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Characterization of an *Azospirillum brasilense* Tn*5* mutant with enhanced N_2 fixation: the effect of ORF280 on *nifH* expression

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Abstract

Disruption of an open reading frame (ORF) of 840 bp (280 amino acids; ORF280) in an *Azospirillum brasilense* Tn*5* mutant resulted in a pleiotrophic phenotype. Besides an enhanced N_2-fixing capacity and altered expression pattern of a *nifH-gusA* fusion, growth on the charged polar amino acids glutamate and arginine was severely affected. ORF280, similar to previously identified ORFs present in *Bradyrhizobium japonicum* (ORF277), *Paracoccus denitrificans* (ORF278) and *Rhodobacter capsulatus* (ORF277), exhibits in its C-terminus a significant similarity with the recently defined family of universal stress proteins.

Keywords: N_2 fixation; Nitrogenase; Oxygen; Tn*5* mutagenesis; *Azospirillum brasilense*

1. Introduction

Azospirillum brasilense is a Gram-negative, microaerobic, N_2-fixing bacterium associated with the roots of many plants [1]. NH_4^+ and O_2 regulation of N_2-fixation in *A. brasilense* is mediated by a complex regulatory cascade. NifA, the key transcriptional activator, activates transcription of *nif* operons which contain σ^{54}-dependent promoters, preceded by upstream activating sequences [2–4]. In *A. brasilense*, *nifA* transcription starts from an unidentified, partly NtrBC-dependent promoter and is largely constitutive [5,6]. NifA is present in an inactive form under conditions incompatible with N_2 fixation [2,7,8]. The NifA protein of *A. brasilense* contains a conserved motif of cysteine residues, which is responsible for the O_2 inactivation of NifA [7,9]. For the regulation of NifA activity in response to the nitrogen status, a specific P_{II} protein (*glnB*) is required [10].

Besides for NifA regulation, P_{II} is involved in attenuating activity of the NtrBC two-component system: in the presence of a combined N-source, the deuridylated form of P_{II} is required for dephosphorylation of NtrC [11]. The NtrBC system is responsible for the nitrogenase switch-off in response to NH_4^+ and anaerobiosis [12]. Furthermore, NtrBC regulates, in concert with the σ^{54} polymerase, genes involved in N-metabolism (NO_3^- utilization) and a gene, *glnZ*, encoding a second P_{II}-like protein (P_z) [11]. The severely abrogated growth of an *A. brasilense* P_{II}/P_z double mutant suggests that both P_{II} and P_z are necessary for optimizing nitrogen and carbon metabolism in any condition [11].

In the present study, we report that Tn*5* mutagenesis of *A. brasilense* open reading frame (ORF) ORF280 results in a pleiotrophic phenotype. *A. brasilense* ORF280 is similar to previously identified ORFs in *Paracoccus denitrificans*, *Bradyrhizobium japonicum* and *Rhodobacter capsula-*

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tus. The C-terminus of *A. brasilense* ORF280 was shown to exhibit a striking similarity to a range of hypothetical proteins from highly distinct origins that have recently been classified as universal stress proteins [13]. To our knowledge, this study is among the first reports describing a phenotype for a member of this newly described protein family.

2. Materials and methods

2.1. Strains and plasmids

A. brasilense and *Escherichia coli* strains and the plasmids used in this work are listed in Table 1.

2.2. Media and growth conditions

The bacterial strains were grown in Luria Bertani medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LB*) at 37°C for *E. coli* strains and at 30°C for *A. brasilense* strains. For the *A. brasilense* Tn*5* mutants, kanamycin was added to a final concentration of 70 μg ml^{-1}. *A. brasilense* wild-type and Tn*5* mutants were also cultivated in NFbHP at 30°C with the appropriate antibiotic concentration as described in [14]. Growth of *A. brasilense* on different nitrogen sources was tested on solid NFbHP supplemented with 5 mM glutamine, arginine, histidine, serine, lysine or glutamate. Conjugation was performed on D-plates (8 g l^{-1} Bacto Nutrient broth, 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1.0 g l^{-1} KCl, 0.01 $MnCl_2$, 2% agar) and, after conjugation, minimal medium AB (MMAB) [15] was used for selection of *A. brasilense* transconjugants. Antibiotics were used at the following concentration unless stated otherwise: tetracycline 10 μg ml^{-1}, ampicillin 70 μg ml^{-1}, kanamycin 25 μg ml^{-1}. Nitrogen-free medium used in the derepression experiments was MMAB without NH_4Cl.

