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The FixM Flavoprotein Modulates Inhibition by AICAR or 5'AMP of Respiratory and Nitrogen Fixation Gene Expression in *Sinorhizobium meliloti*

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AICAR, a purine-related metabolite, was recently shown to inhibit respiratory and *nifA* gene expression in *Sinorhizobium meliloti*. Here, we demonstrate that AICAR has essentially no or little effect in a wild-type *S. meliloti* strain and inhibits respiratory and nitrogen fixation gene expression only in specific mutant backgrounds. We have analyzed in detail a mutant in which addition of AICAR inhibited *fixK*, *fixN*, *fixT*, and *nifA* expression. The corresponding gene, *fixM*, is located just downstream of *fixK*₁ on pSymA megaplasmid and encodes a flavoprotein oxidoreductase. 5'AMP, a structural analogue of AICAR, mimicked AICAR effect as well as the nucleoside precursors AICARiboside and adenosine. The mode of action of AICAR and 5'AMP in vivo was investigated. We demonstrate that AICAR does not affect FixK transcriptional activity and instead regulates *fixK* and *nifA* gene expression. We hypothesize that AICAR and 5'AMP may modulate, possibly indirectly, the activity of the FixLJ two-component regulatory system. The possible physiological roles of AICAR, 5'AMP, and *fixM* in the context of symbiosis are discussed.

The interaction between rhizobia and their host plants culminates in the formation of specialized organs called nodules in which differentiated bacteria fix nitrogen to the benefit of the host plant. Symbiotic expression of most of the nitrogen fixation genes identified so far in *Sinorhizobium meliloti* is under both positive and negative control. A regulatory cascade, controlled by the two-component regulatory system FixLJ, activates expression of nitrogen fixation genes in response to microoxic conditions (David et al. 1988), such as those that prevail inside the nodule (Soupène et al. 1995). Under microoxic conditions, the sensor histidine kinase FixL autophosphorylates from ATP and transfers its phosphate to the FixJ transcriptional regulator protein (Gilles-Gonzalez et al. 1991; Reyrat et al. 1993). Phosphorylated FixJ then activates transcription of two intermediate regulatory genes, *nifA* and *fixK*, that both encode transcriptional regulators. NifA mediates activation of *nif* and *fix* genes involved in nitrogenase biosynthesis and functioning, whereas FixK, a member of the Crp/Fnr family, activates expression of *fixNOQP* genes involved in the synthesis of a respiratory ox-

dase complex (Batut et al. 1989). FixK also controls expression of a gene, *fixT*, that negatively affects expression of FixLJ-dependent genes by inhibiting FixL autophosphorylation (Foussard et al. 1997; Garnerone et al. 1999). The repressor activity of FixT was abolished in an *asnO* mutant of *S. meliloti*, thus suggesting the existence of a yet-to-be identified physiological signal associated with *fixT* (Berges et al. 2001).

Very recently, Soberón and associates (2001) reported that AICAR, a purine intermediate, down-regulates *fixN*, *fixT*, *fixK*, and *nifA* expression in *S. meliloti*, following their initial work on *Rhizobium etli* (Soberón et al. 1997). Previously, infection of bean by *R. etli* was shown to require production of AICAR (Newman et al. 1995). Besides this, little is known about AICAR in prokaryotes as compared with eukaryotes. In animal cells, AICAR (also sometimes called ZMP) stimulates AMP-activated protein kinases (Corton et al. 1995), thereby inhibiting lipolysis and lipogenesis (Henin et al. 1995; Sullivan et al. 1994). AICAR also inhibits glycolysis (Vincent et al. 1992) and gluconeogenesis (Vincent et al. 1991). In plants, AICAR activates NADH-nitrate reductase, sucrose phosphate synthetase, and glutamine synthetase in vivo (Huber and Kaiser 1996; Man and Kaiser 2001; Toroser et al. 1998).

We have analyzed here the effect of AICAR and related molecules on the expression of *fixN*, *fixT*, *fixK*, and *nifA* in *S. meliloti*. We demonstrate the modulation of AICAR response by a flavoprotein oxidoreductase encoded by the *fixM* gene. The possible mode of action of AICAR as well as its physiological significance are discussed.

RESULTS

AICAR inhibits respiratory and nitrogen fixation gene expression only in specific mutant backgrounds.

Following the demonstration by Soberón and associates (2001) that AICAR inhibited *fixN* expression, we investigated the effect of AICAR on *fixN*, *fixK*, *fixT*, and *nifA* expression in different genetic backgrounds, using translational lacZ fusions. We found that a gene of unknown function, *fixM*, (formerly called *orf151* in Batut et al. 1989) located between *fixK*₁ and *fixN*₁ (Fig. 1) was involved in AICAR response.

Expression of *fixN*, *fixK*, *fixT*, and *nifA* was reduced by 9-, 5-, 4-, and 1.7-fold, respectively, when AICAR (0.2 mM) was added to the culture medium of a *fixM* mutant strain (GMI461) (Fig. 2A, B, C, and D). In contrast, AICAR had no effect on the expression level of an *S. meliloti* *hemA* control gene fusion (data not shown).

In the GMI5595 strain that was used by Soberón and associates (2001) in their studies, AICAR led to a fivefold repression

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of *fixN* expression, as expected (Fig. 2E). By contrast, in the wild-type strain (GMI51), AICAR repressed slightly *fixN* and *fixK* expression and had no effect on the expression of *fixT* and *nifA* (Fig. 2A, B, C, D). Therefore, the deletion carried by the GMI5595 strain (Fig. 1), which removes 16 kb of *fix* genes (Renalier et al. 1987), potentiates the effect of AICAR.

In summary, AICAR had little effect on *niffix* gene expression in a wild-type strain, whereas it induced a clear repression of *fixN*, *fixT*, *fixK*, and *nifA* in two mutant backgrounds: GMI5595 and the *fixM* mutant (GMI461). Further experiments performed in a GMI5595 strain mutated for the *fixM* gene (double mutant, GMI5602) showed that the effect of AICAR on *fixN* expression was not enhanced in this strain as compared with the GMI5595 strain (Fig. 2E).

AICAR and 5'AMP have similar effects.

AICAR is an intermediate in the purine biosynthetic pathway. Therefore, we tested whether other purine-related molecules had an effect similar to that of AICAR (Table 1). Adenosine and AICA-riboside reduced *fixN* expression by half in a wild-type strain and by ninefold in the *fixM* mutant strain. In contrast, inosine, guanosine, and their phosphorylated derivatives IMP, GMP, GDP, and GTP had no effect on *fixN* expression in either background. No effect was registered with the cyclic nucleotides cAMP, cGMP, and cAICAR or with the AICAR-related molecule AICA. 5'AMP, a structural and functional analogue of AICAR (Corton et al. 1995), had an effect similar to that of AICAR, whereas no effect was registered with either 2' or 3'AMP or with the di-phosphorylated or the tri-phosphorylated forms. Hence, the active molecule in vivo could be either AICAR or 5'AMP.

Two *fixM*-like genes in *S. meliloti*: one is a pseudogene, the other one does not prevent AICAR effect.

