon) are also found in the respective downstream region of the *Anabaena* sp. strain PCC 7120 sequence (Fig. 4B). Taken together, the data suggest that both ORF1 and ORF2 generally are present in nitrogen-fixing cyanobacteria and are longer than previously described.

The next gene, *fdxH*, comprises 297 bp and encodes a [2Fe-2S] ferredoxin of 98 amino acids with a derived molecular mass of 10.9 kDa. The N-terminal methionine is likely to be removed posttranslationally (3). An upstream ribosome binding site and an inverted repeat sequence downstream of the gene are indicated in Fig. 3. The derived protein sequence has been aligned with the two complete published FdxH sequences (Fig. 6). As a group, these proteins have been compared in descending order of identity with other [2Fe-2S] ferredoxins (Fd) from various sources that represent mostly type 1 (PetF) ferredoxins. The residues conserved in the FdxH group and different from strongly conserved residues in the group of type 1 ferredoxins have been marked with vertical arrows. These comparisons show that FdxH-type ferredoxins form a well-defined separate group. FdxH-specific lysines 10 and 11, important for an interaction with nitrogenase (34), are present as expected in the *Plectonema* FdxH sequence. It is interesting to note that among the type 1 ferredoxins more similar to the FdxH group (about 57 to 60% identity), some originate from eukaryotic algae. Two of the eukaryotic ferredoxins also have lysines at position 10. This fact raises questions concerning their function and evolutionary origin in these organisms. On the other hand, an identity value of not more than 69% between *Anabaena* sp. strain PCC 7120 FdxH and *P. boryanum* PCC 73110 FdxH shows that considerable sequence variation is also possible within this ferredoxin group. Possibly its members are also diverse in function. Alternative nitrogenases found in some cyanobacteria are candidates for such alternate FdxH functions. Recently, genes for an alternative vanadium-dependent nitrogenase (*vnf-DGK*) in *A. variabilis* ATCC 29413 were characterized (44). Different FdxH-type ferredoxins in one cyanobacterium are thus conceivable but have not yet been identified. In the context of alternate functions, it should be mentioned that a protein with significant similarity to nitrogenase reductase, the *frxC* gene product, is involved in a step of light-independent chlorophyll biosynthesis (protochlorophyllide reduction) in *Plectonema* and *Chlamydomonas* strains (11, 12, 43). Perhaps an FdxH-like ferredoxin acts as an electron donor for this reduction by interacting with FrxC.

This is the first report of an *fdxH*-type ferredoxin gene expressed in a cyanobacterium unable to differentiate heterocysts. It follows that *fdxH* expression in cyanobacteria is not in principle confined to heterocysts, except in species differentiating this cell type for nitrogen fixation in an aerobic environment. In *Plectonema* strains, nitrogenase is expressed in all cells of a filament (28). A new small (6-kDa) soluble [4Fe-4S] ferredoxin was recently identified in nitrogen-fixing cultures of *A. variabilis* ATCC 29413 (46). However, its role in nitrogen fixation is unclear at present.

An *fdxB*-type 2[4Fe-4S] ferredoxin gene downstream of *fdxH*. The ORF starting 286 bp downstream from the 3′ end of *fdxH* (Fig. 2 and 3) has not been found in cyanobacteria before.

