Research article

A glutamine-amidotransferase-like protein modulates FixT anti-kinase activity in *Sinorhizobium meliloti*

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Abstract

Background: Nitrogen fixation gene expression in *Sinorhizobium meliloti*, the alfalfa symbiont, depends on a cascade of regulation that involves both positive and negative control. On top of the cascade, the two-component regulatory system FixLJ is activated under the microoxic conditions of the nodule. In addition, activity of the FixLJ system is inhibited by a specific anti-kinase protein, FixT. The physiological significance of this negative regulation by FixT was so far unknown.

Results: We have isolated by random Tn5 mutagenesis a *S. meliloti* mutant strain that escapes repression by FixT. Complementation test and DNA analysis revealed that inactivation of an asparagine synthetase-like gene was responsible for the phenotype of the mutant. This gene, that was named *asnO*, encodes a protein homologous to glutamine-dependent asparagine synthetases. The *asnO* gene did not appear to affect asparagine biosynthesis and may instead serve a regulatory function in *S. meliloti*. We provide evidence that *asnO* is active during symbiosis .

Conclusions: Isolation of the *asnO* mutant argues for the existence of a physiological regulation associated with *fixT* and makes it unlikely that *fixT* serves a mere homeostatic function in *S. meliloti*. Our data suggest that *asnO* might control activity of the FixT protein, in a way that remains to be elucidated. A proposed role for *asnO* might be to couple nitrogen fixation gene expression in *S. meliloti* to the nitrogen needs of the cells.

Background

Sinorhizobium meliloti forms N_2-fixing nodules on the roots of alfalfa (*Medicago sativa*) and closely related plants. Expression of nitrogen fixation genes is under both positive and negative control. This regulation depends on a regulatory cascade, on top of which the two-component regulatory system FixLJ activates expression of nitrogen fixation genes in response to microoxic conditions [1], such as those that prevail inside the nodule [2]. Under microoxic conditions, the sensor histidine kinase FixL autophosphorylates and transfers its phosphate to the FixJ transcriptional regulator protein [3]. Phosphorylated FixJ then activates transcription of two intermediate regulatory genes, *nifA* and *fixK,* that both encode transcriptional regulators [3]. NifA mediates activation of *nif* genes involved in nitrogenase biosynthesis whereas FixK, a member of the Crp/Fnr family, activates expression of genes involved in the synthesis of a respi-

ratory oxidase complex [4, 5]. *fixK* is also indirectly responsible for negative regulation of the cascade since it controls expression of a gene, *fixT*, that negatively affects expression of FixLJ dependent genes (see Figure 6). We have shown recently that the FixT protein negatively affects the expression of *nifA* and *fixK* by inhibiting phosphorylation of the sensor hemoprotein kinase FixL and, by consequence, phosphorylation of FixJ [6]. Whether FixT serves a mere homeostatic function in *S. meliloti* (the level of FixT protein feed-back controlling activity of the FixLJ system) or whether FixT allows integration of a physiological signal by the FixLJ system was so far unknown. We addressed this question by looking for *S. meliloti* mutants in which the FixT protein would not be active in repression.

Here we report the isolation of a *S. meliloti* mutant strain that phenotypically escapes the repressor activity exerted by FixT. The mutation lies in a gene named *asnO* encoding a protein homologous to glutamine-amidotranferases. We discuss the significance of this finding with respect to the regulation of symbiotic nitrogen fixation.

Results and Discussion

Isolation of a S. meliloti mutant strain escaping repression by FixT

We previously observed that in a *S. meliloti* wild-type strain, constitutive expression of *fixT*, driven by the strong neomycin promoter of plasmid pMF10 (Table 1), resulted in strong inhibition of the expression of a *fixK-lacZ* reporter fusion (pMF457 plasmid; Table 1), thus leading to white colonies on X-gal containing plates. We used this observation to screen for *S. meliloti* mutants that would escape repression by FixT. After random Tn5 mutagenesis of a strain overexpressing *fixT*, we isolated blue colonies on X-gal containing plates. These putative mutant colonies were subsequently assayed for β-galactosidase activity in liquid cultures under microoxic conditions. Four independent mutants were isolated, that were characterized by an unrepressed level of expression of the *fixK* gene despite the constitutive expression of *fixT* (Figure 1A; compare lanes 2 and 3). Two of the mutants possessed a Tn5 insertion in the same gene. One of these two mutants, GMI401, was further characterized. Southern-Blot analysis of genomic DNA digested by different restriction enzymes, revealed a single Tn5 insertion in GMI401 (data not shown). Transduction experiments using the N3 phage, showed genetic linkage between the mutant phenotype and the Tn5 insertion since reintroduction of the pMF10 plasmid in the transduced strain confirmed the lack of repression of *fixK* by overexpressed *fixT* (Figure 1A; lanes 5 and 6). This excluded the possibility that a mutation on one of the plasmids, pMF457 or pMF10, could have been responsible for the phenotype observed.