Analysis of the genome sequence revealed the presence of two *fixM*-like genes in *S. meliloti*. Both are located on the

symbiotic pSymA megaplasmid (Fig. 1). One *fixM*-like gene is located in the reiterated *fix* cluster that is deleted in the GMI5595 strain. However, this gene presents a STOP codon 12 amino acids (aa) downstream of the predicted start codon and two frameshifts in the open reading frame, suggesting that this *orf* is not functional. We did not detect transcription of this gene by reverse transcriptase polymerase chain reaction (RT-PCR) analysis. These pieces of evidence strongly suggest that this *fixM*-like gene is a pseudogene.

The second *fixM*-like gene, *SMa1151*, is located 34 kb downstream of *fixM*. *fixM* and *SMa1151* gene products share 60% aa identity. As for the *fixM* gene (discussed below), RT-PCR analysis revealed that *SMa1151* was transcribed in the bacteroid and in free-living bacteria under oxygen limitation (data not shown). We assayed the β -galactosidase activity of the translational *fixN*₁-lacZ fusion in a single *SMa1151* mutant strain (GMI11260) and in a double *SMa1151*-*fixM* mutant strain (GMI11257) in the presence of AICAR (0.2 mM). AICAR did not affect *fixN* expression in the single *SMa1151* mutant strain and the effect of AICAR was not enhanced in the *SMa1151*-*fixM* double mutant strain as compared with the single *fixM* mutant strain (data not shown). Therefore, *SMa1151* is not involved in the repression of *fixN*, *fixT*, *fixK*, and *nifA* by AICAR in *S. meliloti*.

Regulation of *fixM* gene expression.

RT-PCRs were performed on *S. meliloti* RNAs isolated from microoxic free-living bacteria, from oxic free-living bacteria, and from bacteroids isolated 3 weeks after alfalfa inoculation by wild-type *S. meliloti* (GMI211). RT-PCRs were performed with specific primers synthesized from the *fixM* sequence (*Orf151*₁-*Orf151*₂). A *fixM* transcript was detected in symbiotic bacteroids and in microoxic free-living bacteria (Fig. 3A), whereas it was not detected in a *fixJ* mutant or in free-living oxic bacteria (Fig. 3B). This indicates that *fixM* expression is under oxygen and

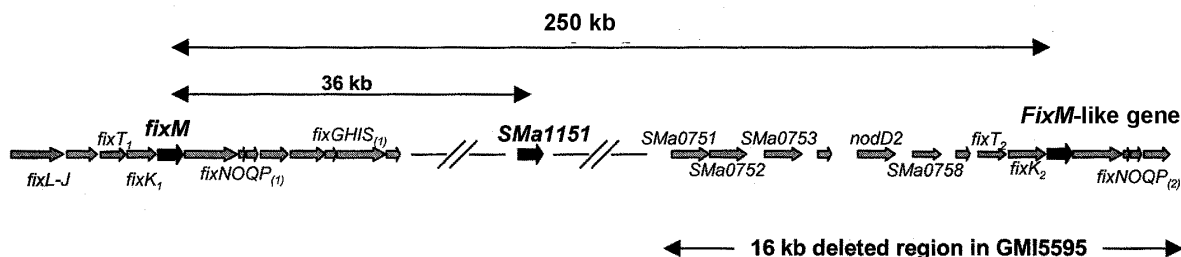


Fig. 1. *fix* Genes organization on *Sinorhizobium meliloti* pSymA megaplasmid.

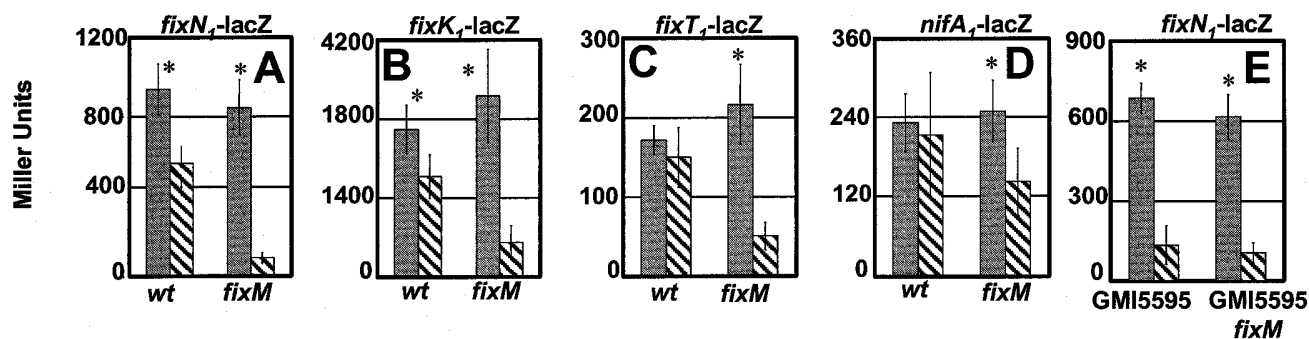


Fig. 2. AICAR effect on *fixN*, *fixK*, *fixT*, and *nifA* expression in *Sinorhizobium meliloti*. A, *fixN*₁-lacZ (pGMI931) expression, B, *fixK*₁-lacZ (pMF457) expression, C, *fixT*₁-lacZ (pGD106) expression, D, *nifA*₁-lacZ (pCHK57) expression, and E, *fixN*₁-lacZ (pGMI931) expression. The β -galactosidase assays were performed in the GMI51 (*wt*) and in the GMI461 (*fixM*) in the absence of (gray) or the presence of (hatched) AICAR (0.2 mM) under microaerobic conditions. Data are means of four to ten experiments. Standard deviations are indicated. Stars indicate that data in the presence of and the absence of AICAR are statistically different ($P < 0.01$).

fixJ control. Northern blot experiments indicated that *fixM* expression was abolished in a *fixK₁-fixK₂* double mutant (GMI942) (data not shown). It remains to be demonstrated whether this is due to a cotranscription of *fixK₁* and *fixM* or whether FixK activates *fixM* expression at its own promoter.

Symbiotic phenotype of a *fixM* mutant.

The disruption of *fixM* by insertion 2.37 previously was reported not to affect nodulation or nitrogen fixation efficiency during the symbiotic association between *S. meliloti* and *Medicago sativa* (Batut et al. 1989). We further analyzed the level of *fixN* and *fixK* expression in *fixM* bacteroids as compared with the level of expression in wild-type bacteroids extracted 5 weeks after alfalfa inoculation. Data showed that *fixN* expression was slightly repressed in *fixM* bacteroids as compared with expression in the wild-type strain (Fig. 4A), whereas *fixK* expression remained unaffected (Fig. 4B). Hence, *fixM* has no or little effect in symbiosis under standard laboratory conditions.