2.3. DNA manipulations, cloning and sequence analysis

General cloning techniques were carried out as described in [16]. Treatment of DNA with restriction enzymes and nucleic acid-modifying enzymes was performed in accordance with the manufacturer's specifications. Southern blot analysis of the *A. brasilense* mutant strains was performed as previously described [17]. DNA sequencing was performed using the dideoxy method (as described in [16]) on fragments subcloned into pBluescript phagemid (Stratagene) with T7 Sequencing kit (Pharmacia) and Sequenase 2.0 kit (USB United Stated Biochemical) using [α-^{32} P]dATP (800 Ci $mmol^{-1}$) or [α-^{35}S]dATP (1000 Ci $mmol^{-1}$) for labeling. Sequence analysis was performed by the programs BLAST and PSI-BLAST [18], CLUSTALW [19], prodom [20] and pfam [21]. Total DNA of the *A. brasilense* Tn*5*-33 mutant was digested with *Eco*RI to completion and hybridized with an internal 1.9-kb *Pst*I DNA fragment derived from Tn*5* transposon (Fig. 1). A 12-kb *Eco*RI DNA fragment was revealed by this probe. A gene bank of enriched 12-kb *Eco*RI DNA fragments of the strain Sp7::Tn*5*-33 was constructed into pUC18 vector and used to isolate the DNA fragment containing the Tn*5* insertion by kanamycin resistance selection. The physical map of this fragment was determined and is shown in Fig. 1.

The nucleotide sequence of the flanking regions of the Tn*5* insertion was determined by subcloning both fragments into pBluescript vector using an internal *Hpa*I restriction site located within the insertion sequence of the Tn*5* region. Both strands of the 0.5-kb *Hpa*I/*Eco*RI and 0.9-kb *Hpa*I/*Sal*I fragments were completely sequenced (Fig. 1).

An *A. brasilense* Sp7 genomic library [22], constructed into pLAFR3 cosmid, was screened using a DNA fragment encompassing *orf280* from Sp7::Tn*5*-33 as a probe. Cosmid pWT1 carrying a 6-kb *Sal*I DNA fragment was isolated and transferred by conjugation into the *A. brasi-*

Table 1
Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
Strains		
A. brasilense		
Sp7	Wild-type strain, ATCC 29145	[37]
Sp7::Tn*5*-33	Sp7 *orf280*Km^R mutant	[23]
Sp7-pFAJ21	Sp7 carrying pFAJ21 plasmid	This work
Sp7::Tn*5*-33-(pFAJ21)	Sp7::Tn*5*-33 carrying pFAJ21 plasmid	This work
E. coli		
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*$^-$ F″[*proAB*$^+$ *lacI*^{q} *lacZΔM15* Tn*10*(tet^r)]	[16]
S17.1	*Thi endA rec*A *hs*dR RP4-2-Tc::Mu-Km::Tn*7*	[38]
Plasmids		
pUC18	Ap^R, cloning vector	[16]
pBluescript	Ap^R, cloning vector	[16]
pFAJ21	Ap^R, pRAJ275 derivative, contains the *A. brasilense nifH* promoter fused to the promoterless *gusA* gene	[39]
pWT1	Tc^R, pLAFR3 derivative contains a 6.0-kb DNA fragment from the *A. brasilense* genome	This work

lense Sp7::Tn*5*-33 mutant. This fragment was also used to complement the sequence of the DNA region containing *orf280*.