In the GMI401 mutant strain, the level of *fixK* gene expression in the absence of pMF10 was the same as in the wild-type strain (Figure 1A; compare lane 1 to lane 4 and lane 5). This result demonstrated that activation of *fixK* gene expression by the FixLJ two-component system was not affected in the mutant strain, but rather that the phenotype was genuinely due to decreased repression by *fixT*. Western-Blot analysis using an antibody directed against the FixT protein indicated that the level of FixT protein was the same in the GMI211(pMF10) wild-type strain and in the GMI401(pMF10) mutant strain (data not shown).

Similar results have been obtained using a *nifA-lacZ* fusion (pCHK57 plasmid). The inhibition of *nifA-lacZ* expression by overexpressed *fixT* that was observed in a GMI211 wild-type strain was not observed anymore in the GMI401 mutant strain (data not shown).

Altogether, these results tend to indicate that, in the GMI401 mutant strain, the absence of repression of *fixK* and *nifA* by *fixT* was due to a reduced inhibition of the FixLJ system by the FixT protein.

The GMI401 mutant strain produced nitrogen fixing nodules on *M. sativa*, as did the GMI211 wild-type strain (data not shown). Whereas the GMI211 parent strain expressing *fixT* constitutively produced non N_2-fixing nodules (Figure 1B), the GMI401 mutant strain was able to induce effective N_2-fixing nodules on alfalfa (*Medicago sativa* cv. gemini) thus enabling the plants to grow in the absence of combined nitrogen. Hence, the gene that modulates repression by FixT activity *ex planta* is also active *in planta*.

The Tn5-insertion maps in an asparagine synthetase-like gene

The Tn5 insertion in GMI401 was positioned on the pSymB megaplasmid of S. meliloti (see Materials and Methods), whereas the fixLJ, fixT and fixK genes are located on pSymA megaplasmid. The genomic DNA flanking the Tn5 insertion was sequenced (Figure 2 and Materials and Methods). Prediction of coding regions around the region of interest was performed using the FrameD program [7]. As shown in Figure 2, this analysis revealed a putative open reading frame encompassing the Tn5 insertion site and another putative orf located just dowstream. On the basis of the sequence analysis, these two genes may belong to the same operon, and the Tn5 insertion may thus affect expression of both genes. During the annotation phase of the S. meliloti genome sequencing project (http://sequence.toulouse.inra.fr/

