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The Involvement of the *nif*-Associated Ferredoxin-Like Genes *fdxA* and *fdxN* of *Herbaspirillum seropedicae* in Nitrogen Fixation

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The pathway of electron transport to nitrogenase in the endophytic β-Proteobacterium *Herbaspirillum seropedicae* has not been characterized. We have generated mutants in two *nif*-associated genes encoding putative ferredoxins, *fdxA* and *fdxN*. The *fdxA* gene is part of the operon *nifHDKENXorf1orf2fdxAnifQmodABC* and is transcribed from the *nifH* promoter, as revealed by *lacZ* gene fusion. The *fdxN* gene is probably co-transcribed with the *nifB* gene. Mutational analysis suggests that the FdxA protein is essential for maximum nitrogenase activity, since the nitrogenase activity of the *fdxA* mutant strain was reduced to about 30% of that of the wild-type strain. In addition, the *fdxA* mutation had no effect on the nitrogenase switch-off in response to ammonium. Nitrogenase activity of a mutant strain lacking the *fdxN* gene was completely abolished. This phenotype was reverted by complementation with *fdxN* expressed under *lacZ* promoter control. The results suggest that the products of both the *fdxA* and *fdxN* genes are probably involved in electron transfer during nitrogen fixation.

***Keywords*:** *H. seropedicae*, nitrogenase, *fdxA*, *fdxN*, ferredoxin

Herbaspirillum seropedicae is an endophytic nitrogen-fixing β-Proteobacterium, which associates with agricultural crops such as rice, maize, sugarcane, and pineapple (Baldani *et al*., 1986; Young, 1992; Cruz *et al*., 2001). Nitrogen fixation in this bacterium and other diazotrophs is highly regulated, requiring the activity of several gene products to produce an active nitrogenase complex (Pedrosa *et al*., 2001).

Nitrogen fixation is a process which requires a high energy input, a characteristic clearly manifested by the need for low-potential reductants that can donate electrons to the Fe-protein. However, the pathway for electron transfer from metabolic intermediates to nitrogenase has been characterized in biochemical and genetic detail in only a few organisms. It has been proposed that the direct electron donors to nitrogenase are flavodoxins or ferredoxins. Yates (1972) showed that flavodoxin hydroquinone acted as a reductant *in vitro* for nitrogenase from *Azotobacter chroococcum*, and in the facultative anaerobe *Klebsiella pneumoniae*, the electron transfer pathway involves the *nifJ* and *nifF* gene products, a pyruvate:flavodoxin oxidoreductase and a flavodoxin, respectively (Shah *et al*., 1983; Deistung *et al*., 1985; Deistung and Thorneley, 1986).

Ferredoxins have been shown to be involved in electron transfer to nitrogenase in several aerobic diazotrophs, such as *Bradyrhizobium japonicum*, *Rhodospirillum rubrum*, *Azoarcus* sp., and *Rhodobacter capsulatus* (Jouanneau *et al*., 1995; Egener *et al*., 2001; Edgren and Nordlund, 2005, 2006; Hauser *et al*., 2007).

In the purple non-sulfur photosynthetic bacterium *R. rubrum* the electron transport to nitrogenase has been both genetically and biochemically investigated and seems to operate through two pathways. The products of the *fix* genes constitute the major pathway under heterotrophic conditions, whereas a pyruvate:ferredoxin oxidoreductase, encoded by *nifJ* gene, seems to provide the main electron donor under darkness and anaerobiosis. In both systems, the ferredoxin N (FdxN) is considered responsible for direct electron transfer to the Fe-protein (Edgren and Nordlund, 2006).

Two ferredoxin-like coding genes located in *nif* operons were identified in *H. seropedicae* and, based on sequence similarity to described genes, named *fdxA* (GenBank accession no. EF666057) and *fdxN* (accession number M60319). The *fdxA* gene is located downstream from the *nifHDKENXorf1orf2* operon and immediately upstream from the *nifQmodA1B1C1* genes (Machado *et al*., 1996; Klassen *et al*., 1999), while *fdxN* is located downstream from the *nifB* gene (Rego *et al*., 2006).
In this work we have analyzed the ferredoxin-coding genes *fdxA* and *fdxN*. Our results indicated that the *fdxN* gene product is essential for nitrogenase function and an *fdxA* mutant showed a significant decrease in nitrogenase activity, suggesting these gene products may be involved in electron delivery to nitrogenase in *H. seropedicae*.

