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Characterization of an *fdxN* mutant of *Rhodobacter capsulatus* indicates that ferredoxin I serves as electron donor to nitrogenase

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Abstract

A mutant of *Rhodobacter capsulatus*, carrying an insertion into the *fdxN* gene encoding ferredoxin I (FdI), has been studied by biochemical analysis and genetic complementation experiments. When compared to the wild-type strain, the *fdxN* mutant exhibited altered nitrogen fixing ability and 20-fold lower levels of nitrogenase activity as assayed in vivo. When assayed in vitro with an artificial reductant, nitrogenase activity was only 3- to 4-fold lower than in the wild type. These results suggested that the FdI-deleted mutant had impaired electron transport to nitrogenase. Immunochemical assay of both nitrogenase components showed that the *fdxN* mutant contained about 4-fold less enzyme than wild-type cells. Results of pulse-chase labeling experiments using [^{35}S]methionine indicated that nitrogenase was significantly less stable in the FdI-deleted mutant. When a copy of *fdxN* was introduced in the mutant *in trans*, the resulting strain appeared to be fully complemented with respect to both diazotrophic growh and nitrogenase activity. Depending on whether *fdxN* expression was driven by a *nif* promoter or a fructose-inducible promoter, FdI was synthesized either at wild-type level or in 10-fold lower amounts. The strain producing 10-fold less FdI did, however, display normal N_2-fixing ability. Analysis of cytosolic proteins by bidimensional electrophoresis revealed that the *fdxN* mutant produced a 14 kDa polypeptide in amounts about 3-fold greater than wild-type cells. This protein was identified by N-terminal microsequencing as a recently purified [2Fe-2S] ferredoxin, called FdV, which cannot reduce nitrogenase. It is concluded that FdI serves as the main electron donor to nitrogenase in *R. capsulatus* and that an ancillary electron carrier, distinct of FdV, is responsible for the residual nitrogenase activity observed in the FdI-deleted mutant.

Keywords: Ferredoxin; Electron transport; Nitrogenase; Nitrogen fixation

1. Introduction

The reduction of molecular dinitrogen by nitrogenase requires ATP and a source of low potential reducing equivalents. Bacteria generate reductants for nitrogen fixation through the oxidation of various substrates, most often organic carbon compounds. The metabolic routes leading from substrate oxidation to the generation of reductants for nitrogenase are expected to differ depending on microorganism physiology. However, little is known on this aspect of N_2 fixation. *Klebsiella pneumoniae* is the only bacterium in which the electron transport pathway linking pyruvate oxidation to nitrogenase function has been clearly established. Two proteins are involved, a pyruvate-flavodoxin oxidoreductase encoded by *nifJ* [1] and a flavodoxin encoded by *nifF* that serves as electron donor to nitrogenase [2]. In photosynthetic prokaryotes, proteins homologous to the *nifF* and *nifJ* gene products are either absent or play marginal role in N_2 fixation. In the heterocystous cyanobacterium *Anabaena* 7120, a NifJ protein has been shown to be required for nitrogen fixation but only under iron-deficient growth conditions [3]. Flavodoxins have been identified in *Anabaena* but none was found to play a role in nitrogen fixation. Besides, when iron is not limiting, another pathway seems to prevail that includes a heterocyst-specific [2Fe-2S] ferredoxin as ultimate donor to nitrogenase [4,5].

In photosynthetic bacteria, a NifJ-like protein has been

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identified in *Rhodospirillum rubrum*, but it appeared to be not functionally related to N_2 fixation [6]. In *R. capsulatus*, a NifF-like flavodoxin has recently been identified, the synthesis of which was repressed by ammonia [7]. Its actual role in N_2 fixation is not yet known. Besides, as many as six distinct ferredoxins have been isolated and characterized from this bacterium [8–11]. Only two of these ferredoxins, designated FdI and FdII, were found to be competent electron donors to nitrogenase, based on their redox properties and electron transfer assays [12–14]. Genetic studies revealed that the *fdxN* gene encoding FdI belonged to a *nif* operon [15], whereas *fdxA*, encoding FdII, presumably had a vital function, unrelated to N_2 fixation [16,17]. Hence, FdI appears as a plausible candidate as physiological electron donor to nitrogenase. In two independent studies, analysis of *fdxN* deleted mutants led to conflicting results. In one case, *fdxN* was found to be absolutely required for N_2 fixation [17] whereas, in the other study, inactivation of *fdxN* resulted in a strain still capable of N_2 fixation, although less efficiently [18].

In this study, the role of FdI has been investigated further by means of complementation analysis and biochemical characterization of the *fdxN* mutant described in [18]. The possible function of FdI in nitrogen fixation is discussed in the light of recent findings on the numerous ferredoxins of *R. capsulatus* and also on the basis of available information on homologous proteins from other diazotrophic bacteria.

2. Materials and methods

2.1. Bacterial strains and culture conditions

R. capsulatus strains and plasmids used in this study are listed in Table 1. Cultures were anaerobically grown in 12 ml screw-capped tubes or 90 ml bottles at 30°C, and exposed to a light intensity of approx. 15 000 lux. Growth medium consisted of RCV mineral salt medium [25] containing 20 mM DL-lactate and either 2 mM or 10 mM NH_4Cl, as carbon and nitrogen sources, respectively. For diazotrophic growth, the source of fixed nitrogen was omitted, and N_2 gas was aseptically bubbled through the culture using a narrow-gauge needle. Concentration of the phosphate buffer was doubled (20 mM) in order to keep the pH of the culture below 8.0. For in vivo labeling experiments with $^{35}SO_4^{2-}$, sulfate concentration was reduced to an estimated 10 μM by appropriate salt replacements in the medium composition. *Escherichia coli* strains, DH5a (Bethesda Research Laboratories) and S17-1 [26] were aerobically grown at 37°C in Luria-Bertani (LB) medium [27].

