

Research article

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

Hélène Bergès, Claire Checroun, Sébastien Guiral, Anne-Marie Garnerone, Pierre Boistard and Jacques Batut*

Address: Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, UMR215 CNRS-INRA, BP27, 31326 Castanet-Tolosan Cedex, France

E-mail: Hélène Bergès - hberges@toulouse.inra.fr; Claire Checroun - checroun@ibcg.biotoul.fr; Sébastien Guiral - guiral@ibcg.biotoul.fr; Anne-Marie Garnerone - garneron@toulouse.inra.fr; Pierre Boistard - boistard@toulouse.inra.fr; Jacques Batut* - jbatur@toulouse.inra.fr

*Corresponding author

Published: 22 May 2001

Received: 4 April 2001

BMC Microbiology 2001, 1:6

Accepted: 22 May 2001

This article is available from: <http://www.biomedcentral.com/1471-2180/1/6>

(c) 2001 Bergès et al, licensee BioMed Central Ltd.

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 N₂-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-amidotransferases. 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 plas-

mids, 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).

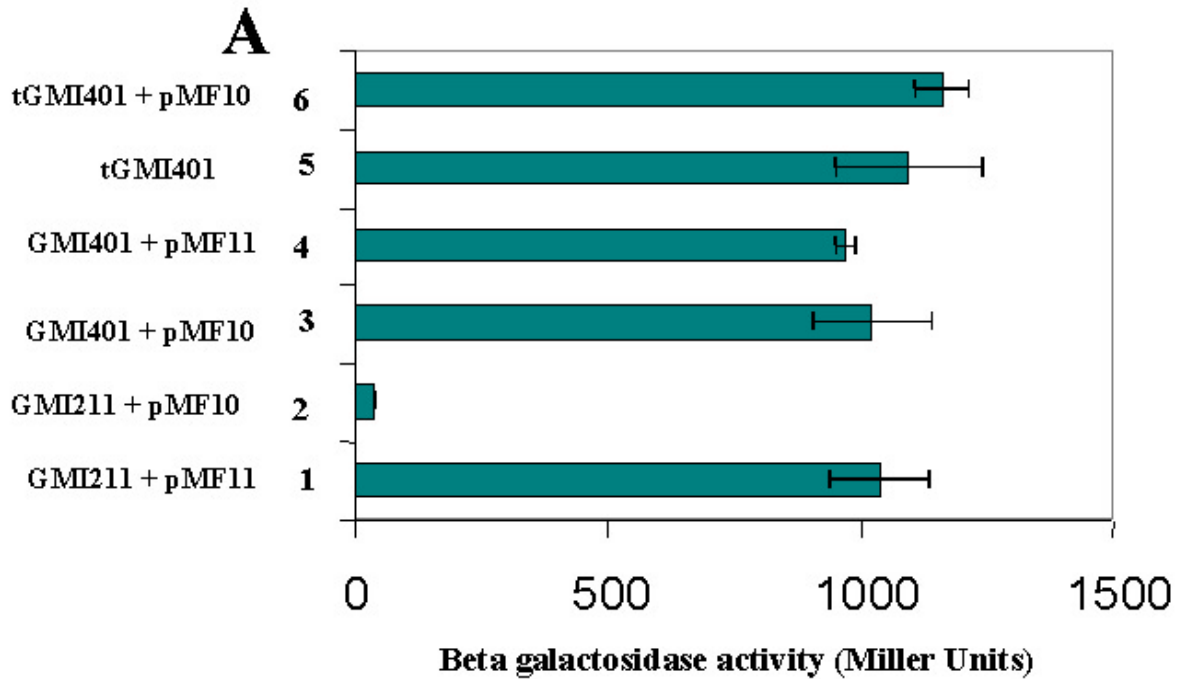
Similar results have been obtained using a *nifA-lacZ* fusion (pCHK57 plasmid). The inhibition of *nifA-lacZ* expression by overexpressed *fixT* that was observed in a GMI211 wild-type strain was not observed anymore in the GMI401 mutant strain (data not shown).