Materials and Methods

Bacterial strains, plasmids, and media conditions

The genotypes of the bacterial strains, and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in LB liquid medium or on LB-agar (Sambrook *et al*., 1989) with appropriate antibiotics (streptomycin 80 μg/ml, ampicillin 100 μg/ml or kanamycin

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristic	Reference
H. seropedicae		
SmR1	Z78; Nif$^+$, Sm^R (wild type)	Souza *et al*. (1999)
SmR7.2	SmR1; Sm^R, Km^R, *fdxN*::, Nif$^-$	This work
KC6	SmR1, *fdxA*::*lacZ*-Km, Sm^R, Km^R	This work
E. coli		
DH10B	Sm^R	Grant *et al*. (1990)
Plasmids		
HS05-MF-037-A04	*H. seropedicae nifN*(3′)-*nifQ*(5′) in pUC18R	Genopar consortium[a]
HS25-MF-013-D09	*H. seropedicae orf1*(5′)-*modA1*(5′) in pUC18R	Genopar consortium[a]
pKOK6.1	Ap^R, Cm^R, Km^R, *lacZ* without promoter	Kokotek and Lotz (1989)
pUC4K	Ap^R, Km^R, cloning vector	GE Healthcare
pMP220	Tc^R, *lacZ* fusion vector	Spaink *et al*. (1987)
pSUP202	Ap^R, Cm^R, Tc^R, *mob*	Simon *et al*. (1983)
pLAFR 3.18	pLAFR3 with pTZ18R polylinker, λcos *lacZ* Tc^R	Machado *et al*. (1996)
pMPnifN-Q	*Bam*HI-*Eco*RI *nifN-nifQ* fragment cloned in vector pMP220	This work
pFGMR2	550 bp *Pst*I fragment containing the *fdxN* gene in pTZ18R, Ap^R	This work
pALI7	Km cassette insertion in *fdxN* gene of the plasmid pFGMR2, Ap^R, Km^R	This work
pALIC1	550 bp *Bam*HI/*Hind*III fragment of the plasmid pFGMR2 containing *fdxN* gene in pLAFR 3.18 , Tc^R	This work
pALFSA	*lacZ*-Km insertion into *fdxA* cloned in vector pSUP202	This work

[a] *H. seropedicae* genome sequencing consortium (www.genopar.org)

50 μg/ml).

H. seropedicae strains were grown at 30°C in NFbHP-malate medium (Klassen *et al*., 1997) supplemented with 20 mM ammonium chloride or the indicated nitrogen source and appropriate antibiotics (kanamycin 500 μg/ml, streptomycin 80 μg/ml or chloramphenicol 100 μg/ml).

Cloning and molecular biology methods

General techniques for DNA isolation, restriction enzyme analysis, cloning procedures and transformations were according to standard protocols (Sambrook *et al*., 1989). Enzymes were obtained from commercial sources and used according to the manufacturers' instructions. DNA sequencing was performed using dye-terminators (ET terminator, GE Healthcare) in an ABI 377 automated DNA sequencer (Applied Biosystems).

Mutagenesis of *fdxA* was performed by subcloning a 1.3 kb *Eco*RI fragment from the plasmid HS25-MF-013-D09, containing a region spanning from the 3′ end of *nifX* to the 5′ end of *nifQ* of *H. seropedicae*, into pSUP202. This plasmid was used to insert the *lacZ*-Km cassette from the plasmid pKOK6.1 into the *Nsi*I site of the *fdxA* gene and the resulting plasmid (pALFSA) was introduced into the *H. seropedicae*