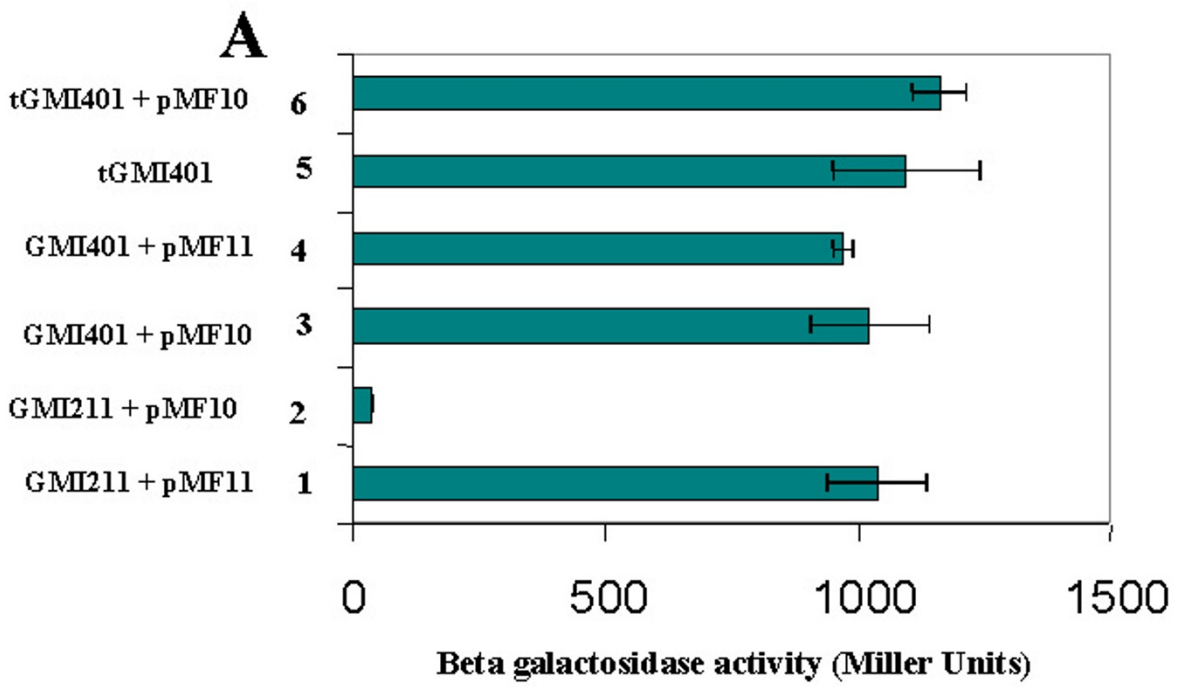

Figure 1

Characterization of the *S. meliloti* mutant strain (GMI401) escaping FixT repressor activity. Panel A : We monitored microoxic expression of a *fixK-lacZ* fusion carried by the reporter plasmid pMF457 in *S. meliloti.* pMF10 allows constitutive expression of *fixT*, pMF11 is a negative control (*fixT* cloned in the antisense orientation). 1 : wild-type strain GMI211(pMF457)(pMF11); 2 : wild-type strain GMI211(pMF457)(pMF10); 3 : mutant strain GMI401(pMF457)(pMF10); 4 : mutant strain GMI401 (pMF457) (pMF11); 5 : transductant strain GMI401(pMF457); 6 : transductant strain GMI401(pMF457)(pMF10). Panel B : *In planta* phenotype of the wild-type GMI211 (pMF10) and mutant strain GMI401 (pMF10). *Medicago sativa* seedlings were inoculated with the bacterial strains and grown for 3 weeks on medium lacking any nitrogen source.

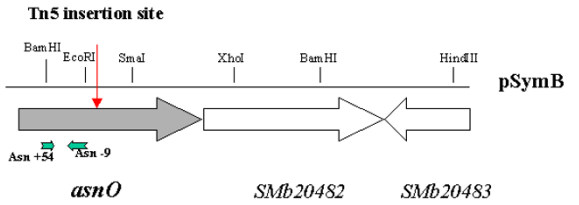

Figure 2
Genetic organisation of the *asnO* region. The genes are shown as thick arrows. The red small arrow indicates the position of the Tn5 insertion in the GMI401 mutant strain. The position and orientation of the specific primers are shown in small green arrows.

meliloti.html) that was run concomitantly to this work, the upstream gene was named asnO, for its homology to the Bacillus subtilis asnO gene. We have thus adopted this annotation in this paper. The deduced amino sequence of the second orf, that was named SMb20482, is similar to an acetyl-transferase in the amino-terminal part of the protein and to a cyanophycin synthetase gene in the carboxy-terminal part of SMb20482.

The deduced amino acid sequence of the gene carrying the Tn5 insertion is similar to that of glutamine-dependent asparagine synthetases of various bacteria including *B. subtilis* and *Escherichia coli* (see Figure 3). These proteins are members of the PurF family of glutamine-dependent amidotransferases. All PurF enzymes possess a conserved amino-terminal cysteine, which is essential for glutamine-dependent amidotransferase activity [8, 9]. This Cys2 residue is also conserved in the *S. meliloti asnO* product. The PurF-type amidotransferases possess 14 additional conserved residues in the amino-terminal glutamase domain [8, 9]. As shown by the alignment of Figure 3, all these residues are present in the *asnO* product of *S. meliloti*. We conclude that the AsnO protein belongs to the PurF family of glutamine amidotransferases. The carboxy-terminal domian of the proteins, carrying the synthetase activity, is less conserved.