When needed, cultures were supplemented with antibiotics at the following concentrations: ampicillin (Ap) 100 mg/l; gentamicin (Gm) 10 mg/l for *E. coli*, 5 mg/l for *R. capsulatus*; spectinomycin (Sp) 10 mg/l; streptomycin (Sm) 100 mg/l; tetracycline (Tc) 10 mg/l for *E. coli*, 0.5 mg/l for *R. capsulatus*. *R. capsulatus* B10S and R3461 were routinely grown with Sm added; cultures of the *fdxN* mutant also contained Gm.

2.2. Plasmids

DNA manipulations followed standard techniques [27]. Plasmids pNI1 and pCD32 were obtained by subcloning the *R. capsulatus fdxN* and *fdxA* genes respectively into vector pNF3 [20]. The pNI1 insert, 347 bp in length, was obtained as a PCR fragment using pCG50 as a template [28] and the following primers: IN11: 5′-GCGCCCT*GGATCC*GCAGCCC-3′ and IN12: 5′-AGGAAACCA*CATATG*GCCATG-3′. PCR was performed with Taq polymerase (Promega) according to the recommendations of the supplier using a Hybaid thermocycler. Primer IN11 introduced three base substitutions (in bold letters) at

Table 1
Rhodobacter capsulatus strains and plasmids

Strain/plasmid	Genotype or description	Source or reference
R. capsulatus		
B10S	Sm^R mutant of strain B10	[19]
R346I	*fdxN*::Gm >	[18]
RCJ1	B10S bearing chromosomal *nifH::lacZ*	This study
RCJ2	R3461 bearing chromosomal *nifH::lacZ*	This study
Plasmid		
pDPT51	Ap^R, Helper plasmid (mobilizes pNF3 derivatives)	[20]
pRK2013	Km^R, Helper plasmid (mobilizes pFRDI)	[21]
pPHU316	Tc^R, *nifH::lacZ*,	[22]
pNF3	$Sm^R/Sp^R/Km^R$, expression vector	[20]
pCD32	Sm^R/Sp^R, 3.2 kb *fdxA*-Gm cassette fragment in pNF3	[23]
pFRDI	Tc^R/Gm^R, *fdxN* fused to the *fru* promoter	[24]
pNI1	Sm^R/Sp^R, 0.35 kb *fdxN* fragment in pNF3	This study

positions 154, 155 and 157 downstream of the last codon of *fdxN*, thus creating a *Bam*HI site (underlined). Primer IN12 led to the insertion of two bases and one substitution (in bold letters) at position −1 relative to the initiation codon of *fdxN*, thus introducing an *Nde*I site (underlined). The PCR fragment was cut by *Nde*I and *Bam*HI and cloned into the corresponding sites of plasmid pNF3; the resulting plasmid pNI1 has the *fdxN* gene at the optimal distance from the *nifH* promoter. The plasmid construction was verified by DNA sequencing.

pCD32 contains a 3.14 kb *Hin*dIII insert. It was obtained by ligation of a 0.85 kb *Hin*dIII-*Eco*RI fragment from plasmid pCD28 that carries *fdxA* [29] and a 2.6 kb *Eco*RI-*Hin*dIII fragment of pWKR189 [30] carrying Gm^r into the *Hin*dIII sites of pNF3. The *fdxA* gene is preceded by its own ribosome binding site and located at a distance of about 100 bp from the *nifH* promoter. Plasmids pNF3, pNI1 and pCD32 were transferred into *R. capsulatus* by conjugation using helper plasmid pDPT51. pFRDI is a pRK404 derivative carrying a copy of *fdxN* fused to the *fruP* promoter, inducible by fructose [24]. It was conjugated into *R. capsulatus* using the pRK2013 helper plasmid.

pPHU316 is a suicide vector carrying Tc^r and a *nifH::lacZ* translation fusion [22]. It was introduced in *R. capsulatus* by conjugation using *E. coli* S17.1. Two clones, RCJ1 and RCJ2, selected for their resistance to tetracycline, are derivatives of B10S and R346I respectively, carrying the *nifH::lacZ* fusion integrated into the chromosome.

2.3. Pulse-labeling experiments

In vivo pulse-labeling of *R. capsulatus* nitrogenase was performed in 80 ml bacterial cultures ($A_{660} = 1.0$) incubated in the light in RCV medium containing 0.5 mM L-alanine in place of NH_4^+. After 2 h of incubation, 5.55 MBq of carrier-free [^{35}S]methionine (Amersham France) was added and 8 ml samples were anaerobically withdrawn with a syringe at the times indicated. At 3 min, protein synthesis was blocked by adding 30 μg/ml of chloramphenicol to the cultures and sampling was prolonged over a 5 h period. Samples were immediately mixed at 0°C with 8 ml of ice-cold stop solution consisting of 0.15 M Tris-HCl (pH 8.0) containing 15 mM EDTA and 330 mg/l of chloramphenicol, and then centrifuged 15 min at 20000 × *g*. Pellets were washed in 1 ml 0.1 M Tris-HCl (pH 8.0), 15 mM EDTA and resuspended in 0.1 ml of the same buffer containing 10 mg/l leupeptin, 125 μM phenylmethylsulfonyl fluoride (both from Sigma) and 0.25 g/l lysozyme. After 15 min at 28°C, bacteria were broken by sonication. Cell-extracts were clarified by centrifugation 15 min at 20000 × *g*, 4°C and half-diluted in SDS sample buffer prior to SDS-polyacrylamide gel electrophoresis.