In further experiments, we engineered a purine biosynthesis mutant (GMI11256) by insertion of a gentamycin resistance cassette in the *purH* gene. The product of *purH* metabolizes AICAR into FAICAR; therefore, a *purH* mutant was expected

to endogenously accumulate AICAR. We assayed the β -galactosidase activity of the translational *fixK₁-lacZ* fusion on 5-week-old bacteroids of a single *purH* mutant (GMI11256) and of a double *purH-fixM* mutant (GMI11255). Results (Fig. 4B) showed that, when the *purH* mutation was associated with the *fixM* mutation, *fixK* expression was repressed 12-fold compared with the expression in a single *purH* mutant. However, we were unable to demonstrate AICAR accumulation in the *purH* mutant using the colorimetric assay of Bratton and Marshall (1939): AICAR concentration in the *purH* mutant and the wild-type strain were both very low (data not shown). Furthermore, the *S. meliloti purH* mutant was prototrophic for purines, indicating that an unknown salvage pathway synthesizes purines from AICAR or another intermediate upstream of AICAR in the purine pathway. Hence, AICAR accumulation in the *purH* mutant is not established. In conclusion, whereas the present data solidify the link between purine biosynthesis and *fixK* gene expression (Soberón et al. 2001), they do not establish that AICAR is the actual effector molecule.

To our surprise, the double *fixM-purH* mutant was Fix⁺ in symbiosis with *M. sativa*, in spite of the marked decrease of *fixK* expression. This clearly indicates that *fixK* expression was not limiting for nitrogen fixation in our assay.

Table 1. Repressed *fixN* expression in a *fixM* mutant strain^a

| Purine metabolites | Wt | <i>fixM</i> |
|---------------------------------|----------|-------------|
| AICAR | 61 ± 10 | 11 ± 4 |
| AICA-riboside | 51 ± 12 | 11 ± 6 |
| Inosine | 117 ± 35 | 130 ± 50 |
| Guanosine | 115 ± 10 | 121 ± 11 |
| Adenosine | 47 ± 16 | 11 ± 2.4 |
| 5'AMP | 32 ± 5 | 15 ± 3 |
| 5'GMP | 117 ± 3 | 102 ± 20 |
| 5'IMP | 102 ± 10 | 104 ± 11 |
| ADP | 96 ± 1 | 141 ± 56 |
| GDP | 85 ± 20 | 101 ± 16 |
| ZDP | 99 ± 1 | 143 ± 13 |
| ATP | 94 ± 22 | 80 ± 36 |
| GTP | 94 ± 30 | 70 ± 10 |
| cAMP | 87 ± 1 | 99 ± 12 |
| cGMP | 87 ± 1 | 87 ± 5 |
| cAICAR | 100 ± 13 | 92 ± 15 |
| AICA | 111 ± 20 | 97 ± 14 |
| Mixed isomer of 3'AMP and 2'AMP | 109 ± 20 | 96 ± 21 |

^a 5'AMP, AICAR, and their nucleoside precursors repress *fixN* expression in a *fixM* mutant strain. Exogenous purine metabolites (0.2 mM) were added to wild-type (Wt) *Sinorhizobium meliloti* strain (GMI151) or to a *fixM* mutant strain (GMI461) culture medium. Cells were incubated for 3 h in oxic conditions before starting microaerobic induction. The β -galactosidase activity of *fixN₁-lacZ* in the presence of each metabolite is expressed in percentage of the activity in the absence of added product.

Molecular target for AICAR.

FixK belongs to the Crp family of regulatory proteins (Batut et al. 1989) and Crp binds the purine-related cAMP effector (Spiro et al. 1990); therefore, we hypothesized initially that AICAR may affect *fixN* expression by modulating FixK transcriptional activity. In order to directly test this hypothesis, we

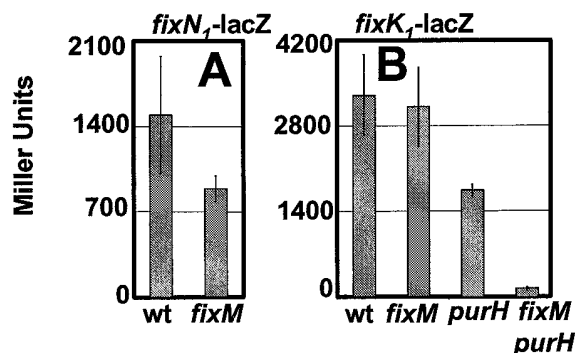


Fig. 4. Endogenously accumulated AICAR affects *fixK* symbiotic expression. **A**, *fixN₁-lacZ* (pGMI931) expression in bacteroids. **B**, *fixK₁-lacZ* (pMF457) expression in bacteroids. Data are the mean of four to ten experiments. Standard deviations are indicated.

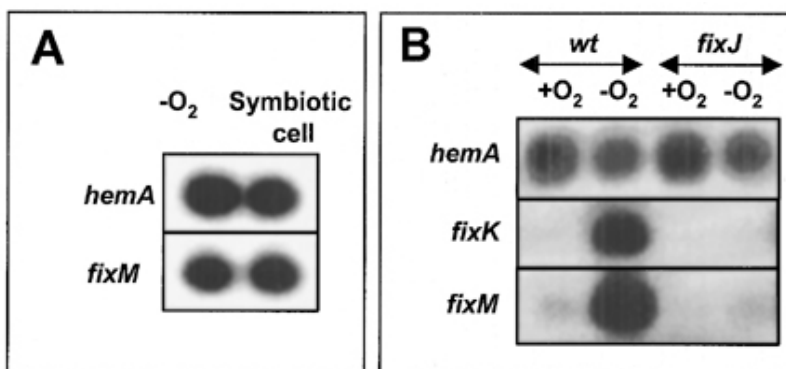


Fig. 3. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of *fixM* gene expression. **A**, RT-PCRs of RNAs isolated from free-living microoxic wild-type strain (GMI211) and from symbiotic bacteroids 3 weeks after alfalfa inoculation. **B**, RT-PCRs of RNAs isolated from free-living wild-type strain and *fixJ* mutant strain (GMI347) under microoxic or oxic conditions.

assayed the effect of AICAR on *fixN* expression in a strain in which *fixK₁* was expressed under the control of a constitutive promoter. We introduced the plasmid in the wild-type strain (GMI51) and in the *fixM* mutant strain (GMI461) and assayed AICAR effect on the activity of a *fixN₁-lacZ* fusion in these strains. As shown in Figure 5, *fixN* expression was not affected anymore by AICAR when *fixK₁* was constitutively expressed. Assays performed in oxic conditions (*fixN* expression does not depend anymore on oxygen in this *fixK^c* strain) confirmed these results (data not shown). Hence, AICAR does not affect FixK protein activity. Instead, AICAR modifies *fixK* and *nifA* expression, by affecting an undetermined target above of *fixK* and *nifA*, possibly FixL-J (discussed below). Inactivation of the two copies of *fixT* in a *fixM* background (GMI1266) did not affect AICAR effect, thus indicating that FixT does not mediate AICAR effect (data not shown).

fixM encodes a flavoprotein oxydo-reductase.