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 N₂-fixing nodules (Figure 1B), the GMI401 mutant strain was able to induce effective N₂-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 downstream. 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/>



B



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.

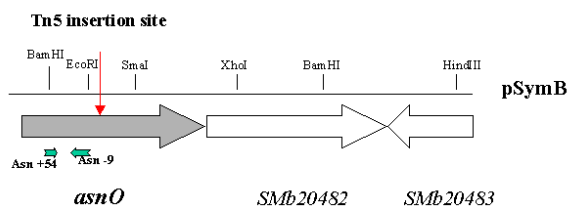


Figure 2
Genetic organisation of the *asnO* region. The genes are shown as thick arrows. The red small arrow indicates the position of the Tn5 insertion in the GMI401 mutant strain. The position and orientation of the specific primers are shown in small green arrows.

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 domain of the proteins, carrying the synthetase activity, is less conserved.

Table 1: Bacterial strains and plasmids used

Material	Characteristics	References
Strains		
<i>S. meliloti</i>		
GMI211	SmR Nod ⁺ Fix ⁺	[20]
GMI401	GMI211 <i>asnO</i> ::Tn5 SmR NmR	This work
GMI5704	GMI211 <i>fixJ2.3</i> ::Tn5 SmR NmR BleoR	[18]
<i>E. coli</i>		
ER	<i>asnA31 asnB32 thi</i>	[15]
MM294	<i>Pro-82 thi-1 hsdR17 supE44 endA1</i>	[21]
DH5 α	<i>endA1 hsdR17 (r_k⁻mk⁺) supE44 thi-1 recA1 gyrA relA1 Δ (lacZYA-argF)U169 deoR (ϕ 80dlacΔ (lacZ)M15)</i>	[29]
Plasmids		
pMF457	pGD926 (IncP broad host range vector) derivative carrying a <i>fixK-lacZ</i> fusion with a mutation in the <i>fixT</i> promoter. TcR	
pCHK57	pGMI41211 (Inc-PI broad host range vector) derivative carrying a <i>nifA-lacZ</i> fusion. TcR	[31]
pMF11	Same as pMF10 but with <i>fixT</i> in reverse orientation. GmR	
pBH1	pBBR1-MCS3 derivative, carrying an HindIII fragment containing the <i>asnO</i> gene and the SMb20482 orf.	This work
pBasn2	pBBR1-MCS3 derivative, expressing <i>asnO</i> under the control of the pLac promoter	This work
pUC23	pUC18 digested with EcoRI, carrying the <i>S. meliloti</i> EcoRI fragment containing the <i>asnO</i> region with the Tn5 insert. Ap ^R , Km ^R .	This work
BAC37	pBeloBAC11 containing a 100 kb <i>S. meliloti</i> genome fragment carrying the <i>asn</i> -like region of pSymB.	[26]
pRK602	PRK600::Tn5, Cm ^r , Nm-Km ^r	[21]