2.4. Coupling of anti-Rc1 and anti-Rc2 antibodies to Sepharose

The IgG fraction from rabbit sera raised against purified Rc1 and Rc2 respectively, was isolated by sodium sulfate precipitation and dialyzed against 0.1 M sodium citrate (pH 6.5). Sepharose CL-6B (Pharmacia) was activated with cyanogen bromide and washed in the same citrate buffer. The IgG fraction (containing 3.0 and 10 mg/ml of protein for the anti-Rc1 and anti-Rc2 antibodies, respectively) was gently shaken with an equal volume of activated-Sepharose at 4°C for 20 h. After protein coupling, the resin was washed with citrate buffer and then incubated in 50 mM glycine for 30 min to block unreacted binding sites. The coupling yield was estimated to be around 95%. The IgG-sepharose hence obtained was washed and stored at 4°C in TSA buffer (10 mM Tris-HCl (pH 8.0), 0.14 M NaCl, 0.025% sodium azide).

2.5. Isolation of nitrogenase components from cell-extracts by immunoadsorption on Sepharose beads

Bacterial cell-free extracts, prepared by lysozyme treatment and ultrasonication, were diluted 10-fold in TSA buffer containing 1% Triton X-100. 0.2 ml samples of such dilutions were incubated 90 min at 4°C with 20 μl of IgG-Sepharose beads in 1.5 ml microtubes placed on a vibration shaker. Beads were collected by centrifugation 5 s at 2000 × *g* and washed twice with 1 ml of 1% Triton X-100 in TSA buffer, followed by two washes in TSA buffer. Adsorbed proteins were released by incubating the beads in 20 μl of SDS sample buffer for 5 min at 95°C, prior to SDS polyacrylamide gel electrophoresis. Alternatively, protein adsorption and bead washings were performed in Costar microfiltration units (0.45 μm pore size) instead of the 1.5 ml microtubes. The latter procedure proved more convenient and reliable.

2.6. SDS-polyacrylamide gel electrophoresis and Western-blotting

Proteins were routinely electrophoresed on 12% polyacrylamide gel in Tris-glycine-SDS buffer [31], using a mini slab gel unit (Mighty Small SE200, Hoefer Scientific Inst.) and revealed by Coomassie blue staining. Radioactive bands were detected by autoradiography of vacuum-dried gels using Hyperfilm-β max (Amersham). Densitometric recording of autoradiographies was performed using a Shimadzu CS-9000 scanner. Western blot analysis was carried out as previously described [29], except that protein transfer was done by semi-dry electroblotting at 2 mA per cm^2 for 20 min in a Biometra Fast Blot apparatus, model B33 (Eurogentec). Anti-Rc1 and anti-Rc2 rabbit antisera were used at a 1:500 dilution. For FdI analysis, protein separation was performed on 18% polyacrylamide gels.

Immunoblot detection of FdI was carried out using anti-FdI rabbit antiserum at a 1:250 dilution.

2.7. Two-dimensional electrophoresis

First dimension (isoelectrofocusing) was performed using 11-cm-long precasted gels containing an immobilised pH gradient in the 4.0 to 7.0 range (Immobilin Drystrips, Pharmacia-LKB Biotech.). Drystrips were rehydrated overnight in a buffer consisting of 8 M urea, 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]propane sulfonate) 0.32% Pharmalytes in the 3.5 to 10 pH range and 0.1 g/l Orange G. Samples were prepared as follows. Bacterial cells were broken by lysozyme treatment followed by sonication in 0.1 M Tris-HCl (pH 8.0), 15 mM EDTA containing 10 mg/l leupeptin and 125 μM PMSF. 15 mM $MgCl_2$ and 50 units per ml of Benzonase (Merck) were added and the extracts were centrifuged 10 min at $90\,000 \times g$ in an Airfuge machine (Beckman). Supernatants were diluted 1:1 in sample buffer consisting of 9 M urea, 2% CHAPS, 0.8% Pharmalytes 3-10, 2% β-mercaptoethanol and 0.02% Bromophenol blue.

Samples were applied and electrophoresed in a Multiphor II unit (Pharmacia LKB) as specified by the manufacturer for a total of 24000 V h. Drystrips were then incubated for 20 min in equilibration buffer (6 M urea, 30% glycerol, 1% SDS, 16 mM dithiothreitol, 0.01% Bromophenol blue in 0.2 M Tris-Tricine (pH 8.2)). Electrophoresis in the second dimension was performed in SDS-containing gels (16 × 14 cm) of 12% polyacrylamide using a Tris-tricine buffer system [32]. Gels were revealed by silver staining [33] or by autoradiography after vacuum drying. For N-terminus sequence analysis of two-dimensional spots, the gel was electroblotted on a ProBlott membrane (Applied Biosystems) in 10 mM Caps-NaOH buffer (pH 11.0) (Caps: 3-[cyclohexylamino]-1-propanesulfonic acid) containing 20% methanol. The membrane was autoradiographied, the spot of interest was localized and cut out from the membrane.

2.8. Other analytical methods

Proteins were assayed using the Coomassie blue G250 stain [34] or the BCA reagent (Bio-Rad). Nitrogenase components were purified and assayed for protein as previously described [9,35]. N-terminal sequence determination of electroblotted polypeptides was carried out on a model 470 automated gas-phase sequencer (Applied Biosystems). Search of sequence homology in data banks was performed using the DNASTAR software.

2.9. Enzymatic assays

Nitrogenase was assayed by acetylene reduction [35]. In vitro assays were performed on toluene-permeabilized cells as previously described [36]. β-galactosidase was assayed on toluene-permeabilized cells by a previously described procedure [24].

