Volume 292, number 1,2, 13–16 FEBS 10303 November 1991

ADONIS 001457939101011R

Transcriptional analysis of two *Rhodobacter capsulatus* ferredoxins by translational fusion to *Escherichia coli lacZ*

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Received 6 August 1991; revised version received 27 August 1991

Plasmids which contained the translational fusion of *Escherichia coli lacZ* to *Rhodobacter capsulatus* ferredoxin genes, *fdxN* and *fdxA*, were constructed. Effects of growth conditions on the expression of each ferredoxin were analyzed by measuring the β-galactosidase activity in *R. capsulatus* which harbored a corresponding plasmid. Transcription of *fdxN*::*lacZ*, the ferredoxin I fusion gene, was regulated at least 100-fold by either NH_4^+ or O_2 but not by illumination, confirming that *fdxN* belongs to the *nif*-gene family. Transcription of *fdxA*::*lacZ*, the ferredoxin II fusion gene, however, was constant under all the conditions surveyed, suggesting that the protein has some constitutive function(s).

Ferredoxin; Nitrogen fixation; Anaerobic respiration; Transcription; *lacZ* fusion; *Rhodobacter capsulatus*

1. INTRODUCTION

Bacterial ferredoxins are classified into several subgroups, according to the number and properties of their iron-sulfur clusters, which are supposed to reflect the evolutional process of the ferredoxin molecule [1,2]. *Rhodobacter capsulatus* is known to possess multiple ferredoxins of distinct subgroups: *R. capsulatus* ferredoxin I is a 2[4Fe-4S] type and has an amino acid sequence similar to those of other photosynthetic bacterial ferredoxins and *nif* gene-related putative ferredoxins, whereas its ferredoxin II is a [4Fe-4S][3Fe-4S] type and has properties very similar to *Azotobacter vinelandii* ferredoxin I [3–6]. Furthermore, two other ferredoxins have been identified, by genetic studies, in this organism [7,8]. Differences of physiological functions among these ferredoxins are of interest, because each structurally differentiated ferredoxins may possess discrete enzymic specificity. Recently, we cloned and sequenced the genes, *fdxN* and *fdxA*, for these two ferredoxins, I and II [9]. In this paper, we report the construction of ferredoxin–β-galactosidase fusion plasmids and the effect of growth conditions on the transcriptional expression of the fusion genes.

2. MATERIALS AND METHODS

Escherichia coli strains used were described previously [9] and were grown in either LB or LC [11]. *R. capsulatus* SB1003 was used as a host for the fusion plasmids. *R. capsulatus* cells were grown at 30°C in either RCV minimal medium [12], RCV medium depleted of $(NH_4)_2SO_4$ (RCV-NF medium, RCV medium in which $(NH_4)_2SO_4$ was substituted by 10 mM glutamate (RCV-E medium) or RCV medium which was supplemented with 50 mM glucose and 40 mM dimethylsulfoxide (DMSO) (RCV-GD medium). The cells were cultured as 16-ml batches in a 16-ml screw-capped test tube for anaerobic growth, in a 2 l Sakaguchi flask shaken at 110 strokes per minute for aerobic growth, and in a 50 ml test tube with 16 ml RCV-NF medium placed in a GasPak anaerobic jar (BBL Microbiology System) for nitrogen-fixing growth. Light was supplied at 3000 lux by tungsten lamps and darkness was achieved by wrapping the culture vessels with aluminium foil. The concentrations of antibiotics added to the culture were the same as those reported previously [9]. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was added with final concentration of 40 μg/ml.

Recombinant DNA techniques were carried out according to established procedures [13], and conjugation of *E. coli* and *R. capsulatus* was performed as described by Young et al. [14]. Plasmids which possessed the *R. capsulatus* and *E. coli lacZ* fusion gene were constructed as described below.