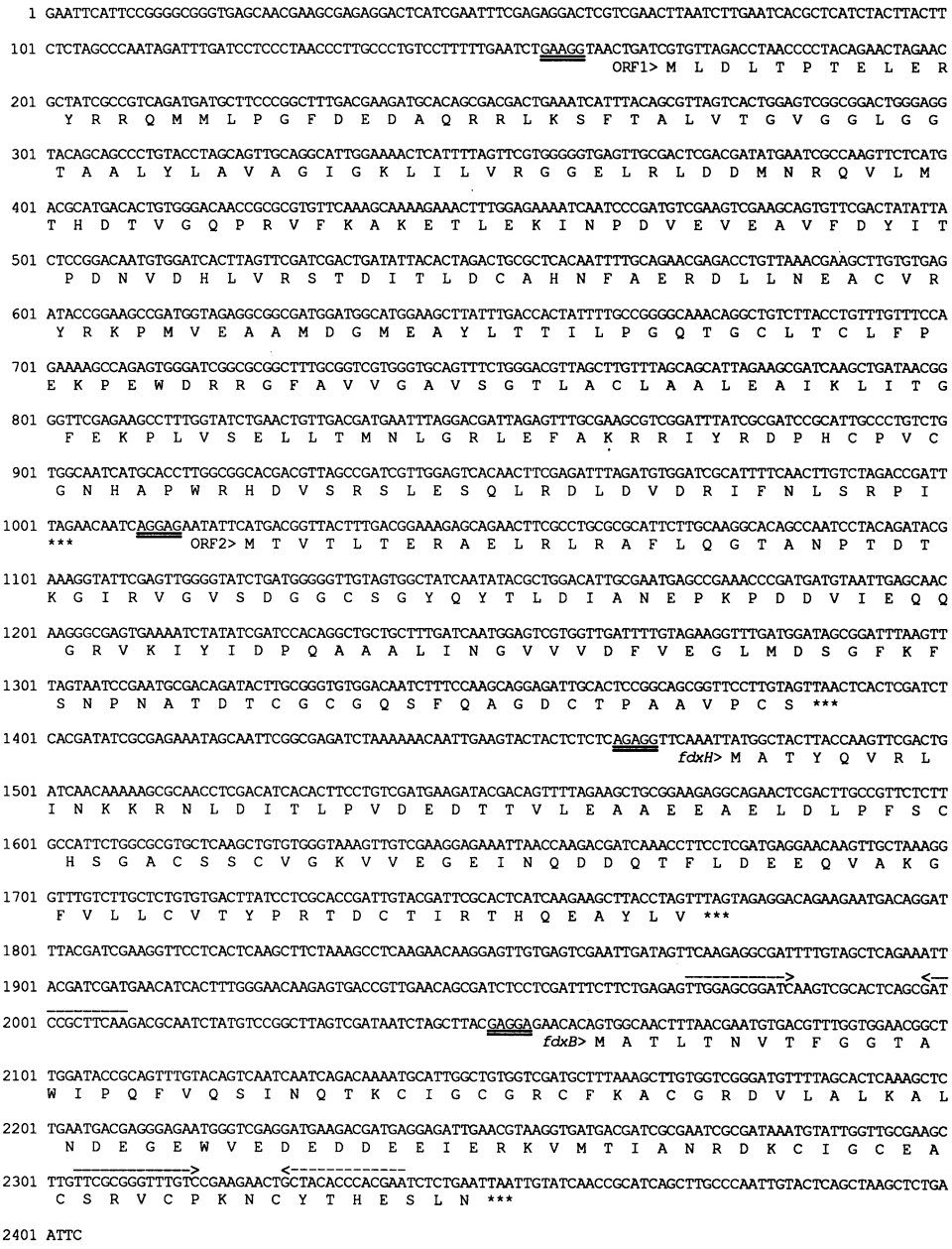

FIG. 3. Nucleotide and derived amino acid sequences of the 2.4-kb *Eco*RI fragment including ORF1, ORF2, *fdxH*, and *fdxB*. Horizontal arrows show inverted repeat sequences. Putative ribosome binding sites are doubly underlined. GTG initiator codons have been translated to methionines.

It was also identified at this position in *A. variabilis* ATCC 29413 (33), but not within 3.4 kb downstream of *Anabaena* sp. strain PCC 7120 *fdxH* (23). Like ORF1, it has a GTG initiator codon preceded by a ribosome binding site and is followed by a downstream inverted repeat. In this case, GTG initiation is also inferred by comparison with the sequence of the *fdxB* gene from *A. variabilis*, which initiates with ATG at this position (data not shown). In Fig. 4C, the derived protein sequence shows regions of extensive similarity when aligned to the protein sequence of ORF5, now called *fdxB*, from the *nif* gene region of *Rhodobacter capsulatus* (25). Their overall identity is 45% (58% similarity). The two protein sequences are especially well conserved in the regions of the two cysteine motifs indicative of a 2[4Fe-4S] (bacterial)-type ferredoxin (93% identity within the first region and 82% within the second region. Cysteine spacing is identical for both proteins.