Table 1: Bacterial strains and plasmids used

Material	Characteristics	References
Strains	**Genotype**	
S. meliloti		
GMI211	SmR Nod$^+$ Fix$^+$	[20]
GMI401	GMI211 *asnO*::Tn5 SmR NmR	This work
GMI5704	GMI211 *fixJ2.3::Tn5* SmR NmR BleoR	[18]
E. coli		
ER	*asnA31 asnB32 thi*	[15]
MM294	*Pro-82 thi-1 hsdR17 supE44 endA1*	[21]
DH5 α	*endA1 hsdR17* ($r_k^-mk^+$) *supE44 thi*-1 recA1 gyrA *relA1Δ* (*lacIZYA-argF)U169* deoR (ϕ 80*dlacΔ* (*lacZ*)M15)	[29]
Plasmids		
pMF457	pGD926 (IncP broad host range vector) derivative carrying a *fixK*-lacZ fusion with a mutation in the fixT promoter. TcR	
pCHK57	pGMI41211 (Inc-P1 broad host range vector) derivative carrying a *nifA*-lacZ fusion. TcR	[31]
pMF11	Same as pMF10 but with *fixT* in reverse orientation. GmR	
pBH1	pBBR1-MCS3 derivative, carrying an HindIII fragment containing the *asnO* gene and the SMb20482 orf.	This work
pBasn2	pBBR1-MCS3 derivative, expressing *asnO* under the control of the pLac promoter	This work
pUC23	pUC18 digested with EcoRI, carrying the *S. meliloti* EcoRI fragment containing the *asn*O region with the Tn5 insert. Ap^R, Km^R.	This work
BAC37	pBeloBAC11 containing a 100 kb *S. meliloti* genome fragment carrying the *asn*-like region of pSymB.	[26]
pRK602	PRK600::Tn5, Cm^r, Nm-Km^r	[21]