2.4. β-Glucuronidase and nitrogenase assays

The acetylene reduction assays on whole cells derepressed for nitrogenase were performed as follows. *A. brasilense* strains were initially grown in LB* medium for 24 h at 37°C with shaking at 250 rpm. Subsequently, 1 ml of each culture was washed twice with sterile 0.85% NaCl solution and 30 μl of each culture was inoculated in 12-ml flasks containing 5 ml of semi-solid NFb medium [23]. Following an overnight incubation at 30°C, 1 ml of acetylene was added to each flask. After an additional incubation for 2 h at 30°C, ethylene production was measured on a gas chromatograph (model CG 3537-D) [23].

For the determination of the nitrogenase activity at different constant O_2 tensions, *A. brasilense* cells were aerobically grown under similar conditions as used for the acetylene reduction assay. After washing cells twice with 0.85% NaCl, cells were suspended in nitrogen-free medium at a cell density of 10^7 cells ml^{-1}. Using a sterile syringe and needle, 1-ml portions of these suspensions were injected into 25 by 200-mm test tubes tightly stoppered with rubber caps containing a gas mixture of N_2 and O_2. The gas mixture was achieved by flushing the test tubes for 20 min with nitrogen and then, using a syringe, air was readmitted to give the appropriated O_2 tensions. An O_2 electrode (ABISS Vak 10) was used to measure the O_2 content in the headspace. Cultures (three independent replicates) were vigorously shaken at 200 rpm at 30°C and after 20 h of incubation, the final O_2 tension was measured.

β-Glucuronidase activity of the culture was determined quantitatively using the substrate *p*-nitrophenyl-β-D-glucuronide as described previously [24]. Each culture was assayed in duplicate and the units are expressed as described by Miller [16].

Nitrogenase activity was measured by the acetylene reduction assay as previously described [23]. Acetylene was injected in the test tubes to a final concentration of 10% after 4 h of incubation. Ethylene production was quantified after 20 h of incubation (total time).

3. Results

3.1. Molecular characterization of the Sp7::Tn5-33 mutant

We have previously isolated an *A. brasilense* Tn*5* mutant named Sp7::Tn*5*-33, characterized by an enhanced N_2-fixing ability (4-fold higher) as compared to the wild-type strain [23]. DNA sequencing of the region containing the Tn*5* insertion revealed one ORF of 840 nucleotides encoding a predicted polypeptide of 280 residues (ORF280). The region preceding this ORF contains a sequence (TCGTCA/TATAAT) showing a striking similarity with −35/−10 promoter sequences. The GAGGGAGAA sequence located 5 bp upstream of the putative ATG of this ORF resembles a ribosome-binding site (Fig. 1). The Tn*5* insertion in the mutant was mapped at position 393 relative to the *Eco*RI site. A sequence with interrupted dyad symmetry ($\Delta G(25°C) = -13.8$ kcal), located 64 bp downstream the stop codon suggests that the ORF may be transcribed as a single unit. The complete nucleotide sequence of the ORF encoding ORF280 has been submitted to the GenBank Nucleotide Sequence Database, under the accession number AF083218 along with the predicted amino acid sequence.

ORF280 exhibits 39.5% similarity with *B. japonicum* ORF277 [25], 42.7% similarity with *P. denitrificans*