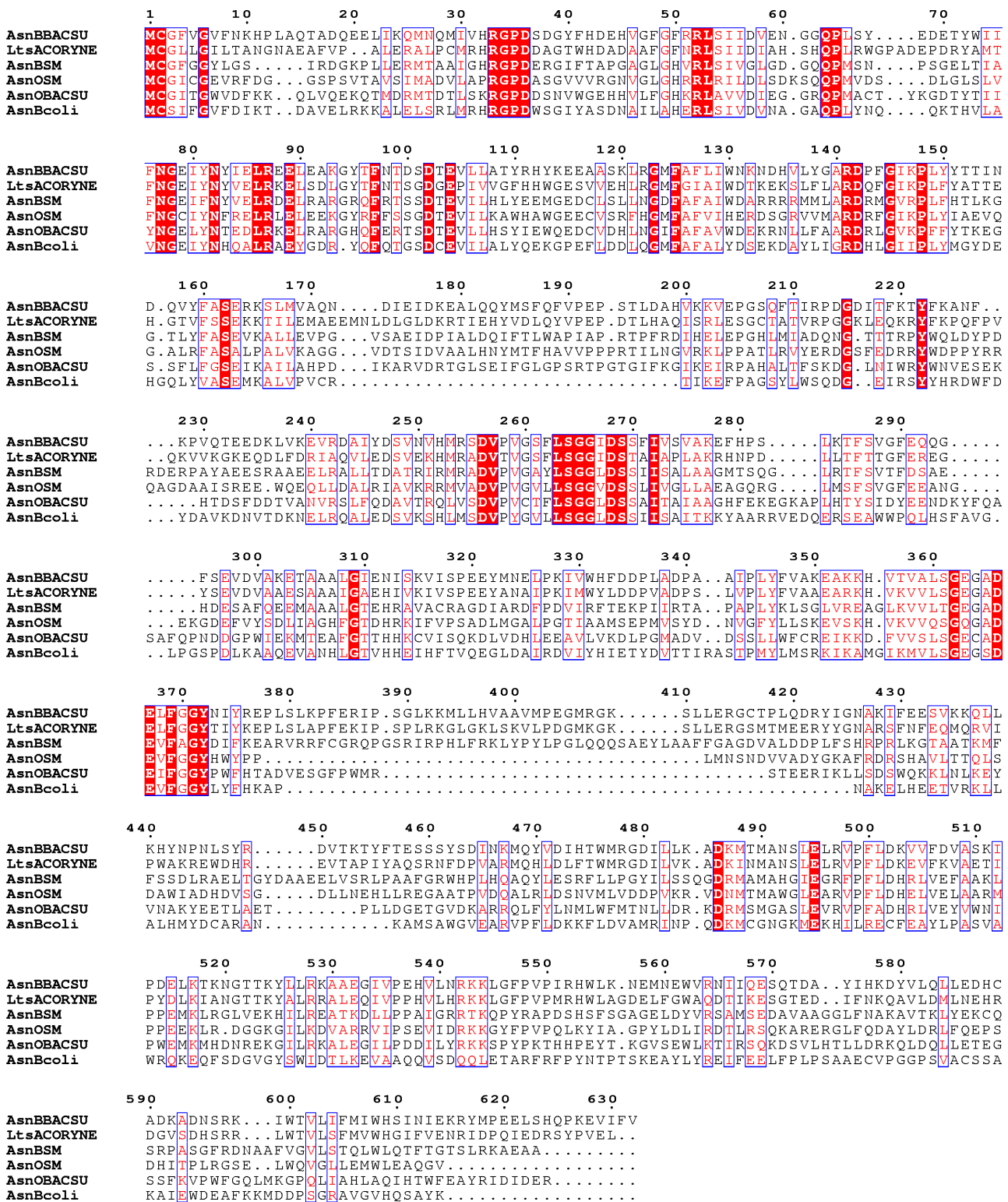


Figure 3
AsnO of *S. meliloti* is related to glutamine-dependent amido transferases. Alignment of amino acid sequences of AsnO and AsnB from *S. meliloti*, AsnO (swissprot accession number: Sp 005272) and AsnB (Sp P54420) from *B. subtilis*, AsnB from *E. coli* (Sp P22106), and LtsA from *C. glutamicum*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

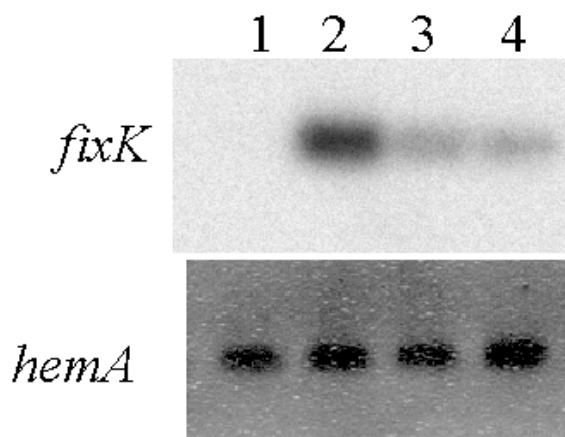


Figure 4

Complementation of the mutant phenotype by the *asnO* gene. RNAs isolated from free-living microoxic cultures (2% O₂) 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 ³²P 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.