We purified the protein encoded by the *fixM* gene as a fusion with intein (discussed below). The protein was yellow throughout all purification steps, suggesting the presence of a

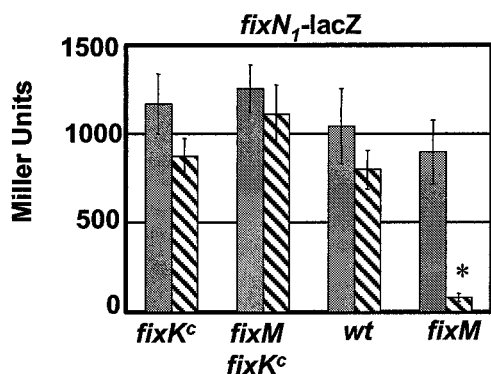


Fig. 5. AICAR did not affect *fixN* expression when *fixK₁* was constitutively expressed. Expression of *fixN* (pGMI931) in the indicated genetic backgrounds in the absence of (gray) or the presence of (hatched) AICAR (0.2 mM) under microaerobic conditions. Data are the mean of four to ten experiments. Standard deviations are indicated. Stars indicate that data in the presence of and the absence of AICAR are statistically different ($P < 0.01$).

chromophore copurifying with the FixM protein. Visible absorption spectra revealed an absorption maximum at 445 nm that bleached upon reduction with sodium dithionite under anaerobic conditions (Fig. 6A). These results were suggestive of a flavin prosthetic group. After denaturation in a 1% sodium dodecyl sulfate (SDS) solution and migration on a PD-10 column (Sephadex G25M; Amersham Pharmacia, Orsay, France), we separated the protein from its yellow prosthetic group, indicating that the FixM apoprotein and its chromophore were not covalently bound. Further separation analyses of the supernatant solution by thin-layer chromatography (TLC) identified a predominant fluorescent species with an Rf characteristic of flavin adenine dinucleotide (FAD) (Fig. 6B). The characteristic absorbance at 445 nm and the direct identification of the FAD cofactor showed that FixM is a flavoprotein.

Sequence comparison of the FixM apoprotein with proteins from the NCBI database revealed that FixM is homologous to Nim proteins of *Bacteroides fragilis* (Trinh and Reyssat 1997), sharing 28% identity at the amino acid level with NimD (Fig. 7). Further FixM homologues have been identified in recently sequenced genomes. Four homologues (BAB52857, BAB52932, BAB54825, and BAB51313) exist in *Mezorhizobium loti* (Kaneko et al. 2000). The strongest hit reaches 51% identity at the amino acid level with BAB52857. Another homologue has been detected in the genome of *Methanobacterium thermoautotrophicum* (Smith et al. 1997) with an identity of 29% with the FixM protein. The alignment performed with the Multalin and ESPript programs (Corpet 1988; Gouet et al. 1999) shows that these proteins, despite the relatively low level of sequence similarity, present several conserved residues at different positions, indicating that they are indeed homologous (Fig. 7). Nim proteins are the only proteins of this family that have been characterized. They are involved in resistance of *B. fragilis* to 5-nitroimidazole drugs upon reduction on this substrate to 5-aminoimidazoles (Carrier et al. 1997; Townson et al. 1994). We thus conclude that FixM is a flavoprotein oxydo-reductase.

DISCUSSION

We report here that the purine intermediate AICAR represses expression of *fixN*, *fixK*, *fixT*, and *nifA* in *S. meliloti* in

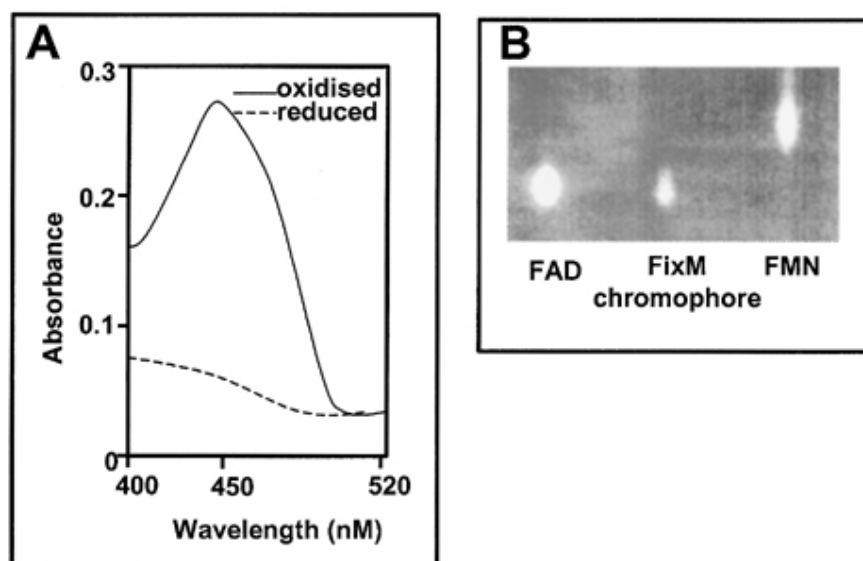


Fig. 6. *fixM* encodes a flavoprotein. **A**, Absorbance spectra of oxidized and dithionite-reduced FixM. **B**, Thin-layer chromatography analysis of the FixM-associated chromophore. Proteins were separated from the prosthetic group by denaturation.

confirmation of the work by Soberón and associates (2001). A surprising finding of this article is that AICAR is not active in a wild-type strain. Instead, AICAR is active in a *fixM* mutant strain and in the GMI5595 deleted strain used by Soberón and associates (2001), although we observed a mean repression (fivefold) weaker than that reported by Soberón and associates (2001) (14-fold). We do not know the reason for this difference. Note, however, that GMI5595 and GMI461 are not strictly isogenic (Table 2). The fact that there is little or no effect of AICAR in a wild-type strain, whereas there is an effect in the 16-kb-deleted strain, suggests that a gene missing in GMI5595 mediates inhibition by AICAR. This cannot be the *fixM*-like gene located in this region, because it is likely a pseudogene. In the GMI5595 background, the AICAR effect is not increased upon *fixM* inactivation. Therefore, *fixM* and the gene missing in GMI5595 that modulates the AICAR effect likely belong to the same pathway rather than to alternate pathways. In the deleted region (Fig. 1), four genes encode putative oxydo-reductases (*SMa0751*, *SMa0752*, *SMa0753*, and *SMa0758*). The products of these genes are good candidates to work together with FixM on the reduction of the AICAR substrate.

FixM, together with Nim proteins and unknown hypothetical proteins found in genome databases, forms a new protein family. Nim proteins exert a reductase activity on 5-nitroimidazole rings but were not known to be flavoproteins (Carlier et al. 1997). We showed here that *fixM* encodes a flavoprotein and suggest that the proteins belonging to this family are all flavoprotein oxidoreductases. The homology of FixM and Nim proteins together with the presence of an imidazole ring in AICAR and 5'AMP suggest that AICAR or 5'AMP is the actual substrate of the FixM reductase. In most flavoproteins described so far, a classical "βαβ" dinucleotide-binding motif known as the Rossmann fold, ensures dinucleotide binding (Rossmann et al. 1974). The proteins that adopt a Rossmann fold share a consensus sequence referred to as the dinucleotide binding motif (Möller and Amons 1985; Wierenga et al. 1986).

Further conserved motifs involved in dinucleotide interaction have also been described, such as the "GD motif" (Eggink et al. 1990), the "GG motif," and the "ATG motif" (Vallon 2000). Analysis of the FixM amino acid sequence and of its homologues did not reveal any of the above-described signatures. Therefore, this new family of proteins may bind its flavinic prosthetic group in a different way than up-to-now-characterized flavoproteins.