No significant identity is seen when the FdxB-type ferredoxins are compared with the FdxN-type 2[4Fe-4S] ferredoxins found to be associated with *nif* gene regions in nitrogen-fixing bacteria and cyanobacteria (26, 32; alignment not shown). The two ferredoxin types characteristically differ in the cysteine spacing of the second (C-terminal) of the two cysteine motifs responsible for coordinating the [4Fe-4S] centers. This motif is split by 7 to 9 amino acids between the second and third cysteines in FdxN proteins. Thus far, a requirement of FdxN for nitrogen fixation has been demonstrated only in *Rhizobium meliloti* (21) and *R. capsulatus* (30a).

The *Rhodobacter* FdxB protein (called Fd III) was recently

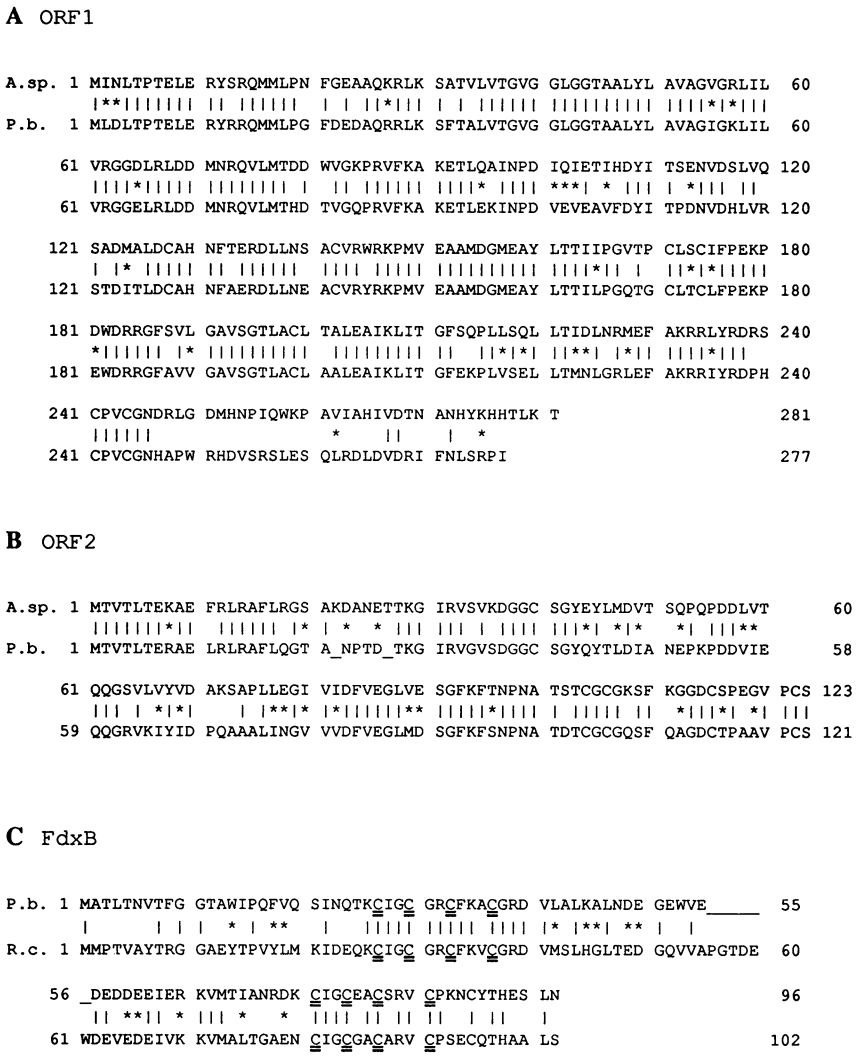

FIG. 4. Alignment of ORF1 (A) and ORF2 (B) from *P. boryanum* PCC 73110 (P.b.) and from *Anabaena* sp. strain PCC 7120 (A.sp.) and of the *fdxB* genes (C) from *P. boryanum* PCC 73110 and *R. capsulatus* (R.c.). Conserved cysteines are doubly underlined. GTG initiator codons have been translated to methionines. Identical amino acids are shown by vertical lines; similar amino acids are indicated by asterisks.