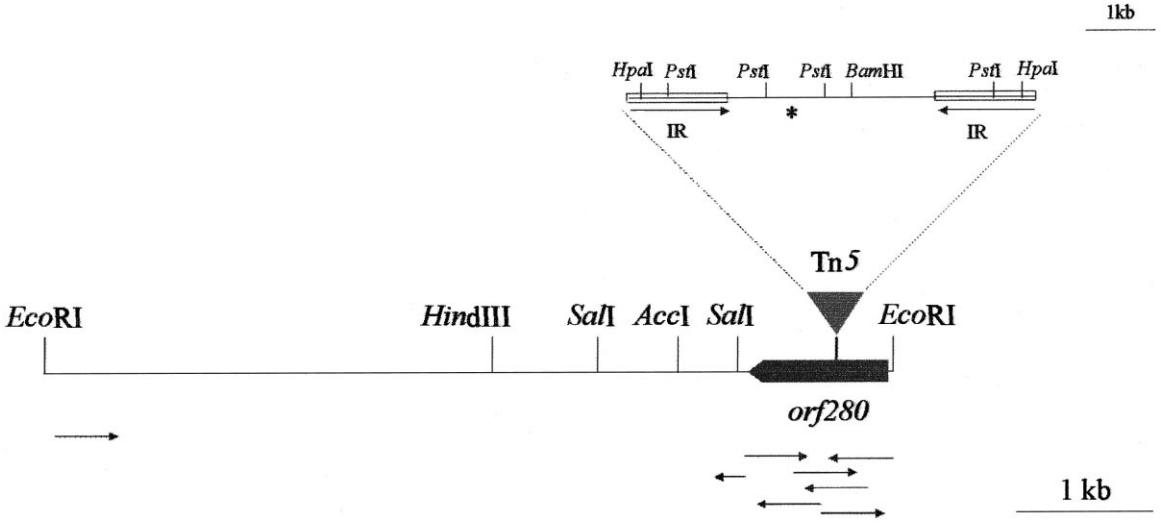

Fig. 1. Physical map of the *A. brasilense* mutated region encompassing *orf280*. The gray box represents the location and transcription direction of the ORF encoding ORF280. Tn*5* insertion into the ORF is indicated by an arrowhead and the *Pst*I fragment used as a probe is marked (*). The sequenced region is marked by arrows.

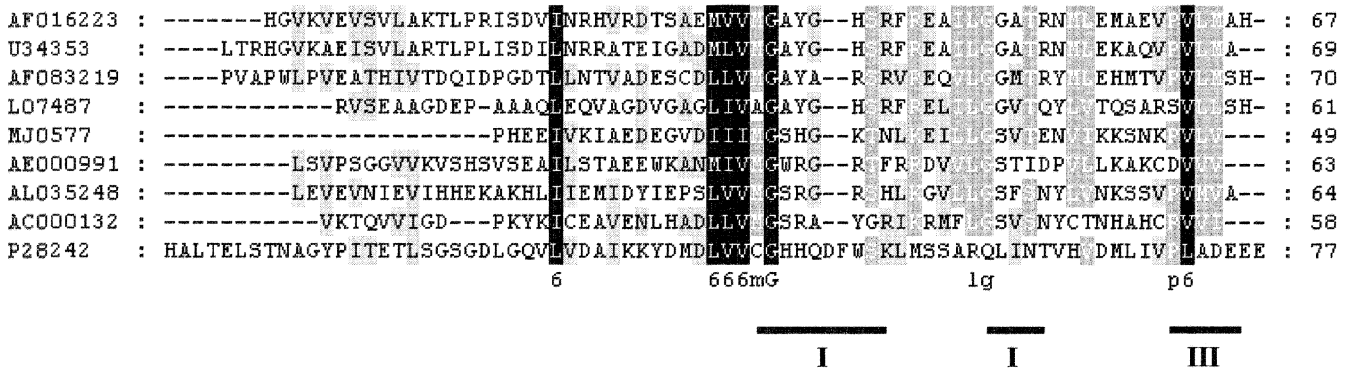

Fig. 2. Multiple alignment of sequences homologous to the C-terminal region of *A. brasilense* ORF280. The alignment was constructed by using PSI-BLAST [18] followed by CLUSTALW [19]. Alignment editing was done using GeneDoc [36]. Black lines below sequences indicate conserved residues interacting with ribose (I), with phosphate (II), and residues in (III) would be located at the dimer interface [29]. Conserved residues are shaded. Black shading: conserved in all aligned sequences; dark gray: conserved in at least 80% of the aligned sequences; light gray: conserved in at least 60% of the aligned sequences. Identical amino acids are marked at the bottom line by the letter code, 6 indicates a conserved replacement (L, I, M, V). AF016223: *R. capsulatus* ORF277; U34353: *P. denitrificans* ORF278; AF083219: *A. brasilense* ORF280; L07487: *B. japonicum* ORF277; MJ0577: *M. jannaschii*; AE000991: *Archaeolobus fulgidus* cationic amino acid transporter; AL035248: *Schizosaccharomyces pombe* universal stress protein family protein; AC000132: *Arabidopsis thaliana*; P28242: UspA *E. coli* universal stress protein.

