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Short Sequence-Paper

Molecular cloning and sequencing of the ferredoxin I *fdxN* gene of the photosynthetic bacterium *Rhodospirillum rubrum*

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Using an oligonucleotide probe derived from the amino acid sequence of *Rhodospirillum rubrum* ferredoxin I, the gene (*fdxN*) was identified, cloned and sequenced. The *FdxN* coding region is 183 nucleotides which codes for a 61 amino acid (7267 Da) protein. Phylogenetic comparisons between the *R. rubrum* FdI and other 8Fe-8S *nif*-coupled ferredoxins showed only moderate degrees of similarity between the amino acid sequences. *R. rubrum* FdI synthesis was stimulated by *nif* derepressing conditions, but was not completely repressed by *nif* repression. Previous reports of an extracellular clostridial-type ferredoxin in *R. rubrum* could not be confirmed.

In the photosynthetic bacterium, *Rhodospirillum rubrum*, four ferredoxins have been reported; ferredoxins I and II are soluble [1,2] and the two other ferredoxins are associated with the photosynthetic membranes [3]. While the cellular role of these low-molecular-weight Fe-S electron carriers is not well understood in phototrophs, the 2[4Fe-4S] ferredoxin I (FdI) was shown to couple electron flow 5-times more efficiently to nitrogenase than did FdII in *R. rubrum* extracts [4]. To better understand their role in cell metabolism, the genes of numerous bacterial ferredoxins designated *fdxN* [5] have been isolated, cloned and sequenced. The expression of *fdxN* in other species has been shown to be coordinated with ferredoxin-like open reading frames and is expressed with various nitrogen fixation (*nif*) genes [6–11]. In *R. meliloti* [11] *fdxN* is essential for nitrogen fixation and in *R. capsulatus* transcription of *fdxN* was turned off when cells were provided with excess fixed nitrogen [12]. Biochemical analysis of ferredoxin in *R. rubrum* has shown that *fdxN* expression is not fully repressed with the *nif* genes which made this system interesting enough to analyze by molecular techniques. The *fdxN* gene from *R. rubrum* was isolated from chromosomal DNA by lysozyme/proteinase K treatment followed by phenol/$CHCl_3$/isoamyl alcohol (50:49:1) extraction, washing with chloroform, and $CsCl_2$ equilibrium centrifugation according to the general methods of Sambrook et al. [13]. The N-terminal amino acid sequence of FdI was determined by automated Edman degradation using an Applied Biosystems Model 477A sequencer. The underlined portion of the sequence (N-ALKITNECISCGACEPECPNEAISVG) was used to synthesize a probe composed of a 96-fold degenerate pool of oligomers having the composition [5′-ATT(CA) ACN ATT(C) GAG(A) TGT(C) AT-3′] with N representing all possible nucleotides. The *fdxN* gene was identified by southern hybridization analysis with this probe; it showed that the *fdxN* sequence was unique in the *R. rubrum* genome as the probe detected a single distinct restriction fragment under high stringency conditions (T_d-5C°) in each of five endonuclease reaction mixtures (data not shown). The hybridizing *Bam*HI fragment was the shortest (approx. 5.7 kb) and was subcloned into pUC19 to give, pU5.7B. A *Sau*3A partial digest of pU5.7B yielded a 1.7 kb fragment that was subcloned (pU5.7B-1.7). An exonuclease III-S1 nuclease generated deletion subclone (using the Erase-A-Base methodology, BRL) was sequenced to reveal the structure of the gene in addition to 106 bp upstream and 31 bp downstream. The pU5.7B-1.7 insert was hybridized against restriction digestions of genomic DNA followed by washings at both high and low stringencies, with the result that under both conditions *fdxN* hybridized only

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The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession number L11914.

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1    GGCTTTGGCGGCTTTCCCCCCGCCTTCCTGCTGGTCCCCTCGGTTCCCTT

51   CAGCAATCGGACGCTTTGGTCCGCAAAAATCTGTCCAGTGGAGTTAGCGT

         Met Ala Leu Lys Ile Thr Asn Glu Cys Ile Ser Cys
101  AAG ATG GCC CTG AAG ATC ACC AAT GAA TGC ATC AGC TGC

     Gly Ala Cys Glu Pro Glu Cys Pro Asn Glu Ala Ile Ser
140  GGC GCC TGC GAG CCG GAG TGC CCC AAC GAA GCG ATT TCC

     Val Gly Asp Gln Thr Phe Thr Ile Asp Pro Ala Lys Cys
180  GTG GGC GAT CAG ACC TTC ACG ATC GAT CCC GCC AAA TGC

     Thr Glu Cys Glu Gly Phe His Asp Gln Pro Gln Cys Val
220  ACC GAA TGC GAA GGC TTC CAC GAT CAG CCC CAA TGC GTG

     Pro Val Cys Pro Val Glu Cys Ile Val Lys Ala END
260  CCG GTT TGT CCG GTG GAA TGC ATC GTC AAG GCG TGA CGC

300  GACGGGTATCAGGGGGGCCCGTCCTTGA
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Fig. 1. Nucleotide sequence of the *R. rubrum fdxN* gene and the corresponding amino acid sequence of FdI. The sequence corresponding to the oligonucleotide probe is underlined. DNA sequencing was according to Sanger [22] by employing the 'Taq Dye Primer and Taq Dye Deoxy Terminator Cycle Sequencing Protocol' developed by ABI (Applied Biosystems Inc., Foster City, CA) using fluorescent labeled primer(s) and labeled dideoxy nucleotides, respectively. The labeled extension products were analyzed on an ABI 373A DNA Sequencer.

to its cognate fragments, suggesting that *R. rubrum fdxN* is a single copy gene.

The nucleotide sequence is shown in Fig. 1. Analysis of *fdxN* and immediate flanking regions showed it to be 60.4% (G + C), which is lower than the 65% estimated for the total genome of *R. rubrum* [14]. Codon usage in the *fdxN* gene appears to be biased towards G or C in the third position as expected in GC-rich bacteria. However, of the seven codons for glutamate, five use GAA and two use GAG; the same glutamate codon usage frequency was reported in Ribsco and B880 holochrome protein from *R. rubrum* [15,16].

The *fdxN* coding region (107–296), which includes start and stop codons, corresponds to a polypeptide of 61 amino acids with a molecular mass of 7267 Da. This compares with the value of 8600 Da determined for the 8Fe-8S holoprotein by sedimentation equilibrium centrifugation [2]. *R. rubrum* FdI has the Cys-X-X-Cys-X-X-Cys-X-X-X-Cys motif near the amino terminus, which is separated by 18 amino acids from Cys-X-X-Cys-(X_7)-Cys-X-X-X-Cys-X-X-X-Cys (Fig. 2). This is typical of the 2[4Fe-4S] ferredoxins that couple to nitrogenase [7]. Ferredoxins from phototrophs have several extra amino acids between the 6th and 7th cysteine residue and an extra cysteine residue at the end of the 2nd cysteine-rich domain when compared to clostridial type ferredoxins (Fig. 2). This motif was first thought to be peculiar to phototrophs [17], but is now recognized in ferredoxins encoded by *nif*-associated genes, and is found in both photosynthetic and non-photosynthetic bacteria [6]. An amino acid sequence alignment of the *R. rubrum fdxN* translation product with other 8Fe-8S bacterial ferredoxin proteins is shown in Fig. 2. The ferredoxins were aligned by inspection because, when they were aligned to maximize the likelihood of conservative amino acid replacement using the Gene-Works program (Intelligenetics), cysteines 7 and 8 of *C. pasteurianum* did not align. A distance tree based on this manual alignment was constructed using the UPGMA program (Intelligenetics) and is shown in Fig. 3. From the ferredoxin amino acid distance tree, it is seen that the *R. meliloti*, *R. palustris* and *R. capsulatus* sequences segregate. However, the latter ferredoxin is less closely related than the other two. *R. rubrum* and *C. limicola* ferredoxins are related to each other and only distantly related to those from the other three purple photosynthetic bacteria. In addition, the *C. pasteurianum* ferredoxin, which is in a different subclass [6], is distantly related to the other sequences (Fig. 3). This alignment assumes equal rates of ferre-

