Molecular evidence for the aerobic expression of \textit{nifJ}, encoding pyruvate:ferredoxin oxidoreductase, in cyanobacteria

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Abstract

Pyruvate:ferredoxin (flavodoxin) oxidoreductase (PFO, EC 1.2.7.1) catalyses the oxidative cleavage of pyruvate and coenzyme A to acetylcoenzyme A and CO$_2$ with concomittant reduction of ferredoxin. PFO occurs in anaerobes and in some aerobic archaea and bacteria. For cyanobacteria, activity measurements indicated the occurrence of PFO in heterocystous forms. The completely sequenced genomes of the unicellular \textit{Synechocystis} sp. PCC 6803 and the heterocystous \textit{Anabaena} sp. PCC 7120 and \textit{Nostoc punctiforme} revealed the existence of one PFO (encoded by \textit{nifJ}) in \textit{Synechocystis} 6803 and \textit{N. punctiforme} but two different PFOs, encoded by \textit{nifJ1} and \textit{nifJ2}, in \textit{Anabaena}. Sequence comparison now indicates that all cyanobacterial PFOs are more closely related to those of anaerobes than to those of aerobes. Reverse transcription-polymerase chain reaction (RT-PCR) experiments show that \textit{nifJ} is transcribed in the presence of saturating iron concentrations in aerobically grown cells of the unicellular \textit{Synechococcus} sp. PCC 6301 and \textit{Synechocystis} 6803. Both \textit{nifJ} genes are transcribed in aerobically grown \textit{Anabaena} 7120. These findings are corroborated by luciferase reporter gene analysis of \textit{nifJ} in \textit{Synechococcus} sp. PCC 7942. The occurrence of PFO in these cyanobacteria is enigmatic. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

In the pyruvate clastic reaction [1], PFO catalyses the oxidative cleavage of pyruvate and coenzyme A to acetylcoenzyme A and CO$_2$ with concomittant reduction of ferredoxin. The enzyme has been described for strictly anaerobic bacteria like \textit{Clostridium} spp. [1], diverse archaea [2] and some eukaryotes [3]. In facultative anaerobes like \textit{Klebsiella pneumoniae}, PFO is expressed under O$_2$-exclusion [4]. The enzyme also occurs in the aerobic, osmototolerant \textit{Halobacterium} [5] and bacteria like \textit{Frankia}, \textit{Myco-bacterium tuberculosis} and \textit{Streptomyces coelicolor} (see Fig. 1) where it may be less O$_2$-sensitive. Depending on the organism and/or the culture conditions, PFO utilises ferredoxin or flavodoxin as electron acceptor. In \textit{K. pneumoniae}, flavodoxin is expressed in parallel with nitrogenase [4]. In \textit{Clostridium}, flavodoxin is synthesised only under Fe-deficiency, and nitrogenase preferentially utilises ferredoxin [6]. The same situation also applies to cyanobacteria [7].

The occurrence of PFO in cyanobacteria has been reported years ago [8]. Biochemical measurements with extracts from \textit{Anabaena cylindrica} showed both a coenzyme A-dependent cleavage of pyruvate as well as a synthesis of pyruvate from acetylcoenzyme A and CO$_2$. This reverse reaction was strictly dependent on reduced ferredoxin [9]. A synthesis of pyruvate via the pyruvate dehydrogenase complex (PDH) with NADH as electron donor was not observed, in accordance with thermodynamic considerations. As the lipoic acid-content was found being too low for a functional PDH, it was concluded that \textit{A. cylindrica} cleaves pyruvate by PFO [10]. In contrast, the unicellular \textit{Synechococcus} 6301 had a high lipoic acid-content. The reduction of NAD$^+$ in dependence of pyruvate and coenzyme A catalysed by PDH was unambiguously demonstrable in extracts of this cyanobacterium [10]. The biochemical measurements did not allow to conclude whether \textit{Synechococcus} 6301 additionally possesses the PFO.

In 1993, two groups, independently from each other, published sequences for \textit{nifJ} encoding PFO in two hetero...
cystous cyanobacteria. In *Anabaena* 7120, *nifJ* was expressed in parallel with flavodoxin under Fe-deficiency [11]. The partially sequenced gene from *Anabaena variabilis* [12] had only about 75% DNA sequence identity with *nifJ* from *Anabaena* 7120. With other genes from *A. variabilis* and *Anabaena* 7120, sequence identities generally amount to at least 95%, indicating the close relationship of these strains (GenBank: AJ003195, X99672). Thus it remained to be shown whether two different PFOs occur in heterocystous cyanobacteria. In the meantime, the completely sequenced genome of *Synechocystis* 6803 [13] revealed the occurrence of one PFO also in this unicellular cyanobacterium. In addition, the genome of *Anabaena* 7120 (www.kazusa.or.jp/cyano/cyano.html) contained homologous sequences obtained from finished and ongoing genome sequencing projects were identified and retrieved through CyanoBase (www.kazusa.or.jp/cyano/cyano.html); the homepage of the DOE joint genome institute (www.jgi.doe.gov) and the TIGR microbial database (www.tigr.org).

In the present study, the PFO sequences from cyanobacteria and from other organisms are compared with each other. Expression of PFOs in unicellular and filamentous cyanobacteria using RT-PCR and bacterial luciferase as reporter system is also demonstrated.

Fig. 1. Sequence similarities of PFO and related oxidoreductases from bacteria, eukarya and archaea. Representative sequences from different taxons were recovered through GenBank (www.ncbi.nlm.nih.gov/BLAST) and the TIGR microbial database (www.tigr.org). The dendrogram depicts sequence similarities calculated from a ClustalX1.8 alignment of approximately 400 amino acid positions (see text) corresponding to (1) the N-terminal part of homodimeric bacterial and eukaryotic pyruvate:ferredoxin oxidoreductases (PFO) and eukaryotic pyruvate:NADP⁺ oxidoreductase (PNO) (black, bold), (2) α-subunits of bacterial and archaeal tetrameric (a) pyruvate and 2-ketoisovalerate oxidoreductases (POR/VOR; grey, bold) and (b) 2-ketoacid oxidoreductases (KOR, including KGOR = 2-ketoglutarate oxidoreductases; grey, bold, dashed), (3) the N-terminal part of α-subunits of archaeal heterodimeric indolepyruvate oxidoreductases (IOR; black) and (4) the C-terminal part of α-subunits of archaeal and bacterial heterodimeric 2-oxoacid (= 2-ketoacid) oxidoreductases (OOR; black, dashed).
2. Materials and methods

2.1. Culture and growth of cyanobacteria

Cells were grown in BG11 medium supplemented with nitrate except for the N₂-fixing *Anabaena* strains which were cultivated in BG11⁺ medium [12]. *Synechococcus* sp. PCC 6301 (= *Anacystis nidulans* SAUG 1402-1) was purchased from the algal collection of the University of Göttingen, Germany, *A. variabilis* was strain ATCC 29413 and *Anabaena* sp. PCC 7119, *Anabaena* sp. PCC 7120, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 (= *A. nidulans* R2) were from the Pasteur Collection. Genetically manipulated strains were selected on chloramphenicol (7.5 μg ml⁻¹) and/or spectinomycin (20 and 2 μg ml⁻¹ on plates and in liquid culture, respectively).

2.2. Isolation and sequencing of *nifJ* from *Anabaena* 7119 and *Synechococcus* 6301

Preparation and restriction of genomic DNA has been described [12]. Using the 3’ part of the formerly sequenced 267 bp segment of *Anabaena* 7119 *nifJ* [12], an 1.1 kb product was generated by inverse PCR with *SspI*-digested and ligated genomic DNA as template and oligonucleotide primers gtacacgcgtcagctacaccc (CYC1) and gtacgactacagcgcgtcagcgc (CYC2), extending the length of the *nifJ* sequence determined for this organism to 1214 bp. Sequencing was performed using the ABI Prism System (PE-Applied Biosystems, Weiterstadt, Germany). Sequencing was performed using the ABI Prism System (PE-Applied Biosystems, Weiterstadt, Germany). *nifJ* from *Synechococcus* 6301 was identified by hybridising a genomic library (bacteriophage λGEM™11, Promega, Mannheim, Germany) with the *Anabaena* 7119 probe. Labelling of probes and detection of hybridisation signals were performed with the DIG Labeling and Detection kit (Roche Diagnostics, Boehringer, Mannheim, Germany).

The sequence data reported for *nifJ* from *Anabaena* 7119 and *Synechococcus* 6301 have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AF307353 and AF307354, respectively.

2.3. RNA isolation, reverse transcription (RT) and PCR

Total RNA from cyanobacteria was isolated using the RNeasy Kit of Qiagen, Hilden, Germany [14]. Isolated RNA was treated with DNase and cDNA was prepared as described [14]. Primers used for the first strand synthesis (2 pmol for each RT reaction) were sequence-specific in the case of *Synechococcus* 6301 (AN-R: gcgcgtcgtcattgtcgaag) and *Anabaena* 7120 (c377R: attttggaggactggact, = *nifJ1*-specific; c244R: gaccacaccaatttagatgcgaag, = *nifJ2*-specific). A primer recognising *nifJ* from cyanobacteria, based on multisequence alignments, was used in the case of *Synechococcus* 6803: NiJJA3 = tc(a/g)tta(t/a/g)gata(alg/t)gtgt-(al/g)cg(a/g)aa(t/a/g)cca. The RT reaction was followed by PCR, using the same (reverse) primers as well as the forward primers AN-F, gcagcttacctcagcaagtc (*Synechococcus* 6301); c377F, ggcttacaccttaacaa (Anabaena 7120, *nifJ1*); c244F, gcacagagcttccagtc (Anabaena 7120, *nifJ2*) and NiJJA1, attc(a/t)attta(c/t)cc(g/c)att(c/t)ttac(a/c/t)cc(a/c/t)tc (*Synechocystis* 6803). Amplification by PCR was performed either with 2 μl of the cDNA obtained by RT, 2 μl of the control (no RTase), 2–5 ng genomic DNA or in the absence of template in a 50 μl assay (100 pmol of each primer, 2 mM MgSO₄, 2 U Taq polymerase from Promega) after 4 min at 95°C running 30 cycles (30 s at 92°C, 1 min at 55°C, 1 min at 72°C) and a final elongation step (7 min at 72°C). PCR products (10 μl of each reaction) were separated on 1% agarose gels.

2.4. Construction of luxAB reporter strains and luciferase measurements

A 700 bp PsI/BamHI fragment (~350 bp of the 5’ untranslated *nifJ* region of *Synechococcus* 6301) was subcloned into *PsI/BamH*-cut pBluescript™IIISK(−). The complete insert was further subcloned by *XbaI/XhoI* restriction and fused to the promoterless bacterial luciferase genes luxAB from *Vibrio harveyi* by introducing the *nifJ* promoter region into the *XbaI/SalI*-cut multicloning site of pAM1580 (see http://ACS.TAMU.EDU/~ssg723/112.html). This pBR322-based vector is routinely used to target specific DNA to the so-called neutral site II of the *Synechococcus* 7942 chromosome via homologous recombination [15]. Strain AMC395 (Sp³), a derivative of *Synechococcus* 7942 carrying the luxCDE genes driven by the *psbA1* promoter in neutral site I of the chromosome and thus expressing the synthetase catalysing the formation of the luciferase long-chain aldehyde substrate, and neutral site II vector pAM1580 were kindly supplied by Susan Golden, Texas A&M University. For transformation experiments, exponentially growing AMC395 (20 ml) was centrifuged (3000 × g, 5 min), the pellet was washed with 10 ml NaCl (10 mM), centrifuged and suspended in 1 ml BG11 medium. Plasmid DNA (0.2–0.5 μg) was added to 300 μl cell suspension. Cells were selected on antibiotics (Cm, Sp)-containing agar plates for 7–10 days.

For luciferase measurements, cells grown in BG11 medium under continuous gassing with air/CO₂ (98/2, by vol) up to an OD₇₅₀ of 0.5–0.8, were diluted to an OD between 0.08 and 0.4 and grown for 18 h (final OD: 0.13–0.65). Cell suspension (200 μl) was added to 200 μl BG11 medium and kept in the dark (3 min) prior to measuring in vivo bioluminescence for five consecutive 30 s intervals in a BIOLUMAT LB 9500 luminometer (Berthold, Wildbad, Germany).

2.5. Sequence comparisons and alignment of PFO with other ketoacid oxidoreductases

Protein sequences were aligned using ClustalX (http://
www.csc.fi/molbio/progs/cluster). The dendrogram inferred from the alignment was generated using TreeView1.6 (http://taxonomy.zoology.gla.ac.uk/rod/treewview.html).

3. Results and discussion

3.1. Two nifJ genes coding for pyruvate:ferredoxin oxidoreductases occur in filamentous Anabaena spp., whereas one is demonstrable in unicellular species

Previously [12], 329 and 267 bp internal segments of nifJ were characterised from A. variabilis and Anabaena 7119, respectively. Now an 1.1 kb nifJ probe, containing part of the earlier characterised 267 bp segment, was generated by inverse PCR from Anabaena 7119 and used in hybridisation experiments of EcoRV/HindIII-digested genomic DNA from A. variabilis. Two hybridisation signals were obtained at 3.5 kb and 3 kb, corresponding to two groups of λ-clones of an A. variabilis genomic library (not shown). This already indicated the existence of an additional nifJ copy in A. variabilis. The completely sequenced genome of Anabaena 7120 contains two nifJ genes, one of which had been described earlier ([11], hereafter named nifJ1). The second copy (nifJ2, located on contig c244, www.kazusa.or.jp/cyano/cyano.html), is more closely related to the partial nifJ sequences from A. variabilis and Anabaena 7119 than to nifJ1 from Anabaena 7120. Using the Anabaena 7119 nifJ probe, this gene has been identified and partially sequenced (615 bp) from Synechococcus 6301. No indications for the existence of additional nifJ copies were obtained for Synechococcus 6301 and Synechocystis 6803, in accordance with sequence data available for the latter organism (www.kazusa.or.jp/cyano/cyano.html).

3.2. Comparison of cyanobacterial PFO sequences

An alignment of the deduced partial PFO sequence from Synechococcus 6301 with PFOs from other cyanobacteria, corresponding to amino acid positions 308–512 of the Synechocystis 6803 sequence, revealed that PFO from Synechococcus 6301 shares 59.8% (67.1%) and 65.2% (72.0%) sequence identity (similarity) with PFO1 and PFO2 from Anabaena 7120, respectively (not documented). Sequence identity (similarity) between both PFOs from Anabaena 7120 is only slightly higher: 68.3% (78.0%).

As observed previously, the sequenced region may contain strain-specific insertions [11]. In Anabaena 7120, nifJ1 contains five tandemly repeated copies of the heptamer CCCCCAGT. The gene of N. punctiforme contains an even larger insert (69 bp), coding for a glycine-rich stretch. The tandemly repeated heptamers are not present. Only three additional bases are inserted in nifJ2 of Anabaena 7120 and 7119. An insert in this region is completely absent in nifJ of Synechocystis 6803 and Synechococcus 6301.

3.3. A dendrogram for PFO and other ketoacid oxidoreductases from archaea, bacteria and eukarya

Most PFOs of mesophilic bacteria and of eukaryotic protozoa are homodimers, whereas enzymes catalysing the oxidative cleavage of pyruvate and/or other ketoacids of hyperthermophilic archaea have a four-subunit structure. A number of organisms, mostly thermophilic archaeal taxaons (Pyrococcus, Archaeoglobus, Methanobacterium), possess tetrameric pyruvate/2-ketoisovalerate/2-ketoacid (POR/VOR/KOR) as well as heterodimeric indolepyruvate oxidoreductases (IOR, see legend to Fig. 1). Methanobacterium and Thermotoga are the only known species, which – in addition to POR, KOR (and IOR) – also contain a heterodimeric 2-oxoacid (= 2-ketoacid) oxidoreductase (OOR). The latter was described for the halophilic Halo bacterium, other aerobic archaea (e.g. Aeropyrum) and bacterial species (Thermus, Thermotoga, several actinobacteria). The heterodimeric OOR is related to homodimeric PFO and tetrameric POR/VOR/KOR. Sequence alignments performed for only some of these oxidoreductase groups are scattered in the literature [2,16–18]. Most of the tetrameric and dimeric oxidoreductase-containing species do not possess a homodimeric PFO. Campylobacter jejuni is an exception as it contains both PFO and a tetrameric oxidoreductase (KOR, Fig. 1). The amino acid sequences corresponding to residues 1–398 in the Synechocystis 6803 PFO are sufficiently similar in all mentioned oxidoreductases to perform an extensive sequence comparison (Fig. 1). The dendrogram depicts that all cyanobacterial PFOs cluster together, being closely related to PFO from Rhodospirillum rubrum (GenBank: X77515) and oth-

![Fig. 2. Agarose gel analysis of polymerase chain reaction products amplified by RT-PCR using nifJ-specific primers. A: Synechococcus sp. PCC 6301; B: Synechocystis sp. PCC 6803. Lane 1: positive controls with genomic DNA as template. Lane 2: negative controls (no addition of reverse transcriptase prior to PCR reaction). Lane 3: cDNA templates. M: 100 bp ladder from Gibco/BRL.](image-url)
er anaerobes and more distantly related to those from aerobes. The present alignment (Fig. 1) confirms [18] that PFO sequences from γ-proteobacteria split into two clusters (Vibrio cholerae and Escherichia coli versus K. pneumoniae and Enterobacter agglomerans). Some organisms possess two or more different PFOs (e.g. Clostridium pasteuriunm contains three PFOs of the homodimeric type, see www.tigr.org). It had been noted earlier that the eukaryotic PFOs are of monophyletic origin [18] and that pyruvate:NADP+ oxidoreductase (PNO) in mitochondria of Euglena gracilis evolved by linking a homodimeric PFO and a flavoenzyme by gene fusion [19].

3.4. nifJ is expressed in aerobically grown cyanobacteria under non-Fe-limitation

Two pairs of specific primers were developed for nifJ from Synechococcus 6301 and Synechocystis 6803 and for each of the two nifJ genes from Anabaena 7120. RT-PCR experiments showed that all these genes are transcribed under aerobic growth conditions (Figs. 2 and 3). To further study nifJ expression in Synechococcus 6301, the putative nifJ promoter region was fused to luxAB and introduced into the chromosome of strain AMC395 (LuxCDE expressing derivative of Synechococcus 7942). Although the two Synechococcus strains 6301 and 7942 are very closely related genetically, Synechococcus 7942 shows a much higher transformability than Synechococcus 6301 and is thus preferentially chosen to study the phenotypic expression of Synechococcus 6301 genes [15]. In vivo luciferase activity of strain SX3 (nifJ::luxAB) was found to be directly correlated with cell density (OD 0.13–0.65, 750 nm) in exponentially growing cultures and was much higher than in the control strain 1580/395 (promoterless luxAB, Fig. 4). This approach also showed that nifJ is expressed in the unicellular Synechococcus 7942, grown aerobically. However, the expression level is low in comparison to that from the bidirectional hydrogenase promoters hoxE and hoxU, which is 2–10-fold higher (O. Schmitz, unpublished).

All these aerobic cultures had been grown under saturating Fe-amounts. We know from long reaching experimental experience [7] that the cells do not contain flavodoxin under these conditions. According to [11], nifJJ is expressed only under Fe-deficiency in Anabaena 7120 as shown by Northern blot experiments. However, nifJ was originally isolated from a heterocyst-specific cDNA library constructed from a non-Fe-deficient culture [11]. The latter finding is corroborated by the present study in which both nifJ genes were shown to be transcribed under normal growth conditions by means of the more sensitive RT-PCR approach. However, nifJ transcription is enhanced when cells are kept overnight in a medium with only 30% of the normal Fe-content (J. Gurke, preliminary data).

3.5. The physiological role of PFOs in cyanobacteria is enigmatic

PFO from many organisms has been reported to be O₂-sensitive. It remains to be shown how PFO can function in aerobic, O₂-evolving cyanobacteria. Heterocysts of cyanobacteria may provide an anaerobic compartment for PFO to function. In these cells, reductants to nitrogenase appear to be mainly generated via glucose-6-P, the hexose-monophosphate shunt, NADPH, NADPH:ferredoxin oxidoreductase and a special type of ferredoxin encoded by fdsH. Mutants in fdsH, however, can still perform N₂-fixation with significant rates [20]. Therefore, an alternative pathway with flavodoxin as an intermediate could exist in heterocysts. The hydroquinone form of all flavodoxins transfers electrons to nitrogenase. A reduction of flavodoxin beyond the semiquinone state (E° = −500 mV for the couple fully reduced/semiquinone by NAD(P)H, E° = −320 mV for NAD(P)/NAD(P)⁺) is thermodynamically non-feasible. When properly reduced to the hydroquinone state, cyanobacterial flavodoxin is able to donate electrons to nitrogenase [7] as in other organisms [4].
Due to the high Fe-demand for nitrogenase biosynthesis, heterocysts may be Fe-limited and may therefore synthesise flavodoxin. The pathway involving pyruvate, PFO, flavodoxin, nitrogenase is thermodynamically feasible and offers an alternative [11].

Outside of heterocysts, PFO has a defined function in fermentative cells. However, fermentation in cyanobacteria is exceptional and restricted to few species which thrive in special habitats [21]. The unicellular and filamentous forms mentioned above are obligate autotrophs or have at best limited fermentative capabilities. The *Synechocystis* 6803 genome contains sequences for the fermentative enzymes phosphotransacetylase and acetate kinase which, surprisingly, may contribute to ATP generation. In addition to PFO, the genomes of *Synechocystis* 6803, *Anabaena* 7120 and *N. punctiforme* contain genes coding for PDH, although the enzyme activity has been reported only for unicellular cyanobacteria [9].

In the present study, transcription of *nifJ* has been detected in *Synechococcus* 6301. The failure to detect *nifJ* in *Synechococcus* 6301 in our preceding publication [12] exemplifies how cautionous negative molecular results should be interpreted. It remains to be shown whether *nifJ* transcription is accompanied by the synthesis of an active PFO in aerobically growing cyanobacteria. Conclusive experiments have not been performed with respect to the O$_2$-sensitivity of PFO in cyanobacteria. Mutant analysis would be a proper tool to resolve the function(s) of the enzyme in cyanobacteria, albeit such studies might be aggrivated by the observation that mutants often do not show distinct phenotypes, as described for mutants impaired in bidirectional hydrogenase [22] or FdxH [20].

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References