Whether AICAR or a closely related metabolite is the real effector of *nifFix* gene repression remains to be determined. In this work, we tested different purine-related molecules and showed that 5'AMP, which is a structural analogue of AICAR, had a similar action to AICAR. In addition, the nucleoside precursor adenosine also repressed *fixN* expression in a *fixM* mutant strain. This last result conflicts with that of Soberón and associates (2001), who reported that *fixN₁-lacZ* expression was not affected by adenosine in the GMI5595 strain. Nucleotides are dephosphorylated by periplasmic nucleotidases before being transported into the cell under their nucleoside form (Zalkin and Nygaard 1996). Therefore, it might not be surprising that the effect observed with AICAR and 5'AMP was also observed with their nucleoside precursors. *SMc04018*, a predicted periplasmic 5'-nucleotidase, is a good candidate for the dephosphorylation of AICAR and 5'AMP before these metabolites enter the cell. *SMc04018* is predicted to be specific for 5'-nucleotides and no other candidate for periplasmic nucleotidase has been detected in the *S. meliloti* genome. Consequently, we cannot rule out that 2' and 3'AMP were inactive in our assays because they could not enter the cell. As did Soberón and associates (2001), we observed that it was necessary to incubate AICAR for at least 1 h in oxic conditions to see a repression of *fixN* expression. This might correspond to the time needed for these metabolites to be transported into the cell.

Experiments performed in a strain in which *fixK* was constitutively expressed showed that AICAR lost its effect when *fixK* expression was not controlled by the FixLJ regulatory system. We thus speculate that AICAR may target the FixLJ

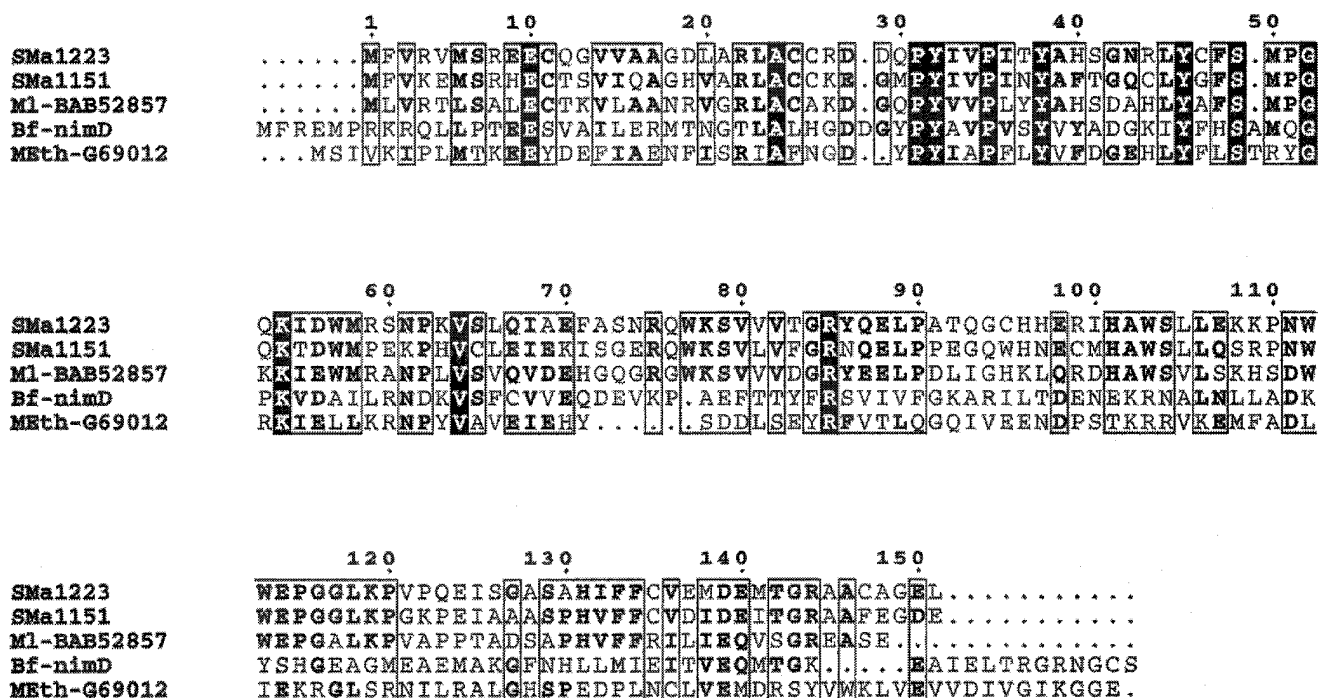


Fig. 7. FixM is homologous to Nim proteins of *Bacteroides fragilis*. The alignment was constructed with the ESPript and multalin programs (Corpet et al. 1988; Gouet et al. 1999). FixM presents 60, 52, 28, and 29% identity, respectively, with *Sinorhizobium meliloti* Sma1151, *Mezorhizobium loti* BAB52857, *Bacteroides fragilis* NimD (CAA544273), and *Methanobacterium thermoautotrophicum* G69012.

two-component system (Fig. 8). However, present attempts to evidence an AICAR or 5'AMP effect in vitro on purified FixLJ proteins remain so far inconclusive. FixL autophosphorylation, FixJ phosphorylation by FixL, FixJ transcriptional activity, FixJ dimerization, and DNA binding at the *fixK* promoter were unaffected by AICAR in vitro (data not shown). Several possibilities may account for this observation. First, it is not certain that AICAR or 5'AMP is the actual effective molecule in vivo. Second, AICAR could alter the intracellular levels of ATP, ADP, or AMP and thereby indirectly affect the activity of the FixL kinase in vivo. Third, the AICAR effect may require a third protein yet to be identified. In plants, AICAR and 5'AMP alike regulate phosphorylated proteins through the interaction with 14-3-3 proteins (Athwal et al. 2000; Finnemann and Schjoerring 2000; Kaiser and Huber 2001; Toroser et al. 1998). The common theme in 14-3-3 proteins action is their ability to associate with phosphorylated target proteins (Bachmann et al. 1996; Moorhead et al. 1999; Tzivion and Avruch, in press). However, 14-3-3 proteins have not been reported so far in any prokaryotic organism including *S. meliloti*.

The physiological significance of the regulation of nitrogen fixation and respiration by AICAR, 5'AMP, or a derived metabolite and the related issue of the biological role of *fixM* remain open questions. The present data, as well as former data (Soberón et al. 1997, 2001), suggest a regulatory link between purine biosynthesis and nitrogen fixation gene expression in *S. meliloti*. Although the possible physiological significance of this link remains unclear, it should be remembered here that, on the plant side, fixed nitrogen (ammonia) assimilation and de novo purine biosynthesis are closely linked in tropical legumes (Schubert 1986).