3. Results

3.1. Diazotrophic growth and complementation analysis of the fdxN mutant R346I

Mutant R346I (*fdxN::Gm*), carries a non-polar insertion into the *fdxN* gene of *R. capsulatus* which results in defective growth on N_2 ([18]; Fig. 1). The growth curve of this mutant is characterised by a lag period of approximately 30 h, and a growth rate more than 2-fold slower compared to the parental strain. The generation times calculated from the growth curves were 13.6 h for strain R346I versus 6.13 h for strain B10S. Since *fdxN* is part of an operon composed of five genes and the Gm-insertion in the R346I chromosome precedes a gene, *rnfF*, essential for nitrogen fixation [18], it was of interest to assess that the R346I phenotype solely resulted from the lack of ferredoxin I, the *fdxN* gene product. For this purpose, complementation experiments were performed using two different plasmids. Plasmid pNI1 carried a copy of *fdxN* placed behind the *nifH* promoter. Plasmid pFRDI is a derivative of a new expression vector in which *fdxN* was fused to P_{fru}, a promoter specifically induced by fructose [24]. Once the plasmids were separately introduced into the *fdxN* mutant, the resultant strains appeared to be fully complemented with respect to growth on N_2 (Fig. 1). In

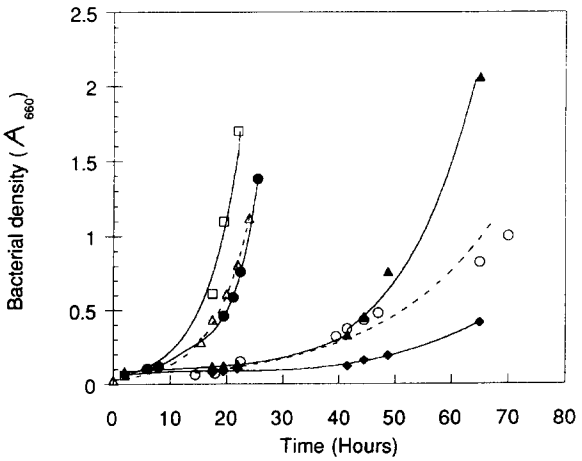

Fig. 1. Diazotrophic growth of the *fdxN* mutant and complemented strains of *R. capsulatus*. Cultures (80 ml) were grown on N_2 as sole nitrogen source and bacterial density was followed by recording optical absorbance at 660 nm. Squares, wild-type strains B10S; open triangles, R346I (pNI1); closed triangles, R346I(pNF3); diamonds, R346I (pCD32). Strain R346I (pFRDI) was grown in the absence (open circles) or in the presence (closed circles) of 11 mM fructose. The growth curve of strain R346I was similar to that of strain R346I (pNF3). Data points were fitted with exponential curves, except for strain R346I (pCD32). A lag time of 30 h was taken into account in the growth curves of strains R346I (pNF3), and R346I (pFRDI) grown without fructose.

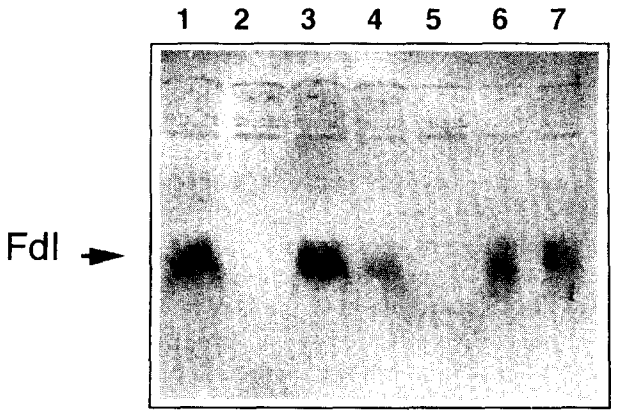

Fig. 2. Comparison of the FdI level in strains obtained by complementation of the *fdxN* mutant. All strains were grown to the same bacterial density (A_{660} = 0.8), then derepressed for nitrogenase in N-free medium supplemented with 0.5 mM alanine. Cell extracts were prepared, adjusted to the same protein content (1.85 mg/ml) and analyzed on a 18% polyacrylamide gel containing SDS. Proteins were electroblotted on nitrocellulose, and FdI was revealed by immunochemical detection. Lane 1, wild-type strain B10S; lane 2, strain R346I (pNF3); lane 3, strain R346I (pNI1); lane 4, strain 346I (pFRDI) supplemented with fructose; lane 5, strain R346I (pFRDI) with no fructose added; lanes 6 and 7 were loaded with the same samples as lanes 1 and 3 respectively, but the protein amount was one-fifth less.

strain R346I(pFRDI), restoration of normal diazotrophic growth appeared to be dependent on the presence of fructose in the culture medium. Immunoblot experiments indicated that FdI was undetectable in mutant R346I and present in the complemented strains ([24] and Fig. 2). Hence, our data demonstrate that the lack of FdI is the unique reason for the impaired nitrogen fixation in mutant R346I.

The residual N_2 fixing ability observed in mutant R346I, suggested that an ancillary protein could mediate electron transfer to nitrogenase. Out of the five other ferredoxins present in *R. capsulatus* cells, only FdII seemed potentially capable of performing such an electron transfer, based on in vitro assays [13,14]. FdII is constitutively synthesized at a level estimated to be 5- to 10-fold lower than FdI. To test whether the in vivo level of FdII somehow limited diazotrophic growth of mutant R346I, we decided to overproduce it. For this purpose, plasmid pCD32 carrying the *fdxA* gene cloned under the *nifH* promoter, was introduced in the *fdxN* mutant. Although cells harboring pCD32 did accumulate FdII to levels 3- to 4-fold greater (data not shown), no beneficial effect was observed on the mutant growth (Fig. 1). On the contrary, it was observed that strain R346I(pCD32) grew very poorly on N_2, suggesting that FdII might compete or interfere with electron donation to nitrogenase in the mutant. The results imply that FdI exclusively fulfills a specific function in N_2 fixation and that FdII cannot replace it.