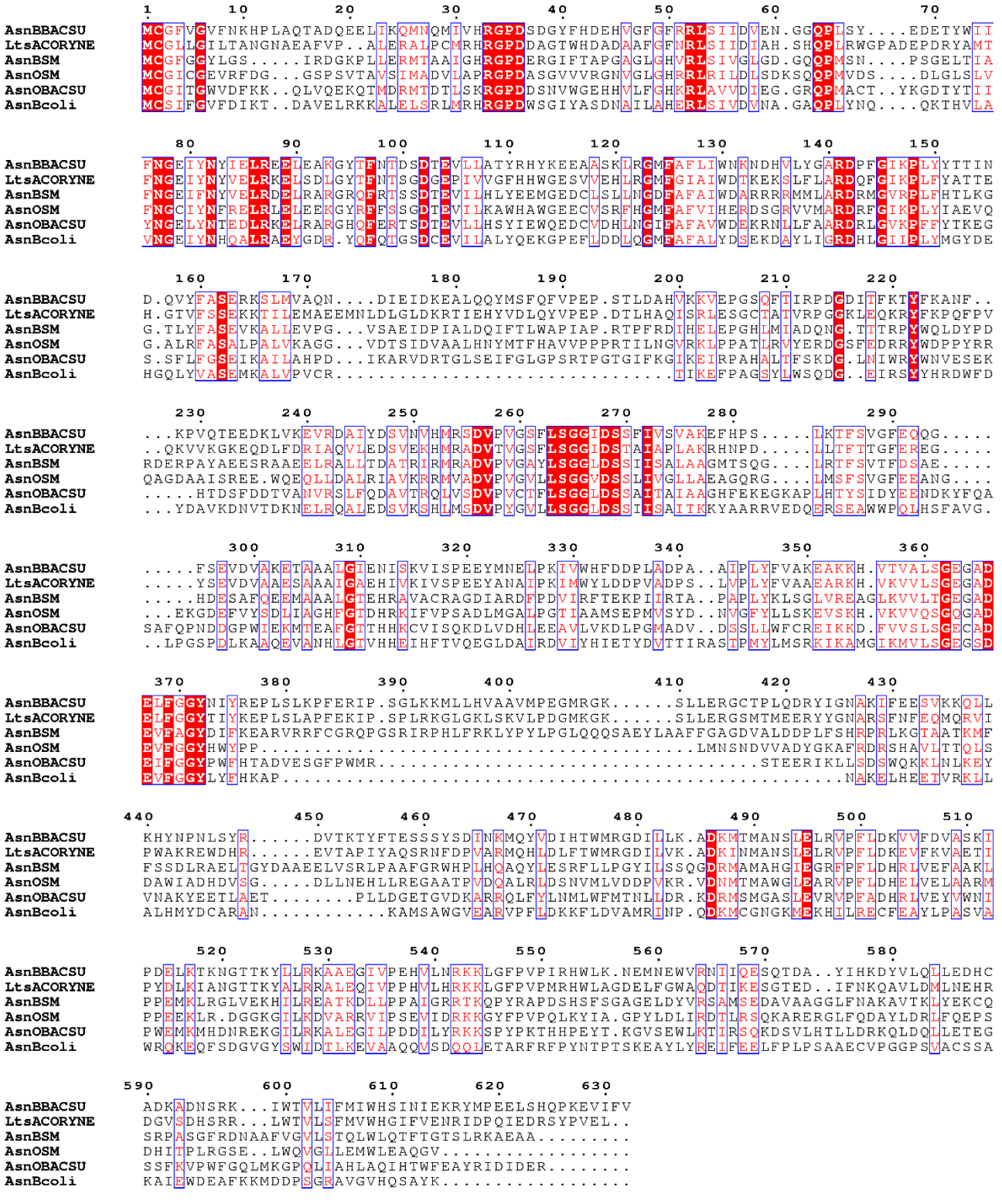

Figure 3
AsnO of *S. meliloti* is related to glutamine-dependent amido transferases. Alignment of amino acid sequences of AsnO and AsnB from *S. meliloti,* AsnO (swissprot accession number: Sp 005272) and AsnB (Sp P54420) from *B. subtilis*, AsnB from *E. coli* (Sp P22106), and LtsA from *C. glutamicum* (Sp BAA89484). The alignement was done by using ClustalW programme [31].

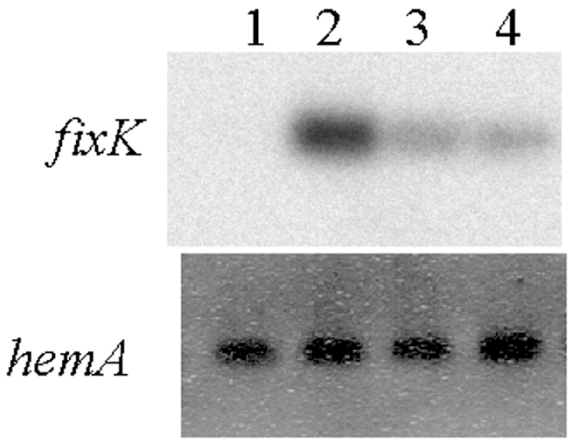

Figure 4
Complementation of the mutant phenotype by the *asnO* gene. RNAs isolated from free-living microoxic cultures (2% O_2) of *S. meliloti* strains grown in M9 minimal medium were amplified by RT-PCR using specific primers. RT-PCR products were separated on agarose gels, blotted onto a nylon membrane and hybridized with the ^{32}P labelled probe of the expected product. Upper panel : *fixK* gene (see Materials and Methods). Lower panel : *hemA* gene (control). Lane 1 = GMI211 (pMF10), Lane 2 = GMI401 (pMF10), Lanes 3 and 4 = GMI401 (pMF10) (pBasn2) of two independent transconjugants.

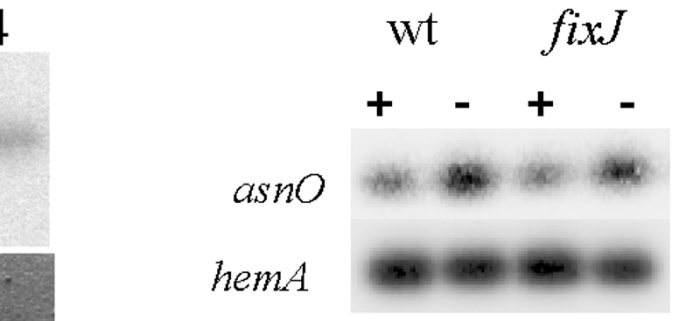

Figure 5
***asnO* gene expression RT-PCR analysis of RNAs isolated from *S. meliloti* GMI211 wild-type strain (wt) or GMI5704 *fixJ* mutant strain (*fixJ*) grown in minimal medium M9 in either oxic (+) or microoxic (2% O2) conditions (-)**. RT-PCR were performed with either *asnO* or *hemA* specific primers and the products were separated on agarose gels, blotted on a nylon membrane and hybridized with the corresponding ^{32}P labelled PCR product and analysed on a Phosphorimager.