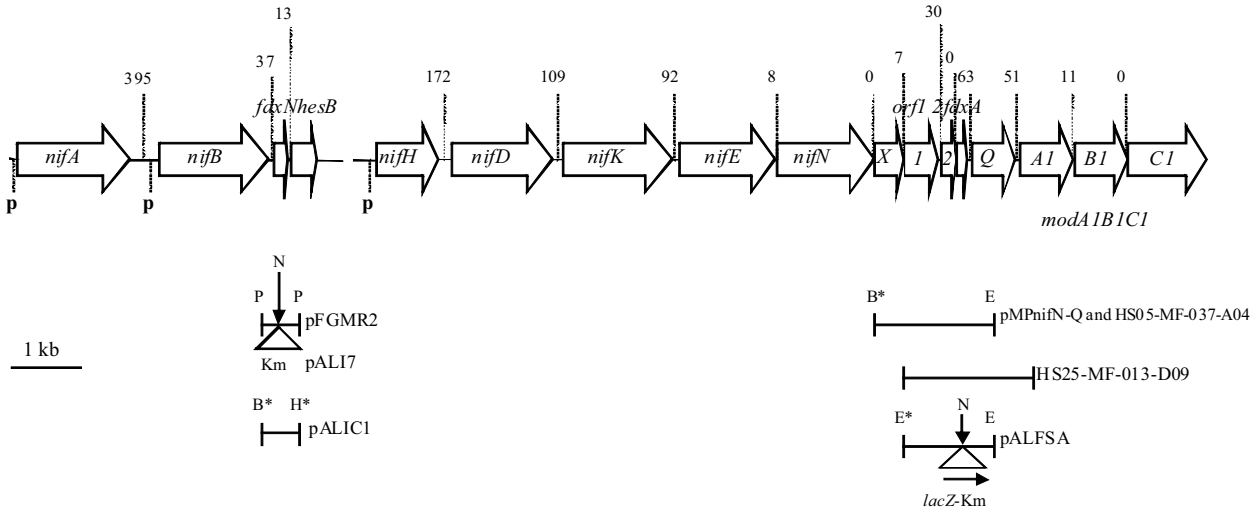

Fig. 1. Genetic map of the *H. seropedicae nif* regions containing the *fdxA* and *fdxN* genes. The large arrows indicate the gene position and orientation. p Indicates the identified promoters upstream from *nifH*, *nifA*, and *nifB*. The length of the intergenic regions are indicated by the dashed lines above the map. Strategies of mutagenesis, including the location and orientation of the inserted Km and *lacZ*-Km cassettes, are indicated. The vertical arrows indicate the *Nsi*I sites used to insert the mutagenic cassettes. Restriction sites: P, *Pst*I; N, *Nsi*I; E, *Eco*RI; H, *Hind*III; B, *Bam*HI. An asterisk indicates a restriction site from the vector polylinker.

SmR1 strain by electroporation, as described (Rego *et al*., 2006) (Fig. 1). A *H. seropedicae fdxA* mutant was selected by kanamycin resistance and was named KC6. Southern blot analysis confirmed the presence of the cassette and disruption of the *fdxA* gene.

To construct a plasmid with the Km cassette insertion into the *fdxN* gene, the Km cassette obtained from pUC4K was inserted into the *Nsi*I site of pFGMR2 resulting in the plasmid pALI7 (Fig. 1). This plasmid was also transformed into the SmR1 strain by electroporation (Rego *et al*., 2006) and the mutant was selected by kanamycin resistance. DNA hybridization using genomic DNA was used to confirm the presence of the cassette in the *fdxN* gene.

To construct a *H. seropedicae*-stable plasmid containing the *fdxN* gene for genetic complementation, the plasmid pFGMR2 was digested with *Bam*HI and *Hin*dIII and the 550 bp fragment containing *fdxN* was cloned into pLAFR 3.18 (Machado *et al*., 1996) resulting in plasmid pALIC1 (Fig. 1).

To generate a *nifN-nifQ:lacZ* gene fusion the plasmid HS05-MF-037-A04 was digested with *Bam*HI and *Eco*RI to produce a 1.6 kb fragment containing a region spanning the 3′ of *nifN* to the 5′ of *nifQ* (Fig. 1). This fragment was then ligated to the vector pMP220 also digested with *Bam*HI and *Eco*RI giving plasmid pMPnifN-Q.