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* *asnO* gene product with known asparagine synthetases, we tested whether the *asnO* gene was involved in asparagine bio-

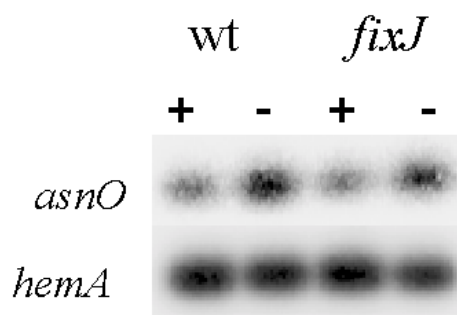


Figure 5

***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 ³²P labelled PCR product and analysed on a Phosphorimager.

synthesis, 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 prokaryotes, only use ammonia as the amino group do-

nor [11, 12]. The AsnB family, which is found in both prokaryotes and eukaryotes, 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 *asnO* 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 *asnO* 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 CaCl₂, 1 mM MgSO₄ 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 OD₆₀₀ = 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 ³²P-labelled probe.

***asnO* cloning and sequencing**

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

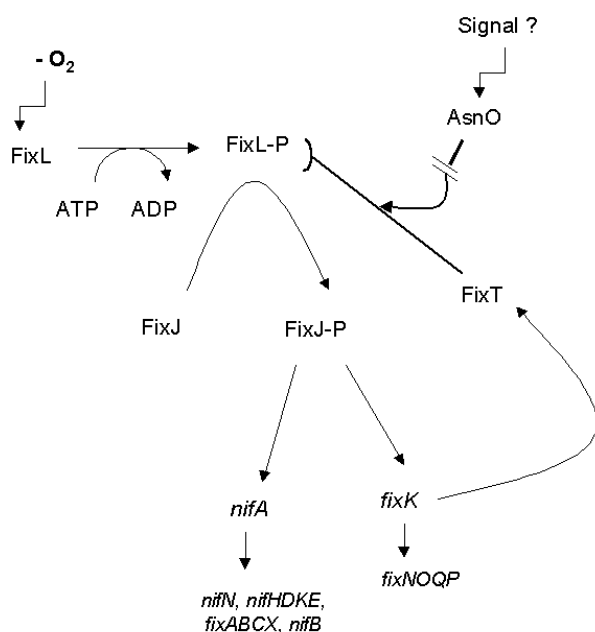


Figure 6
Updated model for the regulation of respiratory and nitrogen fixation gene expression in *S. meliloti*.

fragment into a pUC18 plasmid, resulting in the pUC23 plasmid (see Table 1). However, the presence of a *EcoRI* 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 do-

mains 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'-GTC-GATCGCGTTCTT-3'; hemAf (forward primer) : 5'-TGGATGGGCTGCATCA-3'

For the *asnO* gene: RTA1 (reverse primer) : 5'-TGCG-TATTCTCGACCTG-3'; RTA2 (forward primer) : 5'-TCGCGAAAATTGTAGATG-3'

For the *fixK* gene: KPR (reverse primer) : 5'-CCGAT-TACCAGAAGATGC-3'; KPF (forward primer) : 5'-TATC-TACGCCTCCTTTC-3'

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

Acknowledgements

We thank Dr T. Hirasawa for generous gift of *E. coli* strain ER, Dr Delphine Capela for access to the *S. meliloti* BAC genomic library, Laurent Sauviac for automatic cycle sequencing, Isabelle Gairin for informatic technical assistance, Jérôme Gouzy and Frédéric Ampe for help in sequence analysis. We are grateful to Dr Julie Cullimore and Dr Eliane Meilhoc for critical reading of the manuscript. HB and part of this work were supported by a grant

from the European Union in the frame of the BIOTECH programme (FIXNET, BIOT4-CT97-2319).