The asnO gene complements the mutant phenotype of GMI401

In order to perform complementation trials, we cloned the asnO gene in the pBBRI-MCS3 plasmid [10], a shuttle vector that replicates in both E. coli and S. meliloti. A DNA fragment containing the entire asnO coding region and its 5' flanking promoter region, was cloned downstream of the lac promoter of the plasmid pBBRI-MCS3. We introduced the corresponding pBasn2 plasmid in a strain expressing fixT constitutively and monitored expression of fixK under microoxic conditions by RT-PCR experiments. Data indicated complementation of the GMI401 mutant strain by the asnO transgene (Figure 4). The restoration of the wild-type phenotype (ie inhibition of fixK expression in the presence of pMF10), demonstrated the implication of the asnO gene in the characteristic phenotype observed in the GMI401 mutant strain. However, restoration of the wild-type phenotype was not complete, for a reason that we do not understand.

asnO inactivation in GMI401 does not lead to auxotrophy for asparagine

Because of the homology of the *S. meliloti asn*O gene product with known asparagine synthetases, we tested whether the *asnO* gene was involved in asparagine biosynthesis, by three complementary approaches (data not shown).

First, we observed that the mutant strain GMI401 and the isogenic GMI211 wild-type strain, grew at equal and similar rates in minimal medium with or without asparagine.

Second, we tested the ability of pBasn2 to restore prototrophy of a *E. coli* asparagine auxotroph, a double *asnB/asnA* mutant strain (Table 1). In asparagine-supplemented minimal medium, all strains grew at equal rates. However, in the absence of added asparagine, the *S. meliloti asnO* gene under the control of the plac promoter was unable to complement the asparagine auxotrophy of the *asnA/asnB E. coli* mutant strain.

Third, addition of asparagine at different concentrations in minimal medium, did not lead to complementation of the *S. meliloti* GMI401 mutant phenotype, *i.e* did not restore repression of *fixK* gene expression by overexpressed *fixT*. This observation suggested that the mutant phenotype is not dependent on the level of asparagine, and hence, that the *asnO* gene does not mediate asparagine synthesis.

The reactions catalyzed by asparagine synthetases involve two different family of proteins depending on whether glutamine or ammonia is used as a nitrogen source. Members of the AsnA family, that are found in procaryotes, only use ammonia as the amino group do-

nor [11, 12]. The AsnB family, which is found in both procaryotes and eucaryotes, preferentially uses glutamine as nitrogen source [13, 14]. *E. coli* possesses an *asnA* and an *asnB* gene [15] whereas three asparagine synthetase genes, *asnB*, *asnH* and *asnO*, which all belong to the AsnB family, have been characterized in *B. subtilis*. No member of the AsnA family has been found in *B. subtilis* [16]. Complete genome analysis indicated that *S. meliloti* carries no *asnA*-like gene and two *asnB*-type genes (see http://sequence.toulouse.inra.fr/meliloti.html) one of which is *asn*O and a second gene that was named *asnB*. Sequence analysis is consistent with the possibility that the *asnB* gene of *S. meliloti* might be the ortholog of the biosynthetic *asnB* gene of *E. coli* and *B. subtilis* whereas the *asn*O gene mutated in GMI401 may play a regulatory function, in relationship with nitrogen fixation and microoxic respiration (Figure 3). Similarly, the *asnO* gene of *B. subtilis* has a regulatory role in sporulation [16], whereas the *ltsA* gene of *Corynebacterium glutamicum* is involved in cell wall formation [17].

asnO gene expression is induced in microoxic conditions

We have monitored by RT-PCR experiments expression of the *asnO* gene in a wild-type strain grown in minimal medium, in either oxic or microoxic conditions. The results showed that *asnO* gene expression was slightly enhanced in microoxic conditions as compared to oxic conditions (Figure 5). No effect of a *fixJ* mutation was observed on *asnO* expression using a GMI5704 *fixJ* mutant strain [18].