The 2.0 kbp*Hin*dIII fragment from pHP45Ωsp [15], which contained a spectinomycin resistant (Spc^r) gene flanked by transcription terminators, was cloned in the *Xba*I site of pUC118 by the half-filling method [13]. The resultant plasmid was digested with *Bam*HI and ligated with the 3.1 kbp *Bam*HI fragment which contianed *lacZ* from pMC1871 [16], to produce plasmid pUL12, which is a pUC118 derivative with a *lacZ*-Spc^r cassette at its multi-cloning site. Plasmid pUL12 was digested by *Sal*I, blunted with a Klenow fragment and ligated to produce plasmid pUL13.

A 4.1 kbp *Bam*HI-*Hin*dIII fragment which contained *fdxN* and its upstream, was purified from a cosmid A75 [9] and subcloned into pBR322. The resultant plasmid was digested with *Bcl*I, blunted with a Klenow fragment, and digested further with *Hin*dIII followed by ligation with the *Sma*I-*Hin*dIII fragment which contained the above mentioned *lacZ*-Spc^r cassette from pUL13, to produce plasmid pFAL11, which possessed a *fdxN*::*lacZ* gene. The 5.6 kbp *Sal*I-*Pst*I fragment, containing *fdxN*::*lacZ*-Spc^r, was purified from pFAL11, subcloned into pBR322, and digested with *Sal*I and *Pst*I to construct plasmid pFAL12.

A 1.8 kbp *Hin*dIII-*Sal*I fragment which contained *fdxA* and its upstream, was purified from a cosmid A416 [9] and ligated into pBR322. The resultant plasmid was digested with *Bcl*I, blunted with a Klenow fragment, digested further with *Sal*I, and ligated with the *Sma*I-*Sal*I fragment which contained the above *lacZ*-Spc^r cassette

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from pUL12, to produce plasmid pFBL21, which possessed a *fdxA::lacZ* gene. A 3.1 kbp *Sal*I fragment, which contained *fdxA* and its upstream region, was subcloned from the cosmid A416 into pUC118. The resultant plasmid was digested with *Sal*I, blunted with a Klenow fragment and digested further with *Nsi*I to produce a 1.9 kbp fragment with part of *fdxA* and its upstream region. This fragment was ligated with pFBL21 and digested with *Sca*I and *Nsi*I to yield pFBL20. A *Hin*dIII site was created 28 bp upstream of the *fdxA* initiation codon by site-directed mutagenesis, according to Kunkel's method [17], with an oligonucleotide, pGCTAGGACATAAGCTT-CATGACCTGCGCCAAAGATTG, using the above 1.8 kbp *Hin*dIII-*Sal*I fragment subcloned in pUC118. The sequence of the product was confirmed by the Sanger dideoxy method [18]. A 145 bp *Hin*dIII-*Nsi*I fragment was purified from the engineered plasmid, ligated with pFBL21 and digested with *Hin*dIII and *Nsi*I to construct pFBL22.

R. capsulatus cells were grown to their middle logarithmic phase, chilled on ice, and harvested by centrifugation. The β-galactosidase activity of these cells were measured as described by Biel and Marrs [19] and the amount of protein was determined by the Biuret reaction.

3. RESULTS

Translation fusions of the *fdxN* and *fdxA* gene to the *lacZ* gene were constructed to produce hybrid proteins, which contained the first 33 amino acids of ferredoxin I and the first 79 amino acids of ferredoxin II connected to each other at the 6th position from the amino-terminus of the β-galactosidase. Two plasmids, pFAL11 and pFAL12, in which the length of the *fdxN* upstream regions differed, were prepared with this *fdxN::lacZ* fusion gene (Fig. 1). Both plasmids contained a *nif*-promoter consensus sequence, CTGG-N_8-GTGCT, positioned 129 bp upstream of the *fdxN* initiation codon [20]. For the *fdxA::lacZ* fusion, three plasmids, pFBL20, pFBL21 and pFBL22, each with different length of *fdxA* upstream regions, were constructed (Fig. 1); pFBL20 and pFBL21, but not pFBL22, possessed an *E. coli* type promoter sequence, TTGATT-N_{16}-CTAGGAC, which is located 32 bp upstream of the *fdxA* initiation codon, and two strong palindromic sequences farther upstream [9]. The nearest upstream open reading frame ended at more than 300 bp upstream of the promoter sequence [9]. All the plasmids constructed were introduced into *R. capsulatus* SB1003, in order to investigate the expression of the ferredoxin genes under various growth conditions by measuring the β-galactosidase activity (Table I).

Extracts of *R. capsulatus* cells, which harbored the plasmid pFAL11 and had been grown anaerobically in NH_4^+-free media (RCN-NF), showed significant β-galactosidase activity, and these cells, under such growth conditions, developed a blue color on X-gal-containing solid media. The extent of *fdxN::lacZ* expression was virtually unaffected by the presence or absence of illumination, however, the presence of NH_4^+ reduced the β-galactosidase activity of this strain dramatically. Furthermore, the β-galactosidase activity of this strain was reduced to approximately 30% when the cells were

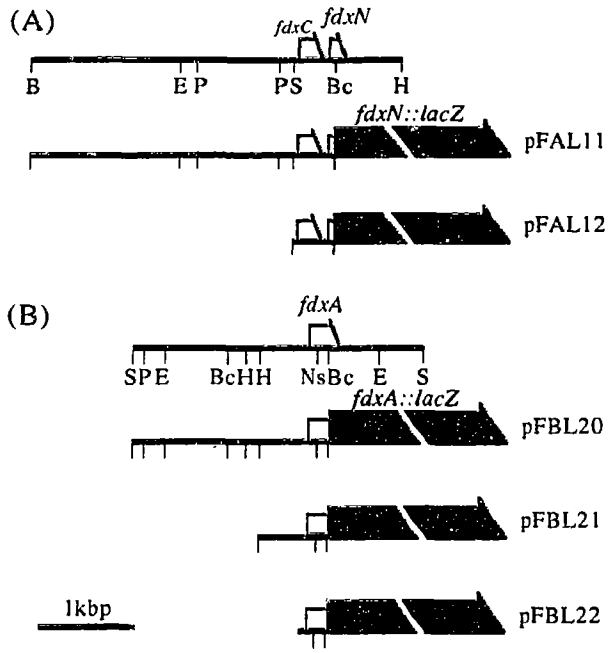

Fig. 1. Restriction maps of *R. capsulatus* genomic DNA around the ferredoxin and translational fusion genes used in this study. These fragments were subcloned in pBR322 derivatives and then introduced into *R. capsulatus* SB1003. (A) Fusions of *fdxN*, the ferredoxin I gene, and *E. coli lacZ*. The plasmids pFAL11 and pFAL12 carry 3.2 kbp and 0.5 kbp of *R. capsulatus* genomic DNA sequences upstream from *fdxN* respectively; *fdxC* shows the chloroplast-type ferredoxin gene [7]. (B) Fusions of *fdxA*, the ferredoxin II gene, and *E. coli lacZ*. The plasmids pFAL20, pFAL21, and pFAL 22 contain sequences which are 3.2 kbp, 1.6 kbp and 32 bp, respectively upstream from *fdxA*; pFAL20 and pFAL21 but not pFAL22, possess an *E. coli*-type promoter sequence upstream from *fdxA*. The solid arrows indicate the regions which originated from *E. coli lacZ*. The restriction enzymes used were: B, *Bam*HI; Bc, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; Ns, *Nsi*I; P, *Pst*I; and S, *Sal*I.

grown with glutamate as a nitrogen source (RCV-E medium). It has been reported that *nif* genes in *R. capsulatus* cells grown on glutamate as a sole nitrogen source are derepressed as much as in the cells grown on molecular nitrogen [21]. In such conditions, the ratio of glutamate to α-ketoglutarate becomes greater to switch on the global transcriptional activators for *nif* genes [22]. These indicate that expression of the fusion gene is regulated more than 100-fold by the presence of NH_4^+ or O_2 in the medium. The *R. capsulatus* cells, which harbored pFAL12, exhibited no significant β-galactosidase activity under the conditions surveyed, which indicates that a NifA-binding site and/or *nif*-promoter for *fdxN* transcription is located in the upstream region possessed by pFAL11 but not by pFAL12.