purified (18). It was isolated as a homodimer and found to be oxygen sensitive. However, it was active neither in electron donation to nitrogenase from the same organism nor in $NADP^+$ photoreduction of spinach chloroplasts. Although this protein was detectable in cells only under nitrogenase-inducing conditions (18), its function remains unclear, as it was not essential for growth under nitrogen-fixing conditions in *Rhodobacter* strains (25). The function of FdxB can now be comparatively investigated in cyanobacteria.

Comparative Northern analysis: *fdxH* versus *petF* transcription. To examine the pattern of *fdxH* expression, total RNA, isolated either from cells grown with nitrate or from nitroge-

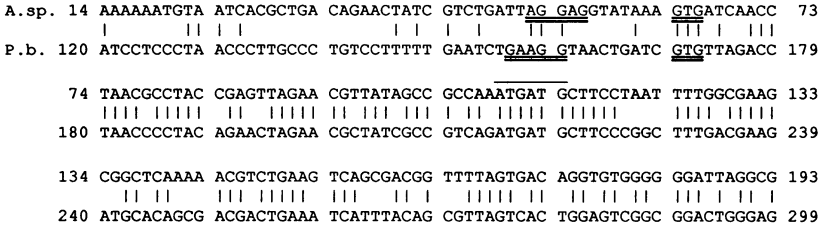

FIG. 5. DNA alignment of the initiator codon regions of ORF1 from *Anabaena* sp. strain PCC 7120 (A.sp.) and from *P. boryanum* PCC 73110 (P.b.). The putative ribosome binding sites and the following GTG codons are both doubly underlined. The previously assumed translation initiation region (two methionine residues) (6) is overlined.

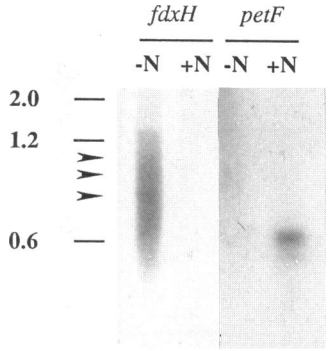

FIG. 7. Northern blot analysis of the *fdxH* and *petF* transcripts in response to the nitrogen source in the growth medium of *P. boryanum* PCC 73110. −N, RNA from nitrogenase-induced cells; +N, RNA from cells grown with nitrate. Arrowheads indicate transcripts of 0.9, 1.0, and 1.1 kb. The *fdxH*- and *petF*-specific hybridization probes are described in Results and Discussion.

nase-induced cells, was hybridized with *fdxH* and *petF* gene probes (the latter was also isolated from *P. boryanum*) (8) under stringent conditions. Figure 7 shows that *fdxH* and *petF* are transcribed in a complementary fashion in *P. boryanum*. A major transcript of about 900 bases and two minor transcripts of 1 and 1.1 kb were seen with the *fdxH* probe exclusively under anaerobic, nitrogenase-inducing conditions. The lengths of the transcripts suggest that *fdxH* can be part of an operon that may include either ORF2 or *fdxB* but apparently not ORF1. This suggestion is in contrast to the situation for *Anabaena* sp. strain PCC 7120, in which ORF1, ORF2, and *fdxH* were shown to be cotranscribed (3, 6). In contrast, *petF* is transcribed in *P. boryanum* only during growth with nitrate. Its single transcript is approximately 600 bases in length, similar to that found in *Anabaena* sp. strain PCC 7120 (1).