Conclusions

FixT is an intriguing protein as it has not been described so far in any other bacterium besides *S. meliloti*. Furthermore its mode of action is original, as it has the capacity to block phosphorylation, and hence activity, of the FixL sensor histidine kinase. They are only a few examples of such anti-kinase proteins in the literature. Lastly, FixT primary sequence did not provide clues to its function. There is thus a great deal of interest in determining the biological role of *fixT* in *S. meliloti*.

The present work argues in favor of a physiological function associated with *fixT*, by showing that mutation of the *asnO* gene impairs repression by the FixT protein. This finding brings support to the previous suggestion that FixT may allow integration of an additional signal by the FixLJ two-component regulatory system whose activity is primarily regulated by oxygen (Figure 6). Multiple signal integration by a single two-component regulatory system is well documented for instance in *B. subtilis* [19].

Further work is required to elucidate the relationship between *fixT*, *fixL* and *asnO*. We propose as a working model that the absence of AsnO may result in an imbalance in the pool of a metabolite (e.g a substrate or a product of AsnO), that would affect the intrinsic repressing activity of FixT or, equally, the interaction between FixT and FixL. Identification of the reaction catalyzed by AsnO and further elucidation of the mode of action of the FixT protein should shed light to this model. Because glutamine, a likely by-product of nitrogen fixation in symbiotic rhizobia, is a predicted substrate of the AsnO protein, it is tempting to speculate that *asnO* and *fixT* may provide a link between the nitrogen status of bacteria -or of the plant cell- and nitrogen fixation activity and reducing power generation. Possibly, such a genetic device may connect the nitrogen needs of the plant to the nitrogen fixation activity of the microsymbiont.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *S. meliloti* GMI211, the symbiotically effective parent strain used in this study is a *lac* Sm^r, Nod+ Fix+ derivative of *S meliloti* RCR2011 [20]. Tn5 transposon mutagenesis of GMI211 was performed using the pRK602 plasmid (Table 1, [21]). In order to isolate random Tn5 insertions in the *S. meliloti* genome, we conjugated *E. coli* MM294(pRK602) donor strain with a *S. meliloti* GMI211(pMF10) recipient strains, and screened for streptomycin (100 μg/ml) and neomycin (100 μg/ml) resistant transconjugants.

S. meliloti strains were grown at 30°C in TY complex medium or in defined M9 medium [22] supplemented with 0.3 mM $CaCl_2$, 1 mM $MgSO_4$ and 2 μM biotin. Sterilised carbon sources were added at 20 mM final concentrations. Microoxic conditions were achieved as described by de Philip *et al* [23] (2% oxygen for 4 h). For testing induction of *fixK-LacZ* expression by microoxic conditions, *S. meliloti* strains were previously grown to OD_{600} = 0.3 in M9 medium. β-Galactosidase assays were performed as described by de Philip *et al*. [23].

Southern Blots

DNA techniques were carried out as described by Sambrook *et al*. [24]. *S. meliloti* genomic DNA was prepared as described by Chen and Kuo [25]. For Southern-Blot analysis, 1 μg of genomic DNA was digested at 37°C for 4 hours with the appropriate enzymes. DNA fragments were separated on a 0.8% agarose gel, transferred onto a nylon membrane (BiodyneA transfer membrane, Pall, East Hills, NY), and hybridized with a Tn5 ^{32}P-labelled probe.

asnO cloning and sequencing

A genomic fragment of *S. meliloti* GMI401 carrying the Tn5 insertion was cloned from a EcoRI-digested genome