Another clear hint we can offer in the context of symbiosis is the well-documented accumulation of 5'AMP in anoxiated pea roots (Glaab and Kaiser 1993) or otherwise environmentally stressed cells (Corton et al. 1994). Nodule plant cells undergo a profound anoxia (less than 30 nM free O₂); therefore, it is possible, although not established to our knowledge, that nodule plant cells accumulate AICAR or 5'AMP. Plant-generated 5'AMP or AICAR may, in turn, inhibit bacteroid gene expression. Alternatively, AICAR or 5'AMP could be generated in bacteroids themselves upon stress induction. In both cases, the function of the FixM protein would be to turn over 5'AMP or AICAR and convert it, together with additional enzymes, into a form that is no longer inhibitory on gene expression. The close physical link between *fixM* and *fixLJT₁K₁* (Fig. 7) as well as *fixM* gene expression under free-living microoxic and symbiotic conditions suggest that *fixM* function is genuinely related to respiration and nitrogen fixation. Nevertheless, a *fixM* mutant is essentially unaffected for symbiotic properties, at least under the laboratory conditions we used, hence suggesting that AICAR or 5'AMP normally does not accumulate to inhibitory levels. However, there is the possibility of AICAR or 5'AMP bursts in the nodule under stressful conditions (extreme anoxia, carbon shortage, and so on) or at specific moments during nodule differentiation, as during infection, as suggested before by Soberón and associates (2001). Hence, AICAR or 5'AMP might act as a signal molecule that finely tunes down respiration, energy production, and nitrogen fixation by the microsymbiont under inappropriate physiological conditions. We also cannot rule out the possibility of other enzymes contributing, independently of

Table 2. Main bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Reference |
|---------------------------------|---|---------------------------------|
| <i>Escherichia coli</i> | | |
| DH5 α | <i>EndA1, gyrSA96, hrd17(rK-mK-), supE44, recA1</i> | Boyer and Roulland-Dussoix 1969 |
| ER2566 | <i>F-λ-fhuA2[lon] ompT lacZ::T7 gene1 gal sulA11 Δ(mrcC-mrr)114::IS10 R(mcr-73::miniTn10--TetS)2 R(zgb-210::Tn10)(TetS) endA1 [dcm]</i> | New England Biolabs |
| <i>Sinorhizobium meliloti</i> | | |
| GMI51 | Wild-type strain, Nod ⁺ , Fix ⁺ | Rosenberg et al. 1981 |
| GMI211 | GMI51 derivative, lac ⁻ , Sm ^r , Nod ⁺ , Fix ⁺ | Niel et al. 1977 |
| GMI461 | Disruption of <i>fixM</i> by insertion 2.37 in GMI51—Sm ^r , Nm ^r , Bleo ^r , Nod ⁺ , Fix ⁺ | Batut et al. 1989 |
| GMI347 | Disruption of <i>fixJ</i> by insertion 2.3 in GMI51—Sm ^r , Nm ^r , Bleo ^r , Nod ⁺ , Fix ⁻ | Batut et al. 1985 |
| GMI942 | Disruption of <i>fixK₁</i> by insertion of a Ω cassette in GMI940—Nod ⁺ Fix ⁻ | Foussard et al. 1997 |
| GMI11260 | Disruption of SMA1151 by insertion of a Ω cassette in GMI51—Spec ^r , Nod ⁺ , Fix ⁺ | This study |
| GMI11257 | Disruption of SMA1151 by insertion of a Ω cassette in GMI461—Sm ^r , Nm ^r , Bleo ^r , Spec ^r , Nod ⁺ , Fix ⁻ | This study |
| GMI11256 | Disruption of <i>purH</i> by insertion of a Ω cassette in GMI51—Gm ^r , Nod ⁺ , Fix ⁺ | This study |
| GMI11255 | Disruption of <i>purH</i> by insertion of a Ω cassette in the GMI461—Sm ^r , Nm ^r , Bleo ^r , Gm ^r , Nod ⁺ , Fix ⁺ | This study |
| GMI708 | Rif ^r , Nod ⁺ , Fix ⁺ | Batut et al. 1985 |
| GMI5595 | GMI708 Δ JB16 Rif ^r , Sm ^r , Nm ^r , Bleo ^r , Nod ^d , Fix ⁺ | Renalier et al. 1987 |
| GMI5602 | Transduction of the 2.37 Tn5 insertion in <i>fixM</i> from GMI461 to GMI5595—Rif ^r , Sm ^r , Nm ^r , Bleo ^r , Nod ^d , Fix ⁺ | Batut et al. 1989 |
| GMI882 | Disruption of <i>fixK₁</i> by insertion 2.81 in GMI51—Nm ^r , Bleo ^r , Nod ⁺ , Fix ⁺ | Batut et al. 1989 |
| GMI11266 | Insertion of the <i>fixT₁</i> mutation in GMI5602 strain Rif ^r , Sm ^r , Nm ^r , Bleo ^r | This study |
| Plasmids | | |
| pUC0441 | pUC19 containing <i>fixM</i> used as a marker to generate a high density map of <i>S. meliloti</i> genome—Ap ^r | Barloy-Hubler et al. 2000 |
| pJQ200KS | Suicide vector used for site directed mutagenesis in <i>S. meliloti</i> —Gm ^r | Quandt et al. 1993 |
| pNPTS139 | Suicide vector used for site directed mutagenesis in <i>S. meliloti</i> —Km ^r | Tsai et al. 2001 |
| pGMI931 | pIJ363 derivative carries <i>fixN₁</i> -lacZ fusion—Tc ^r | David et al. 1987 |
| pMF457 | pGD926 derivative, carries a <i>fixK₁</i> -lacZ fusion and a mutation in <i>fixT₁</i> promoter—Tc ^r | Foussard et al. 1997 |
| pGD106 | pGD926 derivative, carries a <i>fixT₁</i> -lacZ fusion—Tc ^r | Foussard et al. 1997 |
| pCHK57 | pGD926 derivative, carries a <i>nifA</i> -lacZ fusion—Tc ^r | Ditta et al. 1987 |
| pML123 | IncQ broad host range vector—Gm ^r | Labes et al. 1990 |
| pML123- <i>fixK₁</i> | pML123 derivative, carries <i>fixK₁</i> —Gm ^r | This study |
| pHP45(Ω) | pBR322 derivative carries the spectinomycin resistance Ω cassette | Prentki and Krisch 1984 |
| pTYB- <i>fixM</i> | PTYB ₂ derivative, carries the <i>fixM-inteine</i> fusion—Ap ^r | This study |
| pKS+457 | pBluescript-II KS+ derivative carrying the <i>fixT₁</i> mutation | Foussard et al. 1997 |
| pUC1318 | PUC19 derivatives carries the gentamycin resistance cassette—Ap ^r | Brau et al. 1984 |

fixM, to AICAR or 5'AMP detoxification, which would explain why inactivation of the sole *fixM* gene has a limited effect on gene expression.

MATERIALS AND METHODS

Microbiological techniques.

The bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* strains were grown at 37°C in Luria broth (LB) complex medium (Maniatis et al. 1982). *S. meliloti* strains were grown at 30°C in tryptone yeast (TY) complex medium (Truchet et al. 1985) or in defined M9 medium (Miller 1972) supplemented with MgSO₄ (1 mM), biotin (2 μM), and glucose (20 mM). Microoxic conditions were achieved either as described by de Philip and associates (1990) (2% oxygen for 4 h) or using the stoppered tube assay (STA) described by Ditta and associates (1987) (<1% oxygen for 16 h). Matings were performed between recipient strains, donor strains, and the helper strain K12 (pRK2013), when necessary, as described previously (Truchet et al. 1985). N3 phage transduction was performed as described by Finan and associates (1984).

AICAR assay.