Extracts of *R. capsulatus* cells, which harbored either pFBL20 or pFBL21, exhibited considerable β-galactosidase activity (Table I), and these cells displayed a faint, but definite, blue color on the solid media containing X-gal. Although the values fluctuated by up to about 2-fold, the *fdxA::lacZ* gene expression was

Table I

Activities of *E. coli* β-galactosidase fusion to *R. capsulatus* ferredoxins

	Specificity Activity for β-Galactosidase (ng *o*-nitrophenol produced / mg protein)					
	$-O_2$Light			$+O_2$Dark	$-O_2$Dark (+DMSO)	
N-source / Plasmid	NH_4^+	Glutamate	N_2	NH_4^+	NH_4^+	Glutamate
pFAL11	120 (1)	14,000 (117)	46,800 (390)	150 (1.3)	62 (0.5)	12,800 (107)
pFAL12	1,000 (1)	1,300 (1.3)	1,000 (1.0)	n.d.	n.d.	n.d.
pFBL20	3,300 (1)	3,500 (1.1)	2,000 (0.6)	3,700 (1.1)	1,600 (0.5)	1,800 (0.5)
pFB21	1,800 (1)	2,000 (1.1)	2,100 (1.2)	2,500 (1.3)	2,600 (1.3)	1,700 (0.9)
pFBL22	120 (1)	n.d.	n.d.	200 (1.7)	n.d.	n.d.

Values were averaged of at least four independent experiments, n.d., not determined. Values in parentheses are relative values against the activity for the cells grown in RCV medium under illuminated anaerobic conditions.

regarded as remaining constant under all the growth conditions studied. No great increase of β-galactosidase activity in the absence of O_2 and NH_4^+ was observed, unlike the situation with the *fdxN*::*lacZ* gene. The *R. capsulatus* cells, which contianed pFBL22, showed very low β- galactosidase activity, indicating that the promoter for the *fdxA* gene is located in the region included in pFBL21.

4. DISCUSSION

We have demonstrated, by *lacZ* fusion, that expression of *R. capsulatus* ferredoxin I is regulated by either O_2 or NH_4^+, but not by light, and that ferredoxin II expression is constant under all the growth conditions investigated.

The ferredoxin I gene is located in the vicinity of one of the major *nif*-gene clusters in *R. capsulatus* [20], and derepression of its expression under illuminated anaerobic conditions in NH_4^+-depleted media has been demonstrated by both Northern [20] and Western [23] analysis. Moreover, we found recently that *R. capsulatus* strains disrupted of *fdxN* by interposon mutagenesis can hardly grow by nitrogen fixation and that *fdxN* is required to complement such mutants [9]. Not only have the results of this study reconfirmed that ferredoxin I is a member of the *nif*-family proteins, but they have provided evidence that the gene was expressed under dark anaerobic conditions in an NH_4^+-depleted medium, which was supplemented with glucose and DMSO. Our results also have revealed that the *nif*-promoter consensus sequence, situated 129 bp upstream of the *fdxN* initiation codon [20], alone did not induce the functional expression of *fdxN* and that at least a *cis*-acting element, a NifA-binding site, must be located in a region farther upstream. This is consistent with our observation on mutants disrupted of *fdxN* [9].

The results which showed that *fdxA* gene expression remained constant under the various conditions surveyed suggest that ferredoxin II has some constitutive function in *R. capsulatus*. This may be in accordance with the fact that we have been unable to disrupt this gene [9].