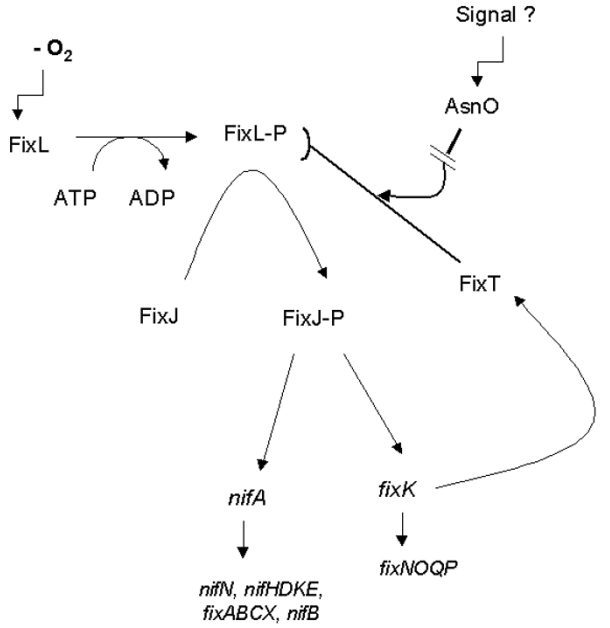

Figure 6
Updated model for the regulation of respiratory and nitrogen fixation gene expression in *S. meliloti*.

fragment into a pUC18 plasmid, resulting in the pUC23 plasmid (see Table 1). However, the presence of a *Eco*RI restriction site in the *asnO* gene (see Figure 2) did not permit to obtain the entire sequence of the gene from this construct. Therefore, a library of 96 BACs covering the *S. meliloti* genome [26], was screened to isolate the full-length *asnO* gene. PCR screening of the BAC library was performed using the oligonucleotide primers ASN+54 and ASN-9 shown in Figure 2. By this method, we could detect the presence of the *asnO* gene on BAC37, that was assigned to the pSymB megaplasmid [26]. Complete sequence of the *asnO* region was determined by a primer-walking approach on the BAC37 and pBH1 plasmid (see Table 1), which was obtained by subcloning a HindIII fragment of BAC37 in pBBR1-MCS3 [10]. Sequencing was performed on a ABI373 automated sequencer (ABI, Columbia, MD), using the ABI PRISM Dye terminator cycle sequencing ready kit (Perkin-Elmer, Oak, Brook, IL).

Database searches

Putative open reading frame were predicted using the FrameD program [7] used for *S. meliloti* whole genome analysis (http://sequence.toulouse.inra.fr/meliloti.html). Protein analysis was done with the NCBI web page using the BLAST2 package program [27] against the NCBI-nr and Swiss-Prot databases. Homologous domains searches were driven using the ProDom database [28].

Plant methods

Medicago sativa cv. Gemini seedlings were aseptically grown on agar slants made up with nitrogen-free Fahraeus medium. Three-day-old plants were inoculated with the different *S. meliloti* strains and grown for 3 weeks before observation.

RNA preparation

The bacteria from a 25 ml culture at 0.4 OD_{600nm} were harvested and RNA prepared with the Qiagen RNeasy kit as described by the manufacturer. DNA was eliminated by addition of 7.5 Units of FPLC-Pure RNase-free DNaseI (Amersham-Pharmacia Biotech). RNA was further extracted with phenol-chloroform and then precipitated with ethanol. After washing with 70% ethanol, the pellet was resuspended in nuclease-free water. RNA was quantified by absorbance measurements at 260 nm. Absence of DNA contamination was verified by PCR amplification.

RT-PCR analysis

RT-PCR reactions were performed according to the manufacturer, using the SuperScript™ One-Step RT-PCR System (GibcoBRL).

The following oligonucleotides were used for reverse transcription of the messenger RNA and amplification of the product to evaluate gene expression :

For the *hemA* gene: hemAr (reverse primer) : 5'-GTCGATCGCGTTCTT-3'; hemAf (foward primer) : 5'-TGGATGGGCTGCATCA-3'

For the *asnO* gene: RTA1 (reverse primer) : 5'-TGCGTATTCTCGACCTG-3'; RTA2 (foward primer) : 5'-TCGCGAAAATTGTAGATG-3'

For the *fixK* gene: KPR (reverse primer) : 5'- CCGATTACCAGAAGATGC-3'; KPF (foward primer) : 5'-TATCTACCGCCTCCTTTC-3'

RT-PCR products were electrophoresed on a 2% agarose gel, blotted onto a nylon membrane and probed with a ^{32}P-labeled DNA probe prepared from the gene of interest. Washing was done with 0.1XSSC, 0.1%SDS at 42°C during 30 min.

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