Nitrogenase and β-galactosidase assays

For nitrogenase assays, cells were grown at 30ºC in semi-solid NFbHP-malate (1.75 g/L agar) medium containing 0.5 mM glutamate at atmospheric pressure (Klassen *et al*., 1999) and acetylene reduction activity was measured by gas chromatography (Dilworth, 1966; Schollhorn and Burris, 1967). Briefly, the flasks containing grown cultures were sealed with a subaseal and acetylene was added to a final concentration of 10% (v/v) of the gas phase. The cultures were incubated at 30°C without agitation. After one hour, samples (0.5 ml) were removed for analysis with a gas chromatograph (Varian model 3400 equipped with a Porapak N column and flame ionization detector) to determine the amount of ethylene produced. Nitrogen was the carrier (20 ml/min) and the temperatures of the column and detector were 120°C and 200°C, respectively. Nitrogenase activity is reported as nmol of C_2H_4 produced per minute per mg protein.

β-Galactosidase activity was assayed as described by Miller (1992). Aliquots (0.1 ml) of the cultures were removed and mixed with 0.9 ml of buffer Z (60 mmol/L $Na_2HPO_4{\cdot}7H_2O$, 40 mmol/L $NaH_2PO_4{\cdot}H_2O$, 10 mmol/L KCl, 1 mmol/L $MgSO_4.7H_2O$, 50 mmol/L β-mercaptoetanol, 0.0027% SDS, pH 7.0). After incubation (30°C for 5 min) *o*-nitrophenyl-β-D-galactoside (4 mg/ml) was added and incubated at 30ºC. The stop solution (Na_2CO_3 1 mol/L) was then added and the absorbance of *o*-nitrophenol (ONP) was measured at 420 nm. The results are reported as nmol of ONP produced per minute per mg protein. Protein was determined as described by Bradford (1976) using bovine serum albumin (BSA) as the standard.

Results and Discussion

Identification of the *fdxA* and *fdxN* genes of *H. seropedicae*

In this work we have identified two ferredoxin-like coding genes associated with the *nif* cluster of *H. seropedicae.* These genes have been named *fdxA* (GenBank accession no. EF666057) and *fdxN* (accession number M60319) based on their similarity to known genes.

The *H. seropedicae fdxA* gene is located downstream from the *nifHDKENXorf1orf2* operon and immediately upstream from the *nifQmodA1B1C1* genes (Klassen *et al*., 1999) (Fig. 1). The *fdxN* gene is located downstream from *nifB* and upstream from a *hesB*-like gene (Rego *et al*., 2006) (Fig. 1). Analysis using the String program (von Mering *et al*., 2007) showed that the genomic neighborhood of *fdxA* and *fdxN* is conserved in several diazotrophs such as *R. rubrum* (Edgren and Nordlund, 2005) and *B. japonicum* (Gottfert *et al*., 2001), suggesting a functional relationship between the proteins coded by these genes (Fig. 2).

The *fdxA* gene codes for a 101 amino acid protein with two typical cysteine clusters (Cys-X_2-Cys-X_2-Cys-X_3-Cys) 41 amino acids apart. It has been suggested that these clusters are involved in the coordination of two [4Fe-4S] iron centers