3.2. Nitrogenase activity is largely dependent on FdI

When grown diazotrophically, strain R346I exhibited nitrogenase activities about 20-fold lower than the parental strain, consistent with the fact that it grew slower under these conditions. The *fdxN* mutant also showed much lower levels of nitrogenase activity when nitrogenase was derepressed either upon nitrogen starvation or in the presence of a poor nitrogen source such as alanine (Table 2).

By introducing a functional copy of *fdxN* in mutant R346I using plasmid pNI1 or pFRDI, strains were obtained that displayed wild-type levels of nitrogenase activity when grown on N_2. When N-starved or N-limited, strains harboring pNI1 or pFRDI had nitrogenase activities from 12 to 20 times as high as in mutant R346I. As the enzyme activities observed in the complemented strains were not quite as high as in the wild-type strain, we wondered whether the observed variations were linked to differences in the FdI content of the cells. Immunoblot detection of FdI in cell extracts revealed that strain R346I (pNI1) contained virtually as much of the ferredoxin as the wild-type strain (Fig. 2). On the other hand, strain R346I (pFRDI), which displayed a lower level of nitrogenase activity when N-limited (Table 2), contained about 10-fold less of FdI. However, when strain R346I(pFRDI) was grown on N_2, high nitrogenase activity was observed (Table 2) although the FdI content was about 10-fold reduced [24]. Hence, there appears to be no obvious corre-

Table 2
Nitrogenase activity in the *fdxN* mutant and complemented strains of *Rhodobacter capsulatus*

Strain	Nitrogenase activity [a]			
	N_2-grown cells	N-starved cells	N-limited cells	in vitro [b]
B10S	84.0	15.0	50.8	1.90
R346I	4.5	1.2	2.60	0.50
R346I(pNF3)	4.8	1.3	1.95	0.61
R346I(pNI1)	–	17.5	42.2	1.59
R346I(pFRDI) [c] + fru	83.0	–	28.3	1.65
R346I(pFRDI) [c] − fru	3.3	–	2.3	0.58

[a] Nitrogenase assays were carried out in duplicate. Activities are expressed as nmol C_2H_2 reduced per min per mg dry weight.
[b] In vitro activities were assayed on permeabilized N-limited cells with dithionite as artificial reductant.
[c] Strain R346I(pFRDI) was grown in the absence (− fru) or in the presence (+ fru) of fructose in the culture medium.

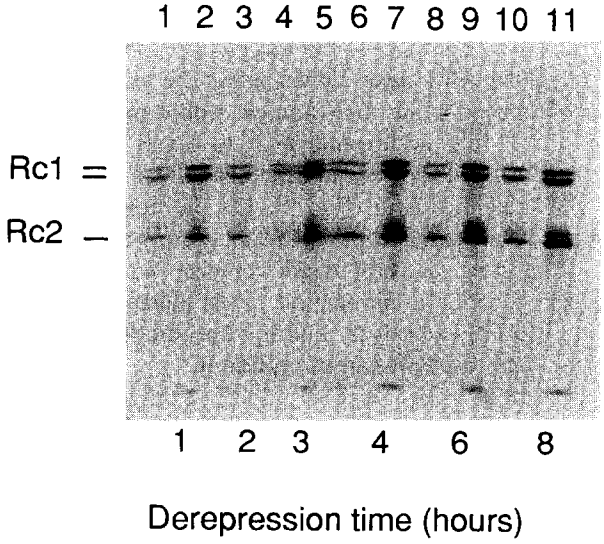

Fig. 3. Accumulation of nitrogenase polypeptides during the course of *nif* derepression as revealed by in vivo ^{35}S-labeling. NH_4^+-grown bacteria (80 ml cultures) were washed and resuspended in N-free medium to a bacterial density of 1.0. Cells were incubated for nitrogenase derepression under argon in the light, in the presence of 7.4 MBq [^{35}S]sulfate added at time zero. Samples (10 ml) were anaerobically withdrawn at the times indicated and centrifuged at 4°C. Cells were broken and nitrogenase polypeptides were extracted by adsorption on beads of specific antibody-IgG-Sepharose as described in Section 2. Labeled polypeptides were analyzed by SDS-gel electrophoresis followed by autoradiography. Each lane was loaded with a protein sample corresponding to approx. 30 μg of cell extract proteins. Samples were from strain R346I (lanes 1, 3, 4, 6, 8, 10) or strain B10S (lanes 2, 5, 7, 9, 11).

lation between nitrogenase activity assayed in vivo and the intracellular FdI concentration.

Nitrogenase assays were also performed in vitro, in permeabilized bacterial cells provided with dithionite as an artificial reductant for nitrogenase. Data presented in Table 2 showed that the *fdxN* mutant exhibited a lower activity level than wild-type cells. In strains harboring plasmid pNI1 or pFRDI, activities almost as high as in the wild-type were restored. Hence, FdI is required for maximal in vitro nitrogenase activity. Besides, the lack of FdI had a much more pronounced effect on the in vivo activity indicating that the ferredoxin actually participates in the electron transport to nitrogenase.