References

- David M, Daveran ML, Batut J, Dedieu A, Domergue O, Ghai J, Hertig C, Boistard P, Kahn D: **Cascade Regulation of nif Gene Expression in Rhizobium meliloti.** *Cell* 1988, **54**:671-683
- Soupeine E, Foussard M, Boistard P, Truchet G, Batut J: **Oxygen as a key developmental regulator of Rhizobium meliloti N2-fixation gene expression within the alfalfa root nodule.** *Proc. Natl. Acad. Sci. U.S.A.* 1995, **92**:3759-3763
- Gilles-Gonzalez MA, Ditta GS, Helinski DR: **A haemoprotein with kinase activity encoded by the oxygen sensor of Rhizobium meliloti.** *Nature* 1991, **350**:170-2
- Reyrat JM, David M, Blonski C, Boistard P, Batut J: **Oxygen regulated in vitro transcription of Rhizobium meliloti nifA and fixK genes.** *J Bacteriol* 1993, **175**:6687-6872
- Batut J, Daveran-Mingot ML, David M, Jacobs J, Garnerone AM, Kahn D: **fixK, a gene homologous with fnr and crp from Escherichia coli, regulates nitrogen fixation genes both positively and negatively in Rhizobium meliloti.** *EMBO J* 1989, **8**:1279-1286
- Foussard M, Garnerone AM, Ni F, Soupeine E, Boistard P, Batut J: **Negative autoregulation of the Rhizobium meliloti fixK gene is indirect and requires a newly identified regulator, FixT.** *Mol. Microbiol* 1997, **25**:27-37
- Garnerone AM, Cabanes D, Foussard M, Boistard P, Batut J: **Inhibition of the FixL sensor kinase by the FixT protein in Sinorhizobium meliloti.** *J Biol Chem* 1999, **274**:32500-32506
- Schiex T, Thébaud P, Kahn D: **Recherche des gènes et des erreurs de séquençage dans les génomes bactériens GC-riches (et autres...)** *Proc. of JOBIM, Montpellier. France.* 2000, :321-328
- Boehlein SK, Richards NG, Walworth ES, Schuster SM: **Arginine 30 and asparagine 74 have functional roles in the glutamine dependent activities of Escherichia coli asparagine synthetase B.** *J Biol Chem* 1994, **269**:26789-95
- Boehlein SK, Richards NG, Schuster SM: **Glutamine-dependent nitrogen transfer in Escherichia coli asparagine synthetase B. Searching for the catalytic triad.** *J Biol Chem* 1994, **269**:7450-7
- Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Ruppel RM, Peterson KM: **Four new derivatives of the broad-host-range cloning vector pBBRI-MCS, carrying different antibiotic-resistance cassettes.** *Gene* 1995, **166**:175-176
- Humbert R, Simoni RD: **Genetic and biochemical studies demonstrating a second gene coding for asparagine synthetase in Escherichia coli.** *J. Bacteriol* 1980, **142**:212-220
- Reitzer LJ, Magasanik B: **Asparagine synthetase of Klebsiella aerogenes: properties and regulation of synthesis.** *J. Bacteriol* 1982, **151**:1299-1313
- Hughes CA, Beard HS, Matthews BF: **Molecular cloning and expression of two cDNAs encoding asparagine synthetase in soybean.** *Plant. Mol. Biol* 1997, **33**:301-311
- Scofield MA, Lewis WS, Schuster SM: **Nucleotide sequence of Escherichia coli asnB and deduced amino acid sequence of asparagine synthetase B.** *J Biol Chem* 1990, **265**:12895-902
- Cedar H, Schwartz JH: **The asparagine synthetase of Escherichia coli. Biosynthetic role of the enzyme, purification, and characterization of the reaction products.** *J. Biol. Chem.* 1963, **244**:4112-4121
- Yoshida KI, Fujita Y, Ehrlich SD: **Three asparagine synthetase genes of Bacillus subtilis.** *J Bacteriol.* 1999, **181**:6081-6091
- Hirasawa T, Wachi M, Nagai K: **A mutation in the Corynebacterium glutamicum ItsA gene causes susceptibility to lys-ozyme, temperature-sensitive growth, and L-glutamate production.** *J Bacteriol* 2000, **182**:2696-26701
- David M, Daveran ML, Batut J, Dedieu A, Domergue O, Ghai J, Hertig C, Boistard P, Kahn D: **Cascade regulation of nif gene expression in Rhizobium meliloti.** *Cell* 1988, **54**:671-683
- Perego M, Hanstein C, Welsh K, Djavakhishvili T, Glaser P, Hoch JA: **Multiple protein-aspartate phosphatases provide a mechanism for the integration of diverse signals in the control of development in B. subtilis.** *Cell* 1994, **79**:1047-1055
- Niel C, Guillaume B, Bechet M: **Mise en évidence de deux enzymes présentent une activité β -galactosidase chez Rhizobium meliloti.** *Can J Microbiol* 1977, **23**:1178-1181
- Finan TM, Hirsch AM, Leigh JA, Johansen E, Kuldau GA, Deegan S, Walker GC, Signer ER: **Symbiotic mutants of Rhizobium meliloti that uncouple plant from bacterial differentiation.** *Cell* 1985, **40**:869-877
- Miller J.H: **Experiments in molecular genetics.** *Cold Spring Harbor, New York* 1972
- de Philip P, Batut J, Boistard P: **FixL is an oxygen sensor and regulates R. meliloti nifA and fixK genes differently in Escherichia coli.** *J Bacteriol* 1990, **172**:4255-62
- Sambrook J, Fritsch EF, Maniatis T: **Molecular cloning A Laboratory Manual.** *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.* 1989
- Chen WP, Kuo TT: **A simple and rapid method for the preparation of Gram-negative bacterial genomic DNA.** *Nucleic. Acids. Res* 1993, **21**:2260
- Capela D, BarloyHubler F, Gatus MT, Gouzy J, Galibert F: **A high-density physical map of Sinorhizobium meliloti 1021 chromosome derived from bacterial artificial chromosome library.** *Proc Nat Acad Sci Usa* 1999, **96**:9357-9362
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: **Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.** *Nucleic. Acids. Res* 1997, **25**:3389-3402
- Corpet F, Gouzy J, Kahn D: **Recent improvements of the ProDom database of protein domain families.** *Nucleic Acids Res* 1999, **27**:263-267
- Woodcock DM, Crowther PJ, Doherty J, Jefferson S, DeCruz E, Noyer-Weidner M, Smith SS, Michael MZ, Graham MW: **Quantitative evaluation of Escherichia coli host strains for tolerance to cytosine methylation in plasmid and phage recombinants.** *Nucleic Acids Res.* 1989, **17**:3469-78
- Thompson JD, Higgins DG, Gibson TJ: **CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice.** *Nucleic Acids Research* 1994, **22**:4673-4680
- Ditta G, Schmidhauser T, Yakobson E, Lu P, Liang XW, Finlay DR, Guiney D, Helinski DR: **Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression.** *Plasmid* 1985, **13**:149-153

Publish with **BioMedcentral** and every scientist can read your work free of charge

"BioMedcentral will be the most significant development for disseminating the results of biomedical research in our lifetime."

Paul Nurse, Director-General, Imperial Cancer Research Fund

Publish with **BMC** and your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours - you keep the copyright



Submit your manuscript here:

<http://www.biomedcentral.com/manuscript/>

editorial@biomedcentral.com