ORF278 [26] and 35% similarity with *R. capsulatus* ORF277 [27]. Using PSI-BLAST, a whole range of hypothetical proteins from prokaryotes, archaea and eukaryotes was found to contain a region of homology with the C-terminus of ORF280. To illustrate the prevalence of this sequence region, an alignment of some representatives is shown in Fig. 2. Using a similar PSI-BLAST strategy with the N-terminus did not reveal homology with proteins in databases.

3.2. The mutation in orf280 is responsible for the altered Nif phenotype in the A. brasilense Sp7::Tn5-33 mutant

The Sp7::Tn*5*-33 mutant was isolated by Tn*5* site-directed mutagenesis aiming to detect additional *nif* functions in the DNA sequences flanking the nitrogenase structural genes *(nifHDK)* of *A. brasilense* [23]. The mutant displays a 4-fold higher nitrogenase activity on semi-solid NFb medium as compared to the wild-type strain (136 nmol min^{-1} mg^{-1} protein and 32 nmol min^{-1} mg^{-1} protein, respectively). Complementation of this mutant with pWT1, carrying *orf280*, restored nitrogenase activity to wild-type levels ($\sim$30 nmol min^{-1} mg^{-1} protein). This suggests that the mutation in ORF280 is responsible for the altered phenotype.

To further characterize the expression of *nif* genes in Sp7::Tn33 mutant, we have analyzed the expression of a *nifH-gusA* fusion (pFAJ21) in the *A. brasilense* mutant. The plasmid pFAJ21 was conjugated into the wild-type and mutant strain. *nifH* expression and nitrogenase activity of both *A. brasilense* strains (Sp7(pFAJ21) and Sp7::Tn*5*-33(pFAJ21)) were determined at different constant O_2 tensions. The maximal nitrogenase activity for both strains was observed at an O_2 concentration of 0.5% (Fig. 3). When the O_2 concentration exceeded 3%, N_2-fixation of both mutant and wild-type was completely abrogated. Therefore, we can conclude that although Sp7::Tn*5*-33 mutant has a higher nitrogenase activity as compared to the wild-type strain (Fig. 3), both strains display the same O_2 tolerance for the N_2-fixation process.

The effect of O_2 on the regulation of *nif* genes in the Sp7::Tn*5*-33 mutant was evaluated by monitoring the expression of a *nifH-gusA* fusion. Compared to the wild-

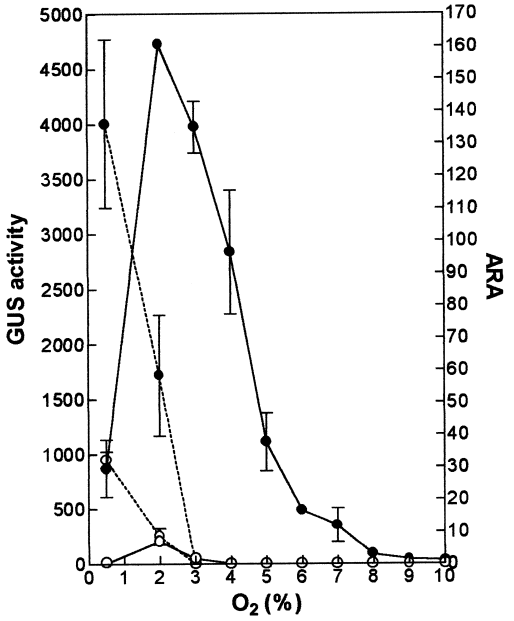

Fig. 3. Expression of the *nifH-gusA* fusion and acetylene reduction activity (ARA) of the *A. brasilense* wild-type and mutant strains as a function of the initial O_2 concentration. β-Glucuronidase activities expressed as Miller units (U) are indicated on the left axis, acetylene reduction activities (nmol ethylene h^{-1} ml^{-1}) are indicated on the right axis. Dashed lines represent ARA activity in wild-type (○) or mutant strain (●) and solid lines represent β-glucuronidase activity in wild-type (○) or mutant strain (●). The values depicted are the means of three independent samples. S.D.s are represented by vertical bars.