Expression of the *fdxH* gene in *E. coli*. The expression of *fdxH* in *E. coli* was examined with pACE1, which carries the 2.4-kb insert fragment shown in Fig. 2 cloned in pUC18 with the *lac* promoter upstream from ORF1. After growth and subsequent cell disruption, a reddish brown band, FdxH, was visible in native PAGE of crude cell extracts and after partial purification; this band corresponded to a strong protein band after Coomassie blue staining (Fig. 8, lane 4). *Plectonema* FdxH was compared with recombinant, partially purified PetF from the same organism (lane 3) as well as with PetF and FdxH from *Anabaena* sp. strain PCC 7120 (lanes 1 and 2). Control extracts from *E. coli* carrying pUC18 without the insert produced no such band (data not shown). These results demonstrate that the *Plectonema fdxH* and *petF* genes are expressed in *E. coli* as holoproteins, as shown previously for *fdxH* and

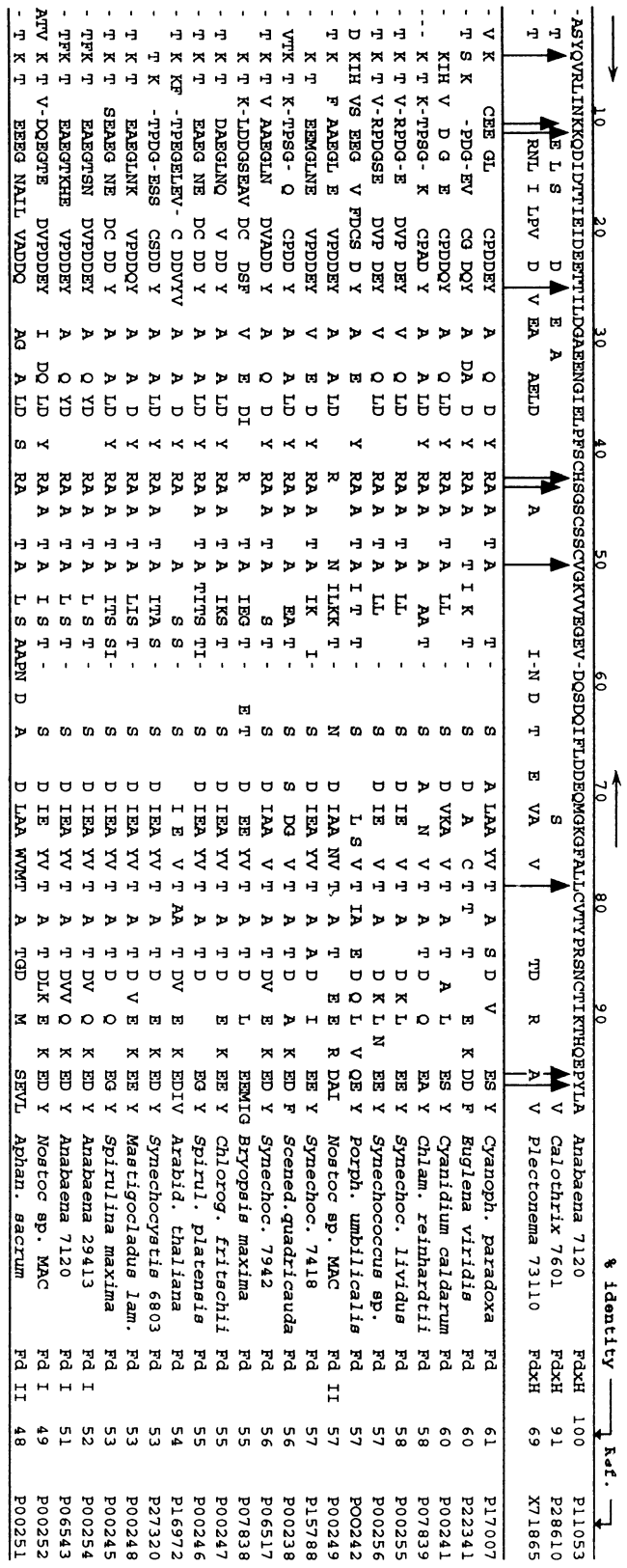

FIG. 6. Amino acid sequence alignment of known FdxH-type ferredoxins and a group of PetF (type 1) (Fd) ferredoxins from various sources. All sequences are compared with the upper sequence set as 100% identity. Residues identical to those in the upper sequence have been omitted for better visibility of differences. Dashes are inserted for proper alignment. Horizontal arrows denote regions from which the *fdxH*-specific PCR primers were derived. The reference (Ref.) numbers show the accession numbers of the respective protein sequences in the Swiss-Prot protein data library. Species abbreviations: *Cyanoph.*, *Cyanophora*; *Chlam.*, *Chlamydomonas*; *Synechoc.*, *Synechococcus*; *Porph.*, *Porphyra*; *Scened.*, *Scenedesmus*; *Chlorog.*, *Chlorogloeopsis*; *Spirul.*, *Spirulina*; *Arabid.*, *Arabidopsis*; *Mastigocladus lam.*, *Mastigocladus laminosus*; *Aphan.*, *Aphanothece.*

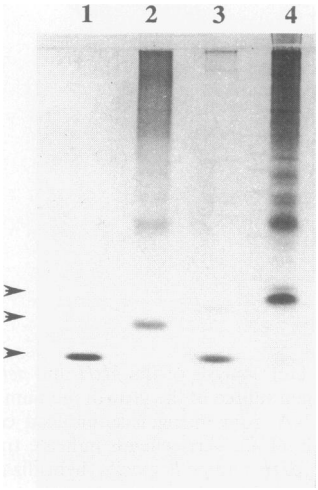