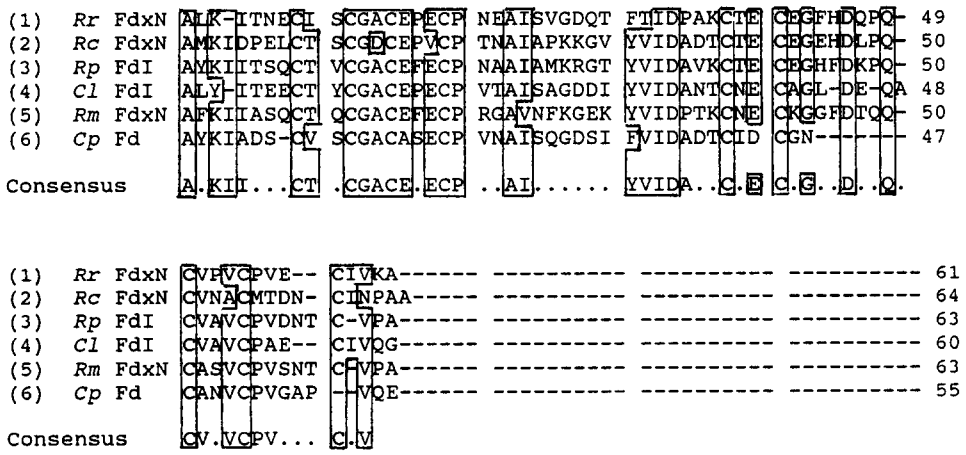

Fig. 2. Alignment of ferredoxin amino acid sequences from: (1) *R. rubrum* (FdxN) (this work); (2) *R. capsulatus* (FdI) [10,12]; (3) *R. palustris* (FdI) [23]; (4) *C. limicola* (FdI) [24]; (5) *R. meliloti* (FdxN) [10] and (6) *C. pasteurianum* [25]. Alignments were based upon visual analysis of the six sequences. It differs from the computerized alignment in that a large gap has been placed at the C-terminal end of the *C. pasteurianum* ferredoxin to get cysteines 7 and 8 to align. The residues enclosed in the boxes represent invariant amino acids in the FdxN protein sequence.

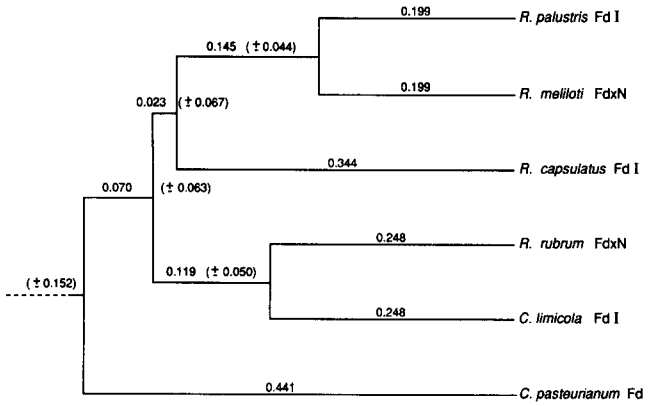

Fig. 3. Phylogenetic distance tree of the ferredoxin amino acid sequences aligned in Fig. 2. Numbers above the branches refer to the corrected estimate of the genetic distance between the putative ancestor gene at the node and the gene at the end of the branch. Hence, the numbers represent the probability that any amino acid will be different between the putative ancestral sequence and the sequence at the end of the node.

doxin gene evolution in all six taxa. However, given the possibility of differential base substitution rates in the different taxa, coupled with selective forces, the alignment may not reflect phylogenetic relationships. The clustering of the *R. rubrum* and *C. limicola* ferredoxins would suggest this. Finally, it remains possible that the long evolutionary distance separating these genes may obscure their true phylogeny.

In *R. capsulatus*, a related non-sulfur phototroph, *nif* repressive conditions completely repress FdI [10,12,20], and linkage of *fdxN* to *nif* loci (*nifN* and *nifE*) has been reported [10,21]. Our work was initiated when it became obvious that *R. rubrum fdxN* was not tightly regulated by the *nif* genes; we saw significant levels of FdI were produced (35 to 50% of the derepressed levels) under *nif* repressing (full NH_4^+) growth conditions (data no shown). This suggests a divergence of gene regulatory elements over time, which is further seen by the fact that *fdxN* maps in different locations in these two organisms (*R. rubrum* and *R. capsulatus*) relative to a particular *nif* gene cluster; cf gene maps in Schatt et al. [10] and Lehman et al. [19]. Both lines of evidence indicate that considerable genetic rearrangements have occurred since their divergence. Molecular phylogenetic analysis of the purple photosynthetic bacteria reveals *R. rubrum* and *R. capsulatus* to occupy distant positions on a denograph [26]. The sequencing of *R. rubrum* FdI gene was under taken in the belief that the difference in expression and location might be accompanied by a fundamental difference in the amino acid sequence and cysteine motif arrangement, but, as shown in Fig. 2, the cysteine motif is identical to other *nif* controlled ferredoxins.

Finally, an 'extracellular' ferredoxin was reported to have been isolated from *R. rubrum* and have a 78% amino acid sequence identity with *C. pasturianum* ferredoxin [18]. However, this protein could not be isolated later from a pure culture of this strain G-9 (Matsubara, H., personal communication), and we could find no evidence that *R. rubrum* strain S1-G excretes a ferredoxin. Furthermore, genetic analysis using a gene probe based on the N-terminal amino acid sequence of *C. pasteurianum* Fd [25], albeit an AT-rich probe, failed to locate a homologous region on the *R. rubrum* genome (Von Sternberg, unpublished data). We conclude that *R. rubrum* does not have a clostridial-type ferredoxin.

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