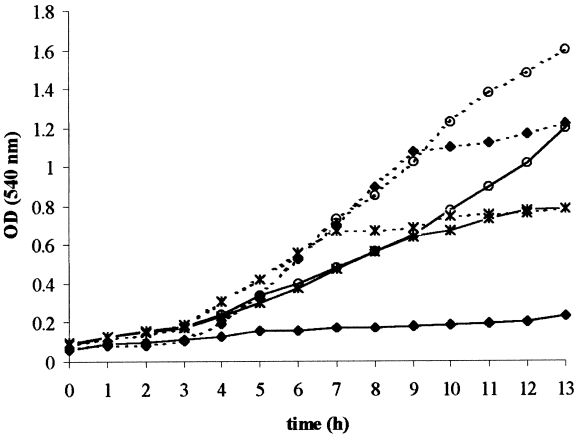

Fig. 4. Effect of nitrogen source on *A. brasilense* growth rates. Bacterial strains were aerobically grown in minimal medium supplemented with 20 mM NH_4Cl (···○··· wild-type and –○– mutant), 2 mM NH_4Cl (···*··· wild-type and –*– mutant) or with 20 mM sodium glutamate (···◆··· wild-type and –◆– mutant) as nitrogen source.

type, the Sp7::Tn*5*-33 mutant showed an approximately 10-fold higher *nifH* expression at O_2 levels up to 3%. Presumably the O_2 level permissive for nitrogenase activity and *nifH-gusA* expression in the *A. brasilense* wild-type coincide (Fig. 3). In the *orf280* mutant, *nifH* expression was detected at O_2 concentrations (ranging from 3 to 7%) apparently not permissive for *nifH* expression in the wild-type background. This can be explained by the higher absolute β-glucuronidase activities in the mutant as compared to the ones detected in the wild-type. Indeed, because of this higher β-glucuronidase activity in the mutant, the detection limit for β-glucuronidase assay is reached at higher O_2 concentrations as compared to the levels where the detection limit is reached in the wild-type. Based on these results, we suggest that the mutation in ORF280 gives rise to increased levels of *nifH-gusA* expression which result probably in a higher N_2-fixing capacity. The Sp7::Tn*5*-33 mutant was growth-impaired in medium containing glutamate or arginine as a nitrogen source.

To analyze the effect of the mutation in ORF280 on other physiological properties of *A. brasilense*, we compared growth of wild-type and mutant in different media. The Sp7::Tn*5*-33 mutant and the wild-type Sp7 were grown in liquid minimal medium (NFbHP) supplemented with NH_4Cl or glutamate as nitrogen source. Compared to the wild-type, growth rate of the mutant slightly decreased, when a concentration of 20 mM NH_4Cl was added to the medium (Fig. 4). However, when glutamate was used as nitrogen source, the growth rate of the Sp7::Tn*5*-33 mutant decreased drastically (Fig. 4). This behavior of the mutant was further investigated: utilization of other amino acids as nitrogen source was analyzed. The growth rate of the Sp7::Tn*5*-33 mutant was not affected on minimal medium when either glutamine, histidine, serine or lysine were used as nitrogen sources. However, when arginine was used, growth of the mutant was completely abolished (data not shown).

4. Discussion

Based on the apparently conserved genomic organization of ORF277-like proteins in the vicinity of cyt*cbb*$_3$ terminal oxidase complexes [25–27], a role for ORF277-like proteins, somehow related to the function of this oxidase complex, was assumed. However, the fact that ORF277 of *R. capsulatus* is required neither for cytochrome oxidase activity nor for respiratory energy production [27] raises the question of a possible function of ORF280, unrelated to the cyt*cbb*$_3$ terminal oxidase complex. In this context, it is worthwhile mentioning that the *A. brasilense orf280* is not located in the vicinity of the *cytNOQP* genes ([28], Marchal (personal communication)).