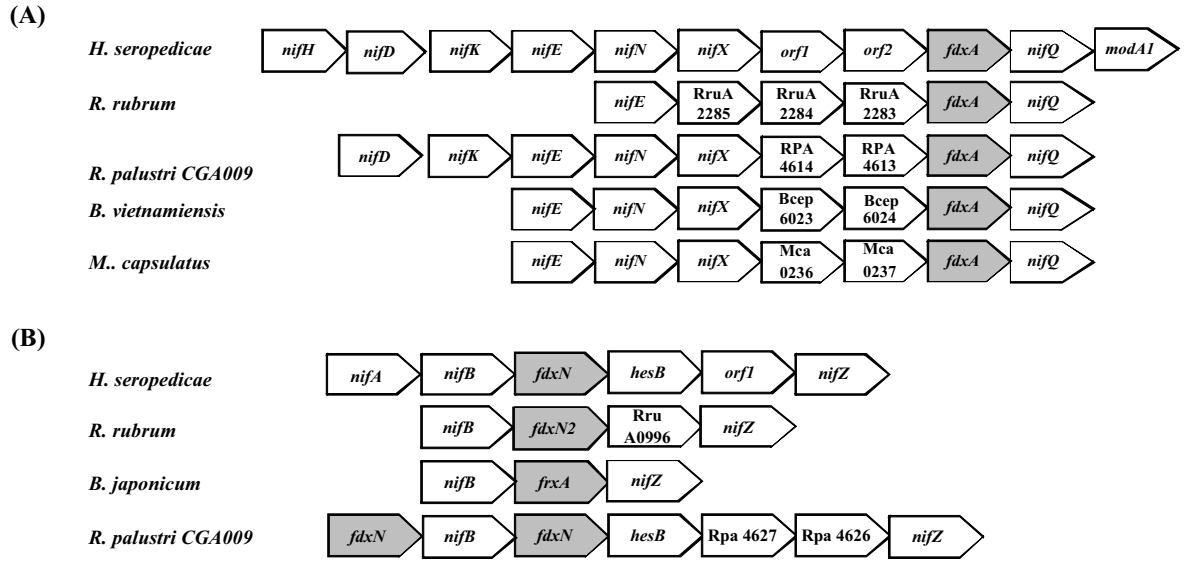

Fig. 2. Neighbourhood analysis of *fdxA* (A) and *fdxN* (B) genes. The analysis was performed using the Program String (von Mering *et al*., 2007). Sequence of the identified regions was retrieved from GenBank and used to draw the diagram. The boxes representing the *fdxA* and *fdxN* genes are shaded.

(Bruschi and Guerlesquin, 1988). Analysis indicated 64% identity (81% similarity) to a 4Fe-4S ferredoxin from *Burkholderia vietnamiensis* G4 (ABO58950) (Menard *et al*., 2007), 56% identity (72% similarity) to FdxA from *Azospirillum brasilense* (AAK51501) (Potrich *et al*., 2001), 55% identity (73% similarity) to Fer3 from *B. japonicum* (AAG60736) (Gottfert *et al*., 2001) and 50% identity (75% similarity) to FdIII (FdxA) from *R. capsulatus* (M26323) (Moreno-Vivian *et al*., 1989). In all these organisms the ferredoxin-coding gene is in the cluster *nifENXorf1orf2fdxAnifQ* (Moreno-Vivian *et al*., 1989; Klassen *et al*., 1999; Gottfert *et al*., 2001; Potrich *et al*., 2001; Menard *et al*., 2007).

The *H. seropedicae fdxN* gene (accession number M60319) codes for a 72 amino acid protein with two cysteine clusters: the first one has a typical Cys-X_2-Cys-X_2-Cys-X_3-Cys motif, while the second one has the motif Cys-X_2-Cys-X_8-Cys-X_3-Cys. It has 86% identity (93% similarity) to FdxN from *Rhodopseudomonas palustris* (GenBank accession no. NP 949963) (Larimer *et al*., 2004), 73% identity (81% similarity) to FrxA from *B. japonicum* (accession number P27394) (Ebeling *et al*., 1988), 66% identity (75% similarity) to FdxN from *B. japonicum* (accession AAG60725) (Gottfert *et al*., 2001), 54% identity (61% similarity) to FdxN2 from *R. rubrum* (accession NC 007643.1) (Edgren and Nordlund, 2005), and 53% identity (63% similarity) to FdxN from *Azoarcus* sp. (accession AF200742) (Egener *et al*., 2001). These genes are located downstream from *nifB* except in *Azoarcus* sp. (Egener *et al*., 2001) where it is located downstream from *nifHDK*. In *B. japonicum,* two homologues of *fdxN* were found, the *frxA* gene is located downstream from *nifB* (Fig. 2), and the *fdxN* gene is located upstream from the *nifDK* cluster and downstream from *aldA* (aldehyde dehydrogenase) (Gottfert *et al*., 2001).