3.3. Stability of nitrogenase is affected in the *fdxN* mutant

The in vitro assays of acetylene reduction suggested that nitrogenase was less abundant in the *fdxN* mutant than in the parental strain. For a quantitative estimation of the enzyme, bacteria were incubated in N-free medium containing [^{35}S]sulfate, and the nitrogenase components were selectively immunoextracted at different times during the course of *nif* derepression. Specific extraction of Rc1 and Rc2 from whole cell proteins was achieved by using Sepharose beads to which antibodies raised against either nitrogenase component, were covalently linked (see Materials and methods). The results of such analyses clearly showed that the *fdxN* mutant accumulated less of the

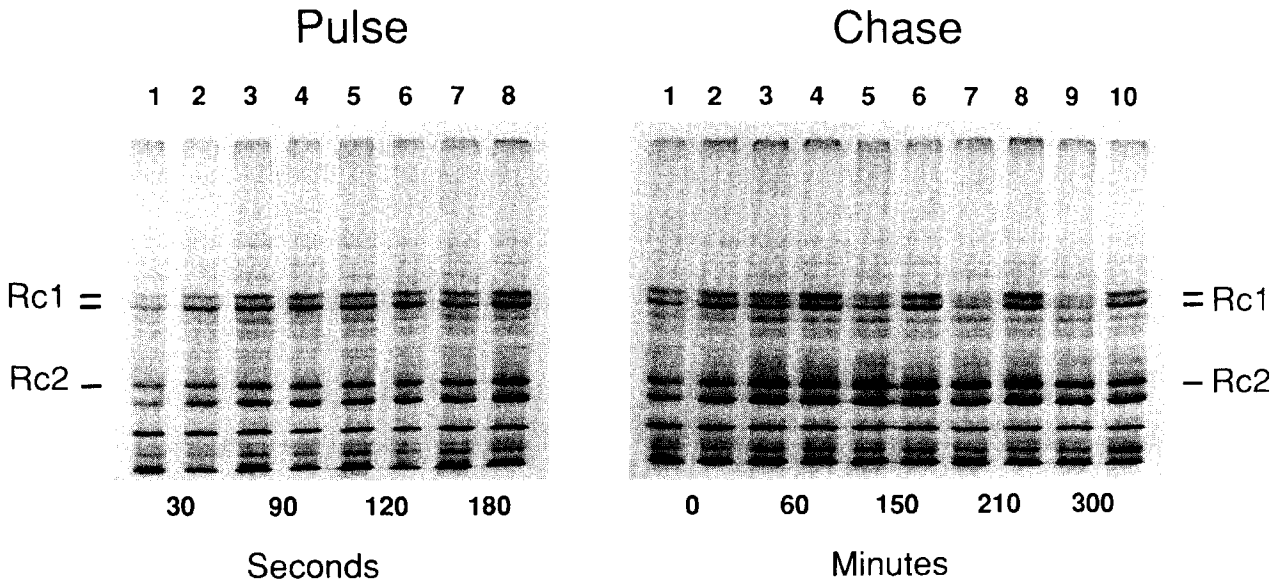

Fig. 4. Stability of nitrogenase polypeptides as judged from in vivo pulse-chase labeling experiments. Bacteria were derepressed for nitrogenase as indicated in the legend to Fig. 3, except that 0.5 mM alanine was added to the incubation medium. At 2.5 h after the beginning of derepression, bacteria were pulse-labeled with [^{35}S]methionine as detailed in Section 2 (left part). After a labeling time of 180 s, protein synthesis was blocked by chloramphenicol and nitrogenase polypeptides were chased for a total time of 300 min. Bacterial samples were withdrawn at the indicated times. Cell extracts were made and analyzed by SDS-gel electrophoresis and autoradiography. Lane numbering: odd numbers refer to the *fdxN* mutant R346I, even numbers to the parental strain B10S. Rc1 and Rc2 markers designate the α and β subunits of nitrogenase component 1 and the γ subunit of component 2, respectively.

nitrogenase polypeptides relative to the wild-type strain (Fig. 3). Comparison of the incorporation of radiolabel indicated that the *fdxN* mutant contained one fourth the wild-type level of both nitrogenase components, in fairly good agreement with the relative enzyme level detected by in vitro assays. Interestingly, the amount of nitrogenase detected in mutant cells steadily increased throughout the derepression period whereas the in vivo activity declined after the second hour of incubation (data not shown). Hence, in contrast to what is observed in wild-type cells, in vivo nitrogenase activity did not reflect the actual level of enzyme in mutant cells.

The fact that the *fdxN* mutant contained less nitrogenase suggested that the absence of FdI affected either the biosynthesis or the stability of the enzyme. The possibility was first considered that the *fdxN* mutation might indirectly have an effect on the transcription of *nifHDK*. To test this hypothesis, a *nifH::lacZ* fusion carried on plasmid pPHU316 was introduced into the chromosome of *R. capsulatus* by selecting for a single cross-over event. Strains RCJ1 and RCJ2 are derivatives of B10S and R346I, respectively, carrying the *nifH::lacZ* fusion. They were incubated on N-free medium, and the activities of nitrogenase and β-galactosidase were measured during the course of *nif* gene derepression. While strain RCJ2 exhibited low levels of nitrogenase activity similar to strain R346I, no significant difference in the levels of β-galactosidase was observed between strains RCJ1 and RCJ2. One can therefore rule out the possibility that *nifHDK* transcription could be altered in the *fdxN* mutant.

We then carried out pulse-chase labeling experiments with [^{35}S]methionine to examine the rates of biosynthesis and decay of nitrogenase in the *fdxN* mutant. Pulse-labeling was performed 2 h after the beginning of derepression at which time, in vivo nitrogenase activity started declining in the *fdxN* mutant while it increased rapidly in the parental strain. Acetylene reduction assays performed a few minutes prior to [^{35}S]methionine labeling, gave nitrogenase activities of 30.3 and 3.2 nmol C_2H_4 min^{-1} (mg dry weight)$^{-1}$ for the B10S and R346I strains, respectively. The radioactivity incorporated into the α and β subunits of Rc1 and the γ subunit of Rc2 was detected by autoradiography after separation of cell extract proteins by SDS-PAGE (Fig. 4). Comparison of the lanes pairwise at different times during the pulse-labeling period showed almost no difference in the Rc1 and Rc2 band pattern between the mutant and control strains. At some time points (30 and 180 s), the Rc1 subunit bands appeared less intense for the mutant. Although this may reflect a slight difference in the rate of biosynthesis of Rc1, this difference is by no means sufficient to explain the 4-fold lower nitrogenase content of mutant cells.