The homology of the ORF280 C-terminus to regions present in proteins from eukaryotes, prokaryotes and archaea is indicative of the physiological importance of proteins containing this domain. Therefore, we were speculating that the current knowledge of this domain in other bacteria could be extrapolated and reveal a clue to the role of ORF280. In this respect, it is worth noting that structural genomics revealed that ORF (MJ0577) of *Methanococcus jannaschii* is an oligomeric ATP-binding protein [29]. The signatures for ATP-binding and dimerization were designated to the conserved residues in the C-terminal part of the protein (Fig. 2), which shows homology to ORF280. ORF (MJ0577) of *M. jannashii* itself did not have an ATPase activity but required other components to stimulate ATPase activity. While preparing this manuscript, the proteins containing this putative domain were classified as a new family of universal stress proteins [13] (for a complete alignment, see pfam [21], prodom [20]). The only member with a defined phenotype is the *E. coli* UspR. UspR is a universal stress protein essential for survival in stationary phase [30]. UspR was shown to be a serine and threonine phosphoprotein of which the phosphorylated form increases during starvation. Phosphorylation of UspR was dependent on another autophosphorylating phosphoprotein [31]. Though the mode of action of UspR is still largely unknown, UspR is assumed to regulate either directly or indirectly the activity of proteins which may be superfluous or even detrimental during stasis. *uspR* mutants are impaired in modulating their C-flow through the central metabolic pathways and exhibit diauxic type of growth [32].

The suggested function for ORF280 as a regulatory stress protein might at least partly explain the pleiotrophic phenotype of an *orf280* mutant. Glutamate has been described as a poor N-source for *A. brasilense* [33]. N-limitation being a stress condition might need activation of specialized biochemical pathways, possibly mediated via

ORF280 for assimilation of these N-sources. However, since N_2-fixation only occurs during severe N-limitation, the enhanced N-fixation capacities in the absence of ORF280 seem puzzling at first glance. However, as is the case in *E. coli*, ORF280 may not only be responsive to N-stress but may act as a global stress regulator. If during the experiment the levels of fixed N, obtained by N_2-fixation, become relative high as compared to the C-source, the lack of C-skeletons and energy may become a factor of limitation overruling N-stress. Hence by down-regulating nitrogenase activity via the activity of ORF280, the cell lowers ATP consumption and attenuates excess degradation of scarcely available C-source. Alternatively, besides its postulated function in stress response, an additional role for ORF280 in regulating N-metabolism or N-sensing cannot be excluded. Invoking a role in N-sensing allows explaining the decreased growth on 20 mM NH_4^+ of the *orf280* mutant. If this mutant does not sense high NH_4^+ levels, high metabolic fluxes adapted to high NH_4^+ availability will no longer be induced and N_2-fixation might be upregulated at the maximal level. Further experiments are under way to distinguish between these possibilities.

How ORF280 interacts with N_2-fixing regulatory cascade remains elusive. However, the enhanced nitrogenase is likely a result of the increased *nifH* expression. Therefore, it is tempting to assume that ORF280 either directly or indirectly modulates *nifH*-regulating proteins. In this context, a possible candidate is unmistakably the transcriptional activator of *nifH*, NifA. Future research will be needed to identify the targets of ORF280 and the mechanism of interaction. However, a possible candidate for interaction with ORF280 would be the NtrBC, P_{II}, P_z regulatory cascade in view of the fact that this cascade links N-metabolism and N_2-fixation and that it has a mode of action based on protein phosphorylation. Interestingly, in *A. brasilense*, the expression of two NtrBC-regulated genes, *glnZ* (P_z) and *amtB* (ammonium transporter), was derepressed in the presence of aspartate. Since this upregulation was dependent on NtrC, it has been assumed that in addition to the general nitrogen status signalling, the presence of aspartate influences the concentration of NtrC-phosphate [11,34]. Furthermore, the recently identified PAS domains indicate that NtrBC may have additional redox regulatory functions [35]. Taken together, the P_{II}, NtrBC, P_z regulatory cascade presumably being a global regulator of N- and C-metabolism [11] might constitute an ideal target of interaction with stress responsive systems. Testing the phenotype of double mutants of ORF280 with possible regulatory proteins may allow to determine at what stage ORF280 interacts with the N regulatory cascade.

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