Transcriptional analysis of the *H. seropedicae fdxA* gene

In *H. seropedicae*, the *nifHDKENXorf1orf2* genes are transcribed from a -24/-12 type promoter located upstream from *nifH* (Machado *et al*., 1996; Klassen *et al*., 1999). *In silico* analysis revealed neither a typical σ^{54}-dependent promoter sequence nor a DNA-binding sequence for the NifA protein in the region upstream from *fdxA* and downstream from *nifH*. Inspection of the whole region *fdxAnifQmodA1B1C1* did not reveal any sequence similar to a *nif* promoter or putative NifA binding site. Furthermore, the intergenic regions are very short and no putative terminator sequences were identified. Earlier, Klassen *et al*. (1999) showed the absence of an active promoter between the *nifK* and *nifE* genes. In order to verify if no cryptic promoter was present upstream from *fdxA*, the region including the 3′ of *nifN* to the 5′ end of *nifQ* was cloned into a *lacZ* fusion vector (Table 2). When this fusion was introduced in the wild type strain of *H. seropedicae*, only background activity was observed under all conditions tested, confirming the absence of a promoter in that region. Moreover, a fragment containing the region between *orf1* and *modA1* was also subcloned into pMP220 producing an *orf1-modA1*::*lacZ* fusion (data not shown) which failed to drive β-galactosidase expression under nitrogen fixation conditions. Together, the results suggest that the gene cluster *nifHDKENXorf1orf2fdxAnifQmodA1B1C1* of *H. seropedicae* forms a single operon under control of the *nifH* promoter.

To confirm that the pattern of *fdxA* expression follows that of a *nif* gene, a mutant strain (KC6) of *H. seropedicae* carrying a *lacZ* reporter gene inserted into *fdxA* in its transcription orientation was constructed. The β-galactosidase activity of this strain in the presence or absence of oxygen and ammonium is shown in Table 2. The results show that *fdxA* is expressed only under nitrogen-fixing conditions (low oxygen and ammonium), suggesting that this gene is under control of a *nif* promoter. Together the results suggest that *fdxA* is expressed from a *nif* promoter located upstream from *nifH,* since no other promoter sequence was found upstream from *fdxA* and the β-galactosidase activity expression pattern of the mutant strain KC6 is comparable to that of a *nifH*::*lacZ* mutant strain (Machado *et al*., 1996).

The *H. seropedicae fdxN* gene is located in another *nif* operon in which *nifB* is the first gene. Sequence analysis did not reveal a *nif*-type promoter or NifA-binding site in the *nifB-fdxN* (37 bp) or *fdxN-hesB* (13 bp) intergenic regions of *H. seropedicae* (Fig. 1). As previously shown by Rego *et al.* (2006), the *nifB* of *H. seropedicae* is expressed by a NifA-/RpoN-dependent promoter under low nitrogen and oxygen conditions (Table 2). Together, the sequence analysis and expression from the *nifB* promoter suggest that the *fdxN* gene is expressed under nitrogen fixation conditions from the promoter located upstream from *nifB* (Rego *et al*., 2006).

Mutation and phenotypic analysis of the ferredoxin genes of *H. seropedicae*

To determine the role of FdxA on nitrogen fixation in *H. seropedicae*, a mutant was obtained by inserting a *lacZ*-Km cassette into the *fdxA* gene, in the same orientation as the gene, yielding strain KC6.

The *fdxA* mutant strain KC6 had nitrogenase activity 70% lower than that of the wild-type (Fig. 3), indicating that ferredoxin FdxA does play an important role in the nitrogen-fixing process. Previously, Klassen *et al*. (1997) showed that a *nifQ* mutant had nitrogenase activity comparable to that of the

Table 2. Expression analysis of the *nifX-nifQ* region and the *fdxA*::*lacZ* mutant strain of *H. seropedicae*. β-Galactosidase activity was determined in *H. seropedicae* SmR1 strain carrying the indicated plasmids and in the mutant strain KC6. +N or -N indicates the presence or absence of 20 mM NH_4Cl, respectively; +O indicates ~20% O_2; –O indicates ~1.5% O_2. Data are the Mean±SD of at least 3 independent assays

Strain (Plasmid)	Conditions	β-Galactosidase activity (nmol ONP/mg protein/min)
SmR1(pEMS140) *nifB*::*lacZ*	+N/+O	3.6±1.2
	+N/–O	7.9±5.6
	–N/+O	6.6±1.3
	–N/–O	815.6±54.3
SmR1(pMPnifN-Q) *nifN-Q*::*lacZ*	+N/+O	6.4±0.6
	+N/–O	7.1±5.0
	–N/+O	0.3±0.1
	–N/–O	12.1±1.2
KC6 *fdxA*::*lacZ*	+N/+O	7.9±3.2
	+N/–O	28.2±14.1
	–N/+O	12.6±7.6
	–N/–O	525.8±19.5