Purine metabolites were purchased from Sigma (St. Louis) and were all dissolved in water at the final concentration of 15 mM. AICAR (Sigma #A1393) was neutralized in 60 mM TRIS-HCL, pH 8.3. Strains were grown for 24 h in TY medium at 30°C with agitation (250 rpm). This culture was used to inoculate 15 ml of minimal M9 medium at an optical density at 600 nm (OD₆₀₀) of 0.05. The strains then were grown at 30°C with agitation to an OD₆₀₀ of 0.4. AICAR (or other purine metabolites) then were added at a final concentration of 0.2 mM. After an additional 4 h of aerated growth at 30°C in the presence of AICAR, the growth conditions were turned to

microoxic for 4 h to induce *fix* and *nif* gene expression. The growth medium (200 μl) was pelleted and resuspended in 700 μl of buffer Z (Na₂HPO₄, 60 mM; NaH₂PO₄, 40 mM; KCL, 10 mM; MgSO₄, 1 mM; β-mercaptoethanol, 50 mM; pH 7). Cells were permeabilized with 100 μl of chloroform and 50 μl of SDS, and β-galactosidase activity was measured as described by Miller (1972). The activity is expressed in nmol of *o*-nitrophenyl-β-D-galactoside hydrolyzed per minute and per OD₆₀₀ unit.

Plant tests.

M. sativa cv. Gemini seedlings were aseptically grown on agar slants made with nitrogen-free Fahraeus medium. Plants (3-day-old) were inoculated with different *S. meliloti* strains. Nodules (0.25 g) were harvested and were immediately frozen in liquid nitrogen 5 weeks after inoculation. Nodules were ground in liquid nitrogen in alumina powder (A5 type, Sigma) with a mortar and pestle, and the paste was resuspended in 1.6 ml of the following buffer: Na₂HPO₄, 60 mM; NaH₂PO₄, 40 mM; KCl, 10 mM; MgSO₄, 1 mM; mannitol, 0.5 M; pH 7. Debris were sedimented by centrifugation for 10 min at *g* × 12,000. β-galactosidase activity was assayed on 400 μl of supernatant of this extract. The activity is expressed in nmol of *o*-nitrophenyl-β-D-galactoside hydrolyzed per minute per mg of proteins. Total protein concentration was determined as described by Bradford (1976). The endogenous β-galactosidase activity of the plant cell was negligible in our experiments.

RT-PCR analysis.

RNA extraction and RT-PCR reactions were performed as described by Cabanes and associates (2000a, 2000b). The amplified products were subjected to Southern hybridization with the specific ³²P-labeled probe generated by PCR on *S. meliloti* total DNA. No RT-PCR product was detected in controls lacking reverse transcriptase during the cDNA synthesis

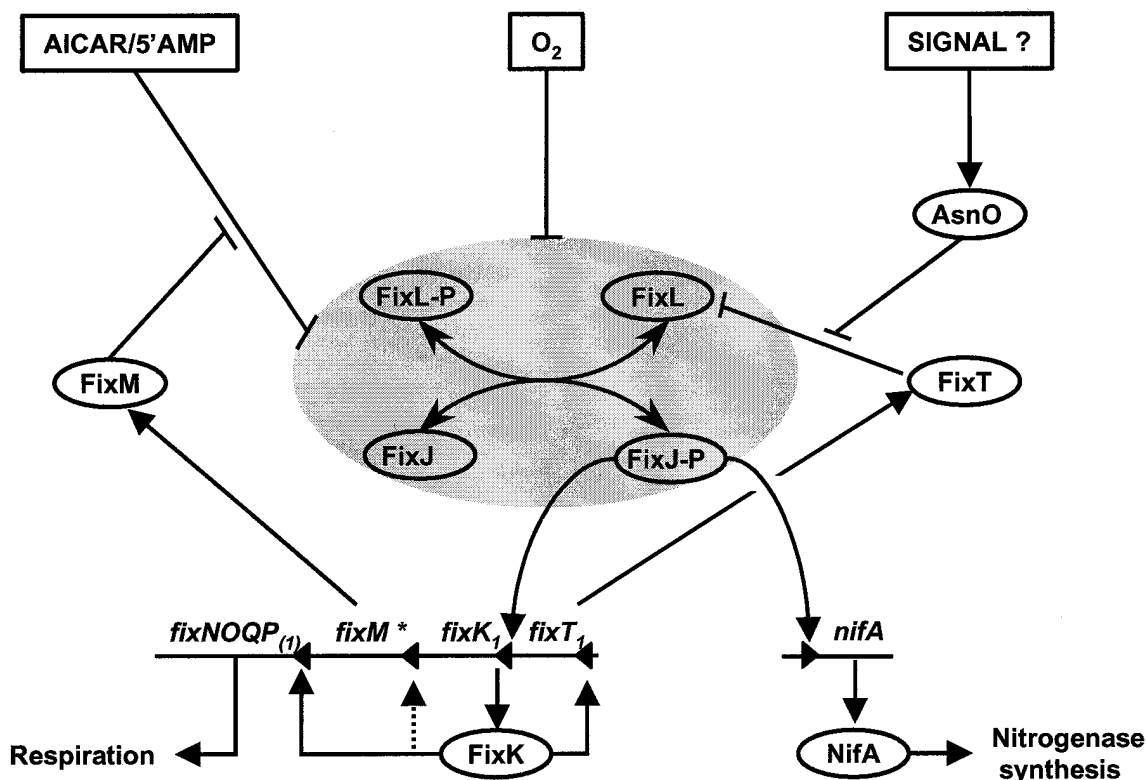


Fig. 8. An updated model for the regulation of *nif* and *fix* gene expression by FixLJ in *Sinorhizobium meliloti*. The dashed arrow indicates the possibility that *fixK* activates the *fixM* promoter. * = *fixM* was annotated as *SMA1223* during the *S. meliloti* sequencing project.

step, thus excluding the presence of DNA contaminating the RNA preparations. As a control of the amount of RNA in the reactions, the *S. meliloti hemA* gene was subjected to RT-PCR procedure with *hemA_{sens}*-*hemA_{antisens}*. The *fixM* transcript was detected with *orf151₁*-*orf151₂*. The *Sma1151* transcript was detected with *orf151₁₀*-*orf151₁₁*. The *fixM*-like gene located in the reiterated *fix* gene cluster was detected with CD₅-CD₉ (Table 3).

Construction of mutants.

The *Sma1151* coding region was amplified in two pieces by PCR with two pairs of primers using pUC0441 (Table 2) as a template. The primers M13 and *Bam151a* generated a 650-bp fragment, and the primers Rev and *Bam151b* generated a 480-bp fragment. *Bam151a* and *Bam151b* were designed with a *Bam*H1 restriction site at the 5' end. The 650-bp and the 480-bp fragment were digested with *Bam*H1 and ligated together. The ligated fragment then was digested with *Kpn*1 and *Pst*1 and cloned in pUC19, generating a new plasmid called pUC0441-*Bam*H1. This plasmid contains the *Sma1151* gene with an extra *Bam*H1 restriction site in which a Ω interposon cassette was inserted as a 1.9-kb *Bam*H1 DNA fragment extracted from pHP45 Ω . A 3-kb *Sst*1-*Pst*1 fragment carrying the Ω interposon inserted in *Sma1151* was subsequently cloned in pJQ200KS (Quandt and Hynes 1993). The cassette then was recombined into the indigenous *Sma1151* gene of the wild-type strain GMI51, selecting on spectinomycin resistance (100 μ g ml⁻¹) conferred by the cassette and sucrose tolerance (elimination of pJQ200KS vector DNA). When necessary, the mutation was moved to other *S. meliloti* backgrounds by N3 phage transduction. PCR analyses were performed to ensure the presence of the Ω interposon at the expected location.