In contrast to our results, Yakunin and Gogotov reported that *R. capsulatus* cells grown under dark anaerobic conditions in the glucose and DMSO-supplemented medium contained high amount of ferredoxin II but not ferredoxin I [5]. In order to reconcile our results and theirs, two sets of assumptions about protein turnover and gene regulation under these conditions must be made. The first assumption is that degradation of ferredoxin I becomes very fast or formation of holo-ferredoxin I needs some unknown component(s), the expression of which does not occur under these conditions. The second is that degradation of ferredoxin II becomes slower to accumulate the protein or an unknown *cis*-acting activator exists at an upstream region, which was not included in our plasmids. At present, we do not think that ferredoxin degradation in a bacterium is so specific or concerted, nor do we think holoferredoxin I formation needs unknown factor(s), because Grabau et al. reported recently that the synthesis of holo-ferredoxin I even occurs in *E. coli* which contains no 2[4Fe-4S] ferredoxin of its own [24]. The existence of an unknown *cis*-acting element in the region farther upstream from *Sal*I site cannot be excluded, however, and the reason why ferredoxin I was absent despite the strong transcriptional expression of *fdxN* must be established. Further genetic and biochemical studies are needed to elucidate the specific reactions in which each ferredoxin is involved.

REFERENCES

[1] Fukuyama, K., Nagahara, Y., Tsukihara, T., Katsube, Y., Hase, T. and Matsubara, H. (1988) J. Mol. Biol. 199, 183–193.
[2] Bruschi, M. and Guerlesquin, F. (1988) FEMS Microbiol. Rev. 54, 155–176.

[3] Saeki, K., Suetsugu, Y., Yao, Y., Horio, T., Marrs, B.L. and Matsubara, H. (1990) J. Biochem. 108, 475–482.
[4] Duport, C., Jouanneau, Y. and Vignais, P.M. (1990) Nucleic Acids Res. 18, 4618.
[5] Yakunin, A.F. and Gogotov, I.N. (1983) Biochim. Biophys. Acta. 725, 298–308.
[6] Jouanneau, Y., Meyer, C., Gaillard, J. and Vignais, P.M. (1990) Biochem. Biophys. Res. Commun. 171, 273–279.
[7] Saeki, K., Miyatake, Y., Young, D.A., Marrs, B.L. and Matsubara, H. (1990) Nucleic Acids Res. 18, 1060.
[8] Moreno-Vivian, C., Hennecke, S., Pühler, A. and Klipp, W. (1989) J. Bacteriol. 171, 2591–2598.
[9] Saeki, K., Suetsugu, Y., Tokuda, K., Miyatake, Y., Young, D.A., Marrs, B.L. and Matsubara, H. (1991) J. Biol. Chem. 266, 12889–12895.
[10] Giuliano, G., Pollock, D., Stapp, H. and Scolnik, P.A. (1988) Mol. Gen. Genet. 213, 78–83.
[11] Marrs, B.L. (1981) J. Bacteriol. 146, 1003–1012.
[12] Weaver, P.F., Wall, J.D. and Gest, H. (1975) Arch. Microbiol. 105, 207–216.
[13] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual. 2nd edn. Cold Spring Habor Lab. Press, New York.
[14] Young, D.A., Bauer, C.E., Williams, J.C. and Marrs, B.L. (1989) Mol. Gen. Genet. 218, 1–12.
[15] Prentki, P. and Krisch, H.M. (1984) Gene 29, 303–313.
[16] Casadaban, M.J., Martinez-Arias, A., Shapira, S.K. and Chou, J. (1983) Methods Enzymol. 100, 301–307.
[17] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[18] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[19] Biel, A.J. and Marrs, B.L. (1983) J. Bacteriol. 156, 686–694.
[20] Schatt, E., Jouanneau, Y. and Vignais, P.M. (1989) J. Bacteriol. 171, 6218–6226.
[21] Hübner, P., Willison, J.C., Vignais, P.M. and Bickle, T.A. (1991) J. Bacteriol. 173, 2993–2999.
[22] Kranz, R.G., Pace, V.M. and Caldicott, I.M. (1990) J. Bacteriol. 172, 53–62.
[23] Hallenbeck, P.C. (1991) Biochim. Biophys. Acta 1057, 97–101.
[24] Grabau, C., Schatt, E. and Vignais, P.M. (1991) J. Biol. Chem. 266, 3294–3299.