FIG. 8. Native PAGE of partially purified ferredoxins expressed in *E. coli* MC1061. Volumes containing from 2 to 5 μg of ferredoxin were loaded. Lanes: 1 and 2, PetF and FdxH, respectively, from *Anabaena* sp. strain PCC 7120; 3 and 4, PetF and FdxH, respectively, from *P. boryanum* PCC 73110. PetF and FdxH from *Anabaena* sp. strain PCC 7120 were expressed from pAn 655.1 and pAn 321, respectively; pVAC1 and pACE1 were used for *Plectonema* PetF and FdxH expression, respectively. Arrowheads indicate location of ferredoxin bands.

petF from *Anabaena* sp. strain PCC 7120 (4). Both *Plectonema* ferredoxins, purified from *E. coli*, show absorption spectra in the oxidized state characteristic of plant-type [2Fe-2S] ferredoxins, with peaks at 330, 420, and 463 nm and a long wavelength shift from 463 to 466 nm for FdxH (data not shown). These data are in accordance with those for the [2Fe-2S] ferredoxins from *Anabaena* sp. strain PCC 7120 and *A. variabilis* ATCC 29413 (4, 5). The differences seen in mobility in Fig. 8 reflect considerable differences in net charges between PetF- and FdxH-type ferredoxins (−16 for both of the PetF-type ferredoxins and −8 and −11 for the *P. boryanum* PCC 73110 and *Anabaena* sp. strain PCC 7120 FdxH-type ferredoxins). These are mainly caused by amino acid differences in two regions (cf. Fig. 6): KK at positions 10 and 11 → EE or EA and PY at positions 95 and 96 → EE or ED. Whereas the first region is important for specific interaction with nitrogenase, the second may provide specificity in interaction with other redox partners. Three consecutive negatively charged amino acids (EEE or EED at positions 94 to 96) in PetF-type ferredoxins were regarded as candidate residues involved in electron-transferring complex formation with ferredoxin-$NADP^+$ reductase (FNR; 20, 22, 48). If so, the question arises as to how an FdxH-type ferredoxin can effectively interact with FNR, as has been demonstrated in a heterocyst thylakoid system with FNR isolated from heterocysts (37). New evidence from site-directed mutagenesis, however, shows that merely E94, besides F65, both of which are also conserved in FdxH-type proteins, are important for ferredoxin-FNR interactions that control electron transfer between the two proteins (16).