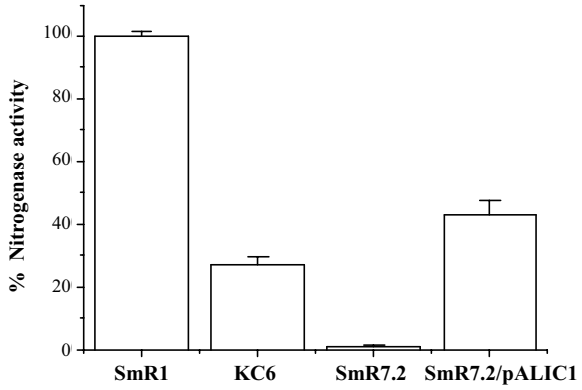

Fig. 3. Relative nitrogenase activities of the *H. seropedicae* wild type (SmR1), *fdxA* mutant (KC6), *fdxN* mutant (SmR7.2), and *fdxN* mutant carrying plasmid pALIC1. Full nitrogenase activity (100%) was approximately 8 nmol of ethylene/min/mg of protein for the wild type strain SmR1. Results are the Mean±SD of at least three experiments.

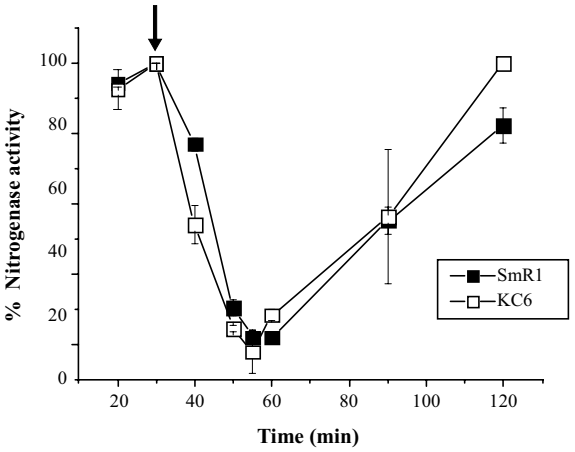

Fig. 4. NH_4^+-dependent nitrogenase switch-off activity of *H. seropedicae* wild type (SmR1) and *fdxA* mutant (KC6) strains. Arrow indicates the addition of 300 μmol/L NH_4Cl. Full nitrogenase activity (100%) was approximately 8 mol of ethylene/min/mg of protein for the wild type strain SmR1 and 70% lower for the *fdxA* strain KC6. Results are the Mean±SD deviation of at least three experiments.

wild type strain under normal molybdenum concentration (7.2 μM). These results suggest that the reduction in nitrogenase activity observed in strain KC6 is probably due to defective electron transport to the nitrogenase and not to a polar effect on the genes upstream from *fdxA*.

Strain SmR7.2 contains the insertion of a kanamycin resistance cassette into the *fdxN* gene. This mutant strain was unable to reduce acetylene (Fig. 3), indicating that the *fdxN* product is essential for nitrogen fixation in *H. seropedicae*. The Nif negative phenotype of the *fdxN* mutant was partially complemented by a plasmid carrying only the *fdxN* gene under control of the *lacZ* promoter (strain SmR7.2/pALIC1). Since the mutation in *fdxN* is polar on *hesB*, either *hesB* is not essential for nitrogen fixation in *H. seropedicae* or its product is being substituted by another protein in the mutant strain. In agreement with the latter suggestion, a similarity search revealed two additional *hesB*-like genes in the *H. seropedicae* genome (unpublished data). Searches of the whole genome sequences database using the String software (von Mering *et al*., 2007) showed that *hesB*-like genes are usually located in the neighborhood of *nifS*-like genes in an organization similar to that observed in the *H. seropedicae* genome (data not shown), suggesting that *hesB* participates, together with NifUS-like proteins, in Fe-S cluster biosynthesis (Dombrecht *et al*., 2002).