At 180 s, protein synthesis was blocked by chloramphenicol and the fate of the labeled nitrogenase subunits was monitored (Fig. 4). The autoradiogram revealed that the Rc1 subunits disappeared more rapidly in the mutant than in the parental strain. Based on densitometric recordings, the half-life of Rc1 in the mutant was estimated to be 2.2 ± 0.2 h versus 7.3 ± 0.7 h in the parental strain. On the other hand, the band corresponding to the Rc2 subunit in

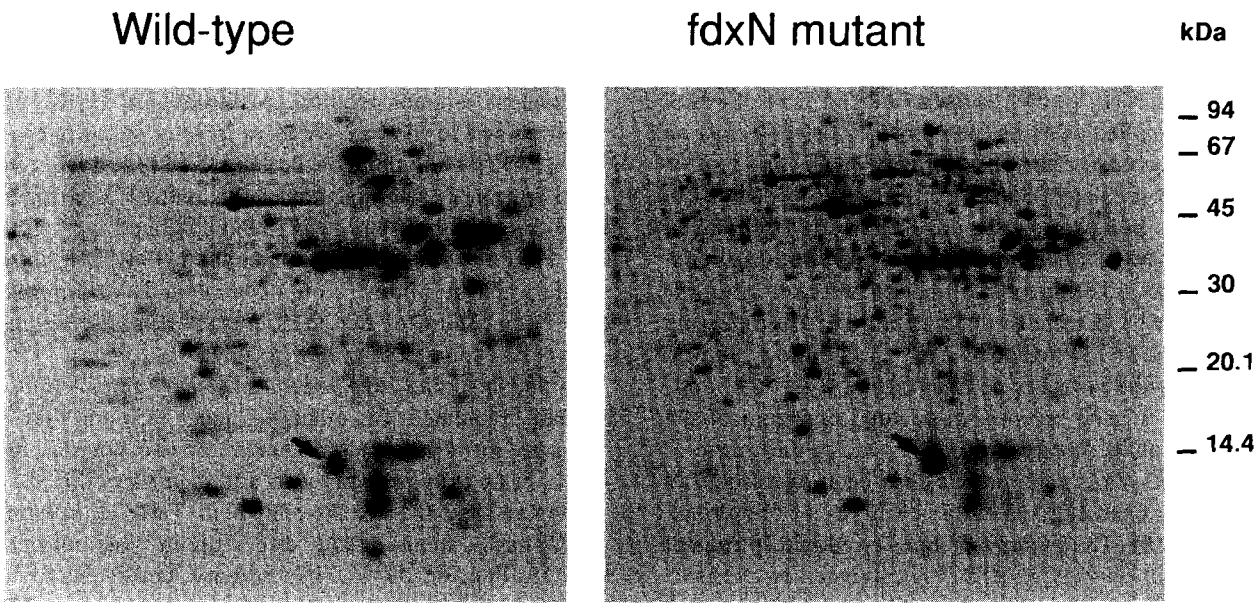

Fig. 5. Comparison of the ^{35}S-labeled proteins from the *fdxN* mutant and wild-type strain as analysed by two-dimensional gel electrophoresis and autoradiography. Strains R346I (*fdxN* mutant) and B10S (wild type) were grown on N_2 as sole nitrogen source in the presence of 92.5 kBq/ml of [^{35}S]sulfate. Cultures (80 ml) were harvested in the mid-log phase of growth. Cell extracts were prepared and subjected to two-dimensional gel electrophoresis. Gels were dried and revealed by autoradiography. pH gradient was 7 to 4 from left to right. Molecular weight marker sizes are indicated on the right side. The arrow points to the overproduced polypeptide detected in the mutant (see text).

the mutant extracts did not show an equivalent decrease in intensity. The possibility was considered that a polypeptide with the same relative mobility as the Rc2 subunit might hinder interpretation of the data. To avoid this problem, Rc2 analysis was performed after selective immunoadsorption on sepharose beads coated with anti-Rc2 antibodies. Results showed no significant differences in the radiolabeling of Rc2 between the mutant and wild-type strains (data not shown). These data suggested that the stability of Rc2 is less dramatically affected than that of Rc1. In several experiments, we noticed that the Rc2 stability in the *fdxN* mutant was variable, and especially depended on the nitrogen status of the cells. It was less stable in N-starved cells (as illustrated in Fig. 3) than in cells supplied with alanine during derepression, as observed in Fig. 4. Although the reason for these differences is unclear, it remains that the stability of the nitrogenase enzyme is significantly altered in the *fdxN* mutant.