A 1.2-kb *purH* fragment gene was generated by PCR using *purH₅* and *purH₁₆* as primers and wild-type *S. meliloti* DNA as template. This fragment was cloned in the PCR product cloning vector pGEM-T (pGEM-T Vector System; Promega, Madison, WI, U.S.A.). A Ω interposon cassette was inserted as a 1-kb DNA fragment extracted from pUC1318 (Brau et al. 1984) into the *Eco*R1 site present in the middle of the *purH* fragment gene. A 2.2-kb *Pst*1-*Apa*1 fragment carrying Ω inserted in *purH* fragment gene subsequently was cloned into the suicide

vector pNPTS139 (Tsai and Alley 2001). The cassette then was recombined into the indigenous *purH* gene of the wild-type *S. meliloti* strain (GMI51), selecting on gentamycin resistance (30 μ g ml⁻¹) conferred by the cassette and sucrose tolerance (elimination of pNPTS139 vector DNA). When necessary, the mutation was moved to other *S. meliloti* strains by N3 phage transduction. PCR analyses were performed to ensure the presence of the Ω interposon at the expected location.

An 880-bp DNA fragment containing the entire *fixK₁* gene and its ribosome binding site was amplified by PCR using *K-rbs* and *K-hind*III primers and *S. meliloti* wild-type DNA as template. The amplified fragment was cloned in pGEM-T. Correct orientation was checked by PCR on colonies using *fixK_r* and *sp6* as primers. A *Hind*III-*Sac*I 880-bp fragment containing *fixK₁* subsequently was cloned in pML123, generating the pML123-*fixK₁* plasmid. This plasmid was introduced in a DH5 α strain by electroporation and the *fixK₁* insert was checked by sequencing. pML123-*fixK₁* was introduced in *S. meliloti* by triparental mating using gentamycin resistance (30 μ g ml⁻¹) as a selective marker.

The *fixT* mutation carried by pKS+457 was introduced into GMI5602 as described by Foussard and associates (1997).

Purification of the FixM protein.

The *fixM* coding region was amplified by PCR from genomic DNA using the *orf151-Nde*I and *orf151-Sma*I primers. These primers have been designed so that they include an *Nde*I and *Sma*I extra restriction sites that permitted in-frame cloning of *fixM* in the pTYB₂ intein vector (New England Biolabs, Beverly, MA, U.S.A.). The ligated product was electroporated into *E. coli* ER2566, selecting on ampicillin resistance (100 μ g ml⁻¹). The *fixM-Intein* insert was entirely sequenced to ensure that no undesired alteration occurred during its construction. The resulting plasmid was named pTYB-*fixM*. ER2566 cells harboring pTYB-*fixM* plasmid were grown at 19°C in 2 l of LB medium supplemented with ampicillin at 100 μ g ml⁻¹. When OD₆₀₀ reached a value of 0.6, IPTG was added to a final concentration of 0.3 mM. After an additional 8 h at 19°C, four fractions of 500 ml of cells were separately pelleted. All subsequent steps were performed at 4°C, essentially as recommended by New England Biolabs. Cells were suspended in 20 ml of buffer S (Tris, 20 mM; EDTA, 1 mM; NaCl, 200 mM) and disrupted by sonica-

Table 3. Primers used in this study

| Genes | Name | Sequence |
|-------------------------|--------------------------------|--|
| <i>fixM</i> | | |
| Forward | <i>orf151₁</i> | 5'-CTGCAGATTGCCGAGTTTGCC-3' |
| Reverse | <i>orf151₂</i> | 5'-CCCCGTCATCTCGTCCATCTCC-3' |
| Forward | <i>orf151-nde1</i> | 5'-GGAATCCATATGTTCGTGAGGGTAATGTC-3' |
| Reverse | <i>orf151-sma1</i> | 5'-TCCCCCGGGTAACCTACCCGCGCAGGC-3' |
| <i>Sma1151</i> | | |
| Forward | <i>bam151a</i> | 5'-CGCGGATCCTCCTGGTAACGG-3' |
| Reverse | <i>bam151b</i> | 5'-GCGGGATCCGCCTGCGGGGC-3' |
| Forward | <i>orf151₁₀</i> | 5'-TACAAGTGTGATTCAGGCAGG-3' |
| Reverse | <i>orf151₁₁</i> | 5'-GCCACTGCCCCCTCAGGC-3' |
| <i>fixM</i> -like gene | | |
| Forward | CD ₅ | 5'-TCTATCGCCGGCC-3' |
| Reverse | CD ₉ | 5'-CAGGGACGGCCAGCC-3' |
| <i>fixK₁</i> | | |
| Forward | <i>fixK_f</i> | 5'-TAT CTA CCG CCT CCT TTC-3' |
| Reverse | <i>fixK_r</i> | 5'-GCATCTCTGGTAATCGG-3' |
| Forward | <i>K-rbs</i> | 5'-CGAACGGGAATAATTCATGTACGCCGCTGC-3' |
| Reverse | <i>K-hind</i> III | 5'-CCCAAGCTTTC AATTGCAAAGCGAGCGCAGCGC-3' |
| <i>hemA</i> | | |
| Forward | <i>hemA_{sens}</i> | 5'-TGGATGGGCTGCATCA-3' |
| Reverse | <i>hemA_{antisens}</i> | 5'-GTGGATCGCGTTCTT-3' |
| <i>purH</i> | | |
| Forward | <i>PurH₅</i> | 5'-ACGATCGTCACCG-3' |
| Reverse | <i>PurH₁₆</i> | 5'-TTCGTCGCGCATCGAACCACCG-3' |

tion. The chitin beads column (50 ml) was equilibrated with buffer S (10 volumes) before adding the crude extract. After extensive washing with 10 volumes of buffer S containing 0.1% triton and after equilibration with 5 volumes of buffer S, the column was incubated overnight with buffer S containing 50 mM of dithiothreitol. Fractions were collected and screened for the presence of FixM protein by SDS-polyacrylamide gel electrophoresis on a Pharmacia Phast gel system. Fractions containing FixM protein were concentrated with a YM10 centricon (amicon bioseparation, millipore). At this final step, the cleaved FixM concentrated protein appeared yellow and insoluble.

Precipitated FixM was analyzed after solubilization in a buffer containing Tris, 20 mM; NaCl, 300 mM; EDTA, 1 mM; SDS, 1%. Absorbance spectra were recorded with a Beckman DU 640B spectrophotometer in the presence or absence of 6 mM sodium dithionite. TLC analyses of the FixM chromophore were performed on Kieselgel 60F254 plates (Merck, Strasbourg, France) in parallel with standards of flavin mononucleotide and FAD, in a buffer containing butanol, acetic acid, and water (12:3:5 vol/vol). The spots were visualized under UV light (312 nm).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Kazusa DNA Research Institute Rhizobia database: www.kazusa.or.jp/rhizobase/
- INRA *Sinorhizobium meliloti* strain 1021 Genome Project: sequence.toulouse.inra.fr/meliloti.html