Mutation of a ferredoxin gene (*fdxN*) of *Azoarcus* sp. which is co-transcribed with the *nifHDK* genes did not abolish its nitrogenase activity (Egener *et al*., 2001), but yielded a phenotype similar to that of *fdxA* mutant of *H. seropedicae*. However, the *Azoarcus* mutant strain was impaired in the ammonium-dependent switch-off of nitrogenase (Egener *et al*., 2001). Although FdxA and FdxN are not homologous proteins, we decided to assay the *H. seropedicae fdxA* mutant for nitrogenase switch-off by ammonium ions since *fdxA* is the only ferredoxin gene located in the *nifHDK* operon. Contrary to *Azoarcus*, no effect on nitrogenase switch-off was observed (Fig. 4): both mutant and wild-type strains exhibited rapid and reversible inhibition of the nitrogenase activity upon addition of ammonium to cells actively fixing nitrogen. Thus, our results indicate that FdxA is not involved in the ammonium-dependent nitrogenase switch-off in *H. seropedicae*. A major difference between nitrogenase activity control in *Azoarcus* sp. and *H. seropedicae* is that in the former the nitrogenase switch-off involves reversible ADP-ribosylation by the DraT and DraG enzymes (Martin and Reinhold-Hurek, 2002). In *H. seropedicae* the nitrogenase switch off/on occurs through an as yet unknown mechanism of post-translational regulation which involves the ammonia channel AmtB (Noindorf *et al*., 2006). The wild type switch-off phenotype of the *fdxA* mutant of *H. seropedicae* may be explained by the distinct mechanisms for nitrogenase post-translational control. Alternatively, *fdxN* from *Azoarcus* and *fdxA* from *H. seropedicae* may not be functionally equivalent despite similar locations in relation to the *nifHDK* genes and the nitrogenase activity phenotype. The latter hypothesis is probably favored since FdxN and FdxA share no significant similarity.

Many studies on the role of ferredoxin-like proteins in nitrogen fixation have been reported in diazotrophs such as *Azoarcus* sp., *B. japonicum*, *R. rubrum*, *K. pneumoniae*, *R. capsulatus* (Shah *et al*., 1983; Ebeling *et al*., 1988; Jouanneau *et al*., 1995; Armengaud *et al*., 1997; Egener *et al*., 2001; Edgren and Nordlund, 2005; Hauser *et al*., 2007). In the photosynthetic bacterium *R. rubrum*, the ferredoxin coded by *fdxN2,* found downstream from *nifB,* is believed to be the primary electron donor to nitrogenase (Edgren and Nordlund, 2005) with the involvement of the *fixABCX* gene products (Edgren and Nordlund, 2006). Two different pathways for electron transfer to nitrogenase have been proposed for this organism: one is dependent on a NifJ-like protein and the other on *fixABCX* gene products. However, both pathways seem to use the FdxN protein as a direct electron donor to the Fe-protein (Edgren and Nordlund, 2006).

In *B. japonicum*, three ferredoxin coding genes are located in the *nif* or *nod* clusters (Gottfert *et al*., 2001): the *frxA* gene, located downstream from *nifB* is not essential for nitrogen fixation (Ebeling *et al*., 1988); the *fixX* gene, located downstream from *fixBC* is required for nitrogen fixation (Gubler *et al*.,

1989); and the *fdxN* gene, located downstream from *aldA* gene, is essential for efficient symbiotic nitrogen fixation and necessary for full nitrogenase activity (Hauser *et al*., 2007).

A recurrent characteristic of the location of *fdxA* and *fdxN* in many diazotrophs is the presence in their neighborhood, usually in the same operon, of *nif* genes involved in the iron-molybdenum cofactor biosynthesis, namely *nifEN* and *nifB*, suggesting their possible participation in FeMoco biosynthesis. This hypothesis is also consistent with the elimination or reduction of nitrogenase activity in the respective mutants.

In *H. seropedicae*, the *fdxA* gene product is important for full nitrogenase activity, but it seems unlikely that FdxA is the primary electron donor to nitrogenase or its activity can be substitute by another protein. On the other hand, the absence of nitrogenase activity in the *fdxN* mutant indicates that FdxN is essential for nitrogenase activity and our results also imply that FdxA cannot replace FdxN in its role in nitrogen fixation in *H. seropedicae*. Whether FdxN is the primary electron donor to nitrogenase in *H. seropedicae* or whether it is necessary for nitrogenase metal cluster assembly, a hypothesis suggested by the conservation of adjacent genes, has yet to be determined.

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