3.4. Another ferredoxin is overproduced in the fdxN mutant

The observation that the *fdxN* mutant grew on N_2 only after a lag time (Fig. 1) led us to the idea that diazotrophic growth in this mutant might require the induction of a protein to partially compensate for the lack of FdI. In order to test this hypothesis, protein extracts from the *fdxN* mutant and the parental strain were analyzed by two-dimensional gel electrophoresis (Fig. 5). Proteins were labeled in vivo by growing the cells on [^{35}S]sulfate, and detected on two-dimensional gels by autoradiography. The comparative analysis revealed that a polypeptide, marked by an arrow on Fig. 5, was more abundant in the mutant extract. This polypeptide exhibited an apparent molecular weight of 14000 and an isoelectric point around 5.2. It was absent in NH_4^+ grown cells, as judged from the lack of a corresponding spot on two-dimensional gels run on extracts of such cells (data not shown), suggesting that its synthesis was regulated like that of *nif* gene products. The nature of this polypeptide was investigated by N-terminal sequence analysis, after electroblotting of a two-dimensional gel and isolation of the spot of interest. A single polypeptide was detected which exhibited the following sequence: PNITFTSPIMKKD. This information allowed us to identify the polypeptide unambiguously as the product of *fdxD*, a gene recently discovered upstream of the *nifHDK* operon in *R. capsulatus* [37]. During the course of this study, the *fdxD* gene product has been overproduced in *E. coli* and characterized has a new [2Fe-2S] ferredoxin, called FdV [10]. The amount of this ferredoxin in mutant cells relative to wild-type cells was estimated by comparing the radioactivity incorporated in the corresponding polypeptide band as separated by one dimension SDS-polyacrylamide gel electrophoresis. Densitometric scanning of the autoradiographies indicated that the *fdxN* mutant accumulated 2.3-fold more of the *fdxD* gene product than the wild-type strain. Similar comparison of the protein patterns obtained from the *fdxN* mutant and parental strain, as detected by silver staining, further confirmed the 2- to 3-fold greater abundance of FdV in the mutant relative to wild-type (data not shown).

4. Discussion

In this study, a biochemical analysis of a *fdxN* mutant of *R. capsulatus* is presented as an approach towards a better understanding of the role of the *fdxN* gene product, ferredoxin I, in N_2 fixation. Results illustrated in Fig. 1 and Table 2 demonstrate that FdI is not absolutely required for nitrogen fixation. However, its absence resulted in a reduced growth rate on N_2 and a dramatic decrease of nitrogenase activity. These findings confirm the observations previously reported by Schmehl et al. [18]. Using a mutant bearing a kanamycin cassette inserted into *fdxN*, Saeki et al. [17] concluded that FdI was indispensable for N_2 fixation. The Nif$^-$ phenotype observed in the latter study, may be due to a polar effect of the kanamycin cassette used for mutagenesis. Indeed, a gene essential for nitrogen fixation has been found downstream of *fdxN* [18]. Hence, although there are phenotypical differences between the mutant described in [17] and that studied in [18] and herein, from all available data we conclude that FdI plays a major role in N_2 fixation.

4.1. Electron transfer to nitrogenase

Several findings argue in favor of FdI as the physiological reductant of nitrogenase. FdI is the more abundant ferredoxin in N_2-fixing cells and is present in roughly equivalent amount relative to nitrogenase [9,12]. It has been shown to mediate electron transfer to nitrogenase in vitro [7,12] while none of the other five ferredoxins except FdII appeared as a competent redox partner of nitrogenase. FdII function, however, was found unrelated to N_2 fixation [16,17]. Besides, raising intracellular FdII concentration using a plasmid-borne copy of *fdxA*, did not improve the N_2-fixing ability of the *fdxN* mutant (Fig. 1), suggesting that FdII cannot replace FdI in vivo.

Genetic studies revealed that the *fdxN* gene is transcriptionally regulated like other *nif* genes [15] and takes part of a group of 10 genes organized in two operons, which have been proposed to code for a *nif*-specific electron transport system [18]. As shown in Table 2, deletion of *fdxN* has a more drastic effect on in vivo nitrogenase activity than on the in vitro activity, consistent with FdI serving as physiological electron carrier to nitrogenase. In addition, the *fdxN* mutation resulted in a lower steady-state level of nitrogenase, due to a decreased stability of the enzyme complex in mutant cells. That the lack of FdI is the unique reason for the phenotypic characters of the

fdxN mutant, is supported by the results of our complementation experiments.

As the *fdxN*-deleted mutant exhibited residual nitrogenase activity sufficient to allow growth on N_2, there must exist another electron carrier providing reductants to nitrogenase. FdV, a [2Fe-2S] ferredoxin which was found to be more abundant in the FdI-lacking mutant (Fig. 5), fails to donate electrons to nitrogenase in vitro, primarily because of its too high mid-point redox potential [10]. A flavodoxin, recently isolated from *R. capsulatus*, appears as a plausible alternative electron donor to nitrogenase [13]. The gene encoding this flavodoxin appeared to resemble *nifF* from *K. pneumoniae* and *A. vinelandii* suggesting that it is involved in N_2 fixation [38]. Analysis of the corresponding mutant should help understanding the role of the NifF-like flavodoxin in *R. capsulatus*.

4.2. Comparison with other FdxN-like ferredoxins

Ferredoxin-like genes similar to *R. capsulatus fdxN* have been found in various diazotrophic bacteria, including *Azotobacter vinelandii* [39], *Bradyrhizobium japonicum* [40], *Anabaena* and *Rhizobium* species [41]. All these genes, except *R. capsulatus fdxN*, happened to be located next to the *nifB* gene, which is required for FeMoCo biosynthesis [42]. Besides, the ferredoxin-like polypeptides predicted from the *fdxN* genes, share substancial sequence similarity [43]. Deletion of *fdxN* in *A. vinelandii* caused a loss of nitrogenase activity similar to what happened in *R. capsulatus* [44]. On the other hand, the *fdxN* gene from *R. meliloti* has been shown to be absolutely essential for N_2 fixation [45]. In the two studies just mentioned, the *fdxN* products were proposed to participate in FeMoCo biosynthesis, based on the presence of genes involved in this process near *fdxN* or cotranscribed with it. Alternatively, FdxN may serve as electron donor to nitrogenase as it is the case in *R. capsulatus*. In this respect, it is of interest that *R. meliloti* FdxN, recently purified in recombinant form, was found capable of transferring electrons to nitrogenase in vitro [46]. Finally, the FdxN products could have different functional specificities depending on bacterial hosts. As an example, the *fdxN* genes from *B. japonicum*, *A. vinelandii* and *R. capsulatus* failed to complement the *R. meliloti fdxN* mutant, despite obvious sequence similarity of the corresponding ferredoxin-like polypeptides [43].

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