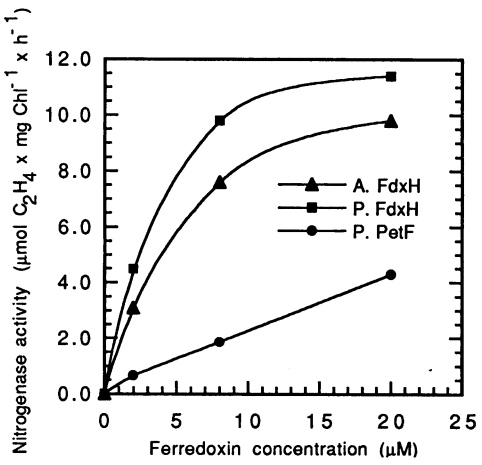

FIG. 9. Reconstitution of nitrogenase activity in heterocyst homogenates from *A. variabilis* ATCC 29413 by the addition of increasing amounts of different ferredoxins. Symbols: ▲, *Anabaena* sp. strain PCC 7120 FdxH; ■, *P. boryanum* PCC 73110 FdxH; ●, *P. boryanum* PCC 73110 PetF. For experimental details, see Materials and Methods. Chl, chlorophyll.

Reconstitution of nitrogenase activity. We established a cell-free system from isolated heterocysts of *A. variabilis* ATCC 29413 with high nitrogenase activity using different physiological electron sources (40). It is suitable for testing the ability of different ferredoxin species from nitrogen-fixing cyanobacteria to donate electrons to nitrogenase. We used anion-exchange chromatography to purify our recombinant ferredoxin species to ≥90% homogeneity before using them in the nitrogenase reconstitution assay. The results shown in Fig. 9 clearly demonstrate that *Plectonema* FdxH is an excellent electron donor for cyanobacterial nitrogenase. It is even somewhat more active than FdxH from *Anabaena* sp. strain PCC 7120. This result may be due to the presence of an additional positively charged residue, arginine at position 12, in *Plectonema* FdxH, improving its interaction with nitrogenase. As shown in Fig. 9, *Plectonema* PetF was more than five times less active at concentrations half saturating for the two FdxH proteins but about four times more active than *Anabaena* sp. strain PCC 7120 PetF (data not shown). Sequence data from the *Plectonema petF* gene (8) indicate that, when compared with FdxH from *Anabaena* sp. strain PCC 7120, *Plectonema* PetF has identity and similarity values (55 and 67%) higher than those of *Anabaena* sp. strain PCC 7120 PetF (51 and 61%); this result may be a reason for the measurable, although limited, activity of *Plectonema* PetF in the nitrogenase assay.

Conclusions. FdxH-type [2Fe-2S] ferredoxins are not restricted to heterocysts but have evolved also in nonheterocystous, nitrogen-fixing cyanobacteria as an additional adaptation to electron donation to nitrogenase. As suggested from the results described before (37) and from the Northern analysis in the present study, it is likely that FdxH-type ferredoxins are able to replace PetF in additional ferredoxin-dependent reactions of a nitrogen-fixing cell. This idea has been confirmed for a light-dependent transhydrogenase reaction (NADH-dependent $NADP^+$ photoreduction via FNR) found to be associated with photosystem I in nitrogen-fixing heterocysts of *A. variabilis* ATCC 29413 (36, 37, 41). Other FdxH-dependent enzymes very likely include glutamate synthase or pyruvate-ferredoxin oxidoreductase. An adaptation to efficient nitrogen fixation may further have led to the coevolution of isoenzymes for some of the above-mentioned ferredoxin-dependent reactions. We will address this question by identifying such enzymes in nitrogen-fixing cells of heterocystous and nonheterocystous cyanobacteria.

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ADDENDUM IN PROOF

Further sequence analysis has revealed an open reading frame with high coding probability located between the *fdxH* and *fdxB* genes (Fig. 2 and 3). It extends from bases 1791 to 2039 with a ribosome-binding site ending at −8 bp from the initiator ATG. The sequence encodes a protein 82 amino acids in length. No significant sequence similarity was detected when compared to other known DNA or protein sequences from the GenBank/EMBL or SWISSPROT data libraries.

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