The NifA-RpoN Regulon of *Mesorhizobium loti* Strain R7A and Its Symbiotic Activation by a Novel LacI/GalR-Family Regulator

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Abstract

*Mesorhizobium loti* is the microsymbiont of *Lotus* species, including the model legume *L. japonicus*. *M. loti* differs from other rhizobia in that it contains two copies of the key nitrogen fixation regulatory gene *nifA*. *nifA*1 and *nifA*2, both of which are located on the symbiosis island ICE*Sym*R7A. *M. loti* R7A also contains two *rpoN* genes, *rpoN*1 located on the chromosome outside of ICE*Sym*R7A and *rpoN*2 that is located on ICE*Sym*R7A. The aims of the current work were to establish how *nifA* expression was activated in *M. loti* and to characterise the NifA-RpoN regulon. The *rpoN*2 and *rpoN*1 genes were essential for nitrogen fixation whereas *nifA*1 and *rpoN*1 were dispensable. Expression of *nifA*2 was activated, possibly in response to an inositol derivative, by a novel regulator of the LacI/GalR family encoded by the *fixL* gene located upstream of *nifA*2. Other than the well-characterized *nif*/*fix* genes, most NifA2-regulated genes were not required for nitrogen fixation although they were strongly expressed in nodules. The NifA-regulated *nifZ* and *fixU* genes, along with *fixO* which was not NifA-regulated, were required in *M. loti* for a fully effective symbiosis although they are not present in some other rhizobia. The NifA-regulated gene *msi158* that encodes a porin was also required for a fully effective symbiosis. Several metabolic genes that lacked NifA-regulated promoters were strongly expressed in nodules in a NifA2-dependent manner but again mutants did not have an overt symbiotic phenotype. In summary, many genes encoded on ICE*Sym*R7A were strongly expressed in nodules but not free-living rhizobia, but were not essential for symbiotic nitrogen fixation. It seems likely that some of these genes have functional homologues elsewhere in the genome and that bacteroid metabolism may be sufficiently plastic to adapt to loss of certain enzymatic functions.

Introduction

*Mesorhizobium loti* is the natural microsymbiont of *Lotus* species, including the model legume *L. japonicus*. The genes required for nodule formation and nitrogen fixation in *M. loti* strain R7A are located on a 502-kb chromosomally located symbiosis island [1,2], which was subsequently named ICE*Sym*R7A [3] as it belongs to the family of mobile genetic elements collectively termed integrative and conjugative elements (ICEs) [4]. Sequence analysis of ICE*Sym*R7A revealed that it shares 248 kb of DNA with the 611-kb symbiosis island of the sequenced *M. loti* strain MAFF303099 [5], including all the genes likely to be required for Nod factor synthesis and the formation of a functional nitrogenase enzyme. In addition, it contains mobility genes, a type IV secretion system similar to that of the *vir* system from *Agrobacterium tumefaciens* and a diverse range of regulators, metabolic genes, and transporters that may contribute to nodule function [6,7].

Compared to several other rhizobial species, very little is known about how *M. loti* genes required for symbiotic nitrogen fixation are regulated. In most nitrogen-fixing bacteria, the NifA protein binds to an upstream activating sequence (UAS) and acts in association with the RNA polymerase sigma factor RpoN (σ54) to activate *nif* gene expression and, in rhizobia, the expression of several other symbiotic genes [reviewed in [8]]. *M. loti* differs from other rhizobia examined to date in that it contains two copies of the *nifA* gene, *nifA*1 and *nifA*2, both of which are located on ICE*Sym*R7A. The *nifA*1 gene is most similar to and in the same genomic context between *fixL* and *nifB* as *nifA* from *Bradyrhizobium eli*, *R. leguminosarum*, *Rhizobium sp.* strain NGR234 and *Sinorhizobium meliloti* [9,10,11,12,13]. In contrast, *nifA*2 is most similar to *nifA* from *Bradyrhizobium japonicum* and is not located adjacent to known nitrogen fixation genes. The two genes are not functionally redundant as *M. loti* *nifA*2 mutants form Fix− nodules [14,15] whereas *nifA*1 mutants are not symbiotically impaired [14].

NifA activity in rhizobia is oxygen-sensitive and it is thought that conserved cysteine residues present within NifA are involved in sensing and reacting to the cellular oxygen status [reviewed in [8]]. In addition, in most rhizobia the *nifA* gene is subject to transcriptional regulation although the mechanisms vary depending on the rhizobial strain. In *S. meliloti* *nifA* expression is activated by the FixI/J two-component regulatory system in response to low oxygen tension. In addition *nifA* is located downstream of *fixABCX* genes and it is possible that NifA could influence expression of these genes by serving as a negative regulator of *fixABCX* genes.

The authors have declared that no competing interests exist.

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Citation: Sullivan JT, Brown SD, Ronson CW (2013) The NifA-RpoN Regulon of *Mesorhizobium loti* Strain R7A and Its Symbiotic Activation by a Novel LacI/GalR-Family Regulator. PLoS ONE 8(1): e53762. doi:10.1371/journal.pone.0053762

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Editor: Valerie de Crécy-Lagard, University of Florida, United States of America

Received September 28, 2012; Accepted December 4, 2012; Published January 7, 2013

Funding: This work was supported by a grant from the Marsden Fund administered by the Royal Society of New Zealand and grants from the University of Otago. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.
and nifA expression is enhanced by NifA-mediated expression via the fixC promoter [8]. In B. japonicum the fixK-nifA operon is controlled by the redox-responsive two-component system RegSR acting on the fixR promoter [16]. In R. leguminosarum bv. viciae strain UPM791, nifA is expressed only under symbiotic conditions, through autoregulation via the promoter which precedes the 3′-end of fixY [17]. For R. etli, expression of nifA occurs independently of cellular oxygen status and no genetic regulatory elements have been identified. However nifA expression is upregulated under symbiotic conditions, suggesting that it may be under some form of symbiosis-specific regulation [18].

RpoN recognizes and binds a −24/−12 promoter sequence with the consensus 5′-TGGCACG-N4-TTG-3′. The G situated at position −24 and C situated at −12 relative to the transcription start site (shown in bold in consensus) are almost invariant although the nifH promoters of M. loti and R. etli have an A instead of C at the −12 position. Sixteen candidate NifA-regulated promoters were defined on ICEMIsymR7A on the basis of their containing a potential NifA upstream activator sequence (TGT-N10-AGA) and a −24/−12 promoter sequence [6]. Of these, 15 are located upstream of annotated genes, including eight that precede known nif/fix genes clusters (Table 1). One potential promoter region was found upstream of msi281 but in reverse orientation, facing a 2.3-kb region that contains a fragment of the nodulation gene nosL but no annotated complete genes. The msi320-321 cluster is the only potential NifA-regulated cluster present on ICEMIsymR7A that is not present in MAFF303099 [6]. Whether the M. loti genes in the putative NifA-regulated clusters other than the well-characterised nif/fix genes are required for symbiotic nitrogen fixation remains unknown. However many of them have predicted functions that may be of symbiotic relevance (Table 1).

M. loti also contains two tpsN genes, tpsN1 (mll3196 in strain MAFF303099) located on the chromosome outside of the symbiosis island and tpsN2 that is located on the island (msi335 in strain R7A). R. etli also contains tpsN1 and tpsN2 genes and RpoN1 is required for the metabolism of C4-dicarboxylic acids and several nitrogen sources during free-growing [19] while RpoN2 is involved in symbiotic nitrogen fixation. The tpsN2 gene is part of a prxS-tpsN2 operon that is activated by NifA. An additional symbiosis-specific weak promoter is located between prxS and tpsN2 [20,21]. In M. loti the tpsN2 gene is also downstream of prxS as part of a potential NifA-regulated operon (Table 1). B. japonicum also has two tpsN genes but both RpoN1 and RpoN2 are functional in free-living and symbiotic conditions [22]. In contrast, S. meliloti and R. leguminosarum bv. viciae strain VF398SM have a single copy of tpsN that in R. leguminosarum is negatively autoregulated [23,24].

A transcriptome macroarray analysis based on the M. loti MAFF303099 genome revealed clusters of genes within the symbioses island that were up-regulated during symbiosis compared to free-growing growth, whereas genes outside the island were in general down-regulated. The up-regulated genes included island genes involved in metabolism as well as nif/fix genes and the duplicate fixNOQP genes outside the symbiosis island [25].

The aims of the current work were to characterise the NifA-RpoN regulon in M. loti and to establish how nifA expression is activated. In addition we wished to determine the symbiotic phenotypes of selected metabolic genes found to be up-regulated in nodules and to determine whether their expression depended on NifA. We show that symbiotic gene expression in M. loti is under novel regulation and identify several new symbiotic genes. The availability of the M. loti mutants described in this paper should

| Gene or operon         | −24/−12 promoter seq. | Putative gene/operon function                                      |
|------------------------|-----------------------|-------------------------------------------------------------------|
| omp2b (msi036)         | TTGGCACGCTATTTGGG     | Outer-membrane porin (Omp2 family)                                 |
| msi071-msi064          | TTGGCACAGGTCTTGGG     | Diterpenoid synthesis                                              |
| msi158                 | TTGGCACGACATTTGGG     | Outer-membrane porin (OmpW family)                                 |
| msi262-msi263          | CTGGCACACCTCTTGGG     | Ms262 iron-sulfur cluster assembly, HesB family, IscN; Ms263 FeS   |
| gacD (msi273)          | TTGGCACGGTACATGCT     | 1-aminocyclopropane-1-carboxylate deaminase                        |
| fixV frag, hypC frag, msi276-274 | CTGGCATGACGTCTTGGT     | Ms276 DUF683 (found in nif clusters); Ms275 FdxB Ferredoxin III [4Fe-4S], nif-specific; Ms274 partial similarity (SynA superfamily) |
| msi280                 | CTGGCACGCCCTTGAGC     | L-lysine 6-monoxygenase                                             |
| Nt msi281              | TTGGCACGGCTTGGT       | no annotated genes                                                 |
| nifHDKEXN-msi288       | TTGGCACGAGTTTGGG      | Nitrogenase enzyme synthesis; msi288 unknown function DUF269, NifX-associated protein |
| nifH frag, msi321-320  | TTGGCACGAGTTTGGG      | Ms321 methyltransferase; Ms320 unknown                             |
| nifQ frag, msi332-331  | TTGGCACGACTTTTGGG     | Ms332 cytochrome P450 monoxygenase; Ms331 DUF1271 superfamily, possible ferredoxin |
| prxS-tpoN2 (msi334-msi335) | ATGGCACGCCCTTGGG     | Peroxiredoxin; Sigma 54 subunit of RNA polymerase                   |
| nifS-nifW (msi340-341) | TTGGCACCGTCTTGGG      | NifS cysteine desulphurase, iron-sulfur cluster synthesis; NifW nitrogenase-stabilizing/protective protein |
| fixB-ACX-nifA1 (msi342-346) | TTGGCACAGATGCT | Electron transport to nitrogenase; Nif-regulatory protein          |
| nifB-fdxN-nifN-fdxU-msi351 (msi347-351) | TTGGCATATCTCTTGGG | Nitrogenase synthesis; Ms351 conserved hypothetical, prokaryotic sirtuin-like family |
| ccrB (msi380)          | TTGGCACGACTTTTGGT     | Cytochrome c peroxidase                                             |

Results

NifA2 but not NifA1 is required for symbiotic nitrogen fixation although nifA1 encodes a functional protein

Previous work showed that *M. loti* nifA2 mutants form Fix− nodules [14,15] whereas nifA1 mutants are not symbiotically impaired [14]. For the current work, marker exchange deletion mutants JS01 (ΔnifA1::Δkan) and JS02 (ΔnifA2::Δkan) were constructed. As expected, JS01 formed Fix− nodules on *Lobus corniculatus* whereas JS02 was Fix+ (Table 2). Plasmid pJS100 that contained nifA2 and the preceding 626 bp cloned into vector pFAJ1700 (Table 3) complemented strain JS02 to Fix+, confirming that the Fix− phenotype of the nifA2 mutation was not due to a polar effect on downstream genes.

To determine whether nifA1 was expressed in nodules, an insertion-duplication (IDM) mutant with a transcriptional fusion between the 5′-end of the mutated gene and lacZ was constructed by integration through homologous recombination of the suicide vector pFUS2 containing a cloned internal fragment of the gene. Examination of expression of the lacZ fusion in JS03 (ΔnifA1::lacZ) revealed that nifA1 was expressed. However the same fusion was not expressed in a nifA2 mutant strain (Table 4), suggesting that nifA1 transcription initiated from the fixA promoter and not the region immediately upstream of nifA1. To determine if nifA1 encoded a functional protein, the region upstream of nifA2 spanning from the 3′ end of *msi360*, the gene that precedes nifA2, to the nifA2 start codon was joined to the fixA gene at the start codon by extension overlap PCR. The product was cloned into pFAJ1700 and the resulting plasmid pJS101 complemented the nifA2 mutant JS02 to a fully Fix+ phenotype. Taken together these results indicate that nifA1 is functional in nodules formed by R7A, but its expression is dependent on NifA2.

RpoN2 but not RpoN1 is required for symbiotic nitrogen fixation

To determine the roles of the two *M. loti* genes that each encode the sigma factor RpoN, an IDM mutant of *rpoN1* (strain JS04) and marker exchange deletion (R7AΔ*rpoN2::Δkan*) strain JS05A) and IDM (rpoN2::lacZ strain JS05B) mutants of *rpoN2* were constructed in the R7A background. When plated on RDM agar containing sucinate as carbon source, growth of the *rpoN1* mutant was severely reduced whereas the *rpoN2* mutants grew at the wild-type rate. The *rpoN1* mutant formed microcolonies on the plates after 7 days, presumably as a result of scavenging carbon sources present in the agar. Growth was restored by plasmid pJS102 that contains the *rpoN1* gene and the preceding 118 bp cloned into pFAJ1700. When assayed on *L. corniculatus*, the *rpoN1* mutant formed Fix+ nodules whereas the *rpoN2* mutants were Fix−. These results suggested that *rpoN2* was not expressed in free-living *M. loti* but was essential for symbiotic nitrogen fixation.

The *prxS* gene that encodes an atypical 2-Cys peroxiredoxin is located immediately upstream of the *rpoN2* gene and is preceded by a potential NifA-regulated promoter (Table 1). A *prxS* IDM mutant JS06A formed Fix− nodules. To ascertain if the Fix− phenotype of the *prxS* mutation was due to a polar effect on *rpoN2* expression, a *prxS* markerless in-frame deletion mutant JS06B was constructed. This strain formed Fix+ nodules. The 570-bp region preceding the *prxS* start codon was then amplified by PCR and fused to the *rpoN2* gene to give plasmid pJS103. This plasmid complemented both mutant strains JS06A and JS06B to a Fix+ phenotype, confirming that *prxS* was not required for an effective symbiosis and that *rpoN2* was expressed from the *prxS* promoter.

FixJK and RegSR are not required for symbiotic nitrogen fixation

In order to determine if genes known to mediate *nifA1* expression in other rhizobia were involved in regulating *nifA2* in *M. loti*, IDM mutants were constructed for the R7A fixK (mll6606 in MAF303099), fixJ (mll6578), regR (mll5308), and regS (mll5307) genes. The resulting mutants, strains JS08 to JS11, all formed Fix+ nodules. Double mutants JS13 (ΔregR fixK::lacZ and JS14 (ΔregR fixJ::lacZ) mutants were then constructed and also formed Fix+ nodules. Southern hybridization analysis carried out to confirm the mutants suggested that only a single copy of each of these genes was present in the R7A genome, as is the case for MAF303099 [5].

Taken together, the above results indicate that the regulation of *nifA1* expression in *M. loti* differs from that established for other rhizobial species examined to date. The results are most similar for those found with *R. etli* where no regulators of *nifA1* expression have yet been found.

*nifA2* expression is not autoregulatory

The intergenic region (ICEMSymR7A coordinates 436876−437433) between *msi360*, the gene upstream of *nifA2*, and *nifA2* comprises 536 bp (Fig. 1A, 1C). BlastN [26] searches carried out using this region as a query showed that it shared 70% nucleotide identity from bp 120-466 with another region conserved between the R7A and MAF303099 symbiosis islands (Fig. 1B). BlastX analysis showed the presence of two gene fragments spanning bp 182-465 (ICEMSymR7A coordinates 437058−437341) sharing highest similarity (approximately 45% amino-acid identity) with the N-terminal end of the *msi19* gene product that encodes a putative sugar epimerase (COG4130) (Fig. 1A, C).

To delineate the *nifA2* promoter region, a series of *nifA2-lacZ* nested promoter fusion strains were constructed using the suicide vector pFUS2. The pFUS2 clones were constructed using a series of nested PCR products amplified using a primer nifA2CMDR located within the 5′ end of *nifA2* and a series of 5 primers (*nifA2CMDL1-5) located at intervals between the 3′ end of *msi360* and the 5′ end of *nifA2* (Fig. 1A). Insertion of the plasmids into the genome created five cis-merodiploid (CMD) strains. In these strains the full intergenic region along with the 5′-end of the *nifA2* gene was fused to lacZ while the amplified promoter region was fused to an intact copy of *nifA2* downstream of the inserted plasmid.

The shortest clone that gave a Fix+ phenotype was JS15 that contained a 536-bp region preceding the *nifA2* start codon, whereas strains JS16 and JS17 that contained 426-bp and 293-bp regions preceding the start codon respectively were Fix− (Fig. 1A). This indicated that the *nifA2* promoter was located upstream of the gene fragments homologous to *msi19* located in the *msi360-nifA2* intergenic region. β-galactosidase assays carried out on bacteroids extracted from nodules of plants 14 days post-inoculation with strains JS15, JS16 and JS17 revealed no significant differences in expression measured from the intact *nifA2* promoter-lacZ fusion in the strains. The Fix− strain JS15 that contains the full-length promoter in front of both the *nifA2* gene and the *nifA2-lacZ* fusion gave 307.5±55.6 Miller Units. In comparison, the Fix+ strains JS16 and JS17 that contain the same *nifA2-lacZ* fusion but an inactive *nifA2* gene gave 304.8±263.2 and 337.2±103.9 Miller Units, respectively. These data showed that *nifA2* was not autoregulated, consistent with the absence of NifA and RpoN binding sites within the putative *nifA2* promoter region.
| Strain Background | Description* | R7A mutant Fix phenotypeb |
|-------------------|--------------|--------------------------|
| JS01              | ΔnifA1: Δkan, gene replacement deletion | + |
| JS02              | ΔnifA2: Δkan, gene replacement deletion | - |
| JS03              | nilA1::lacZ, pFUS2 IDM | + |
| JS04              | nifV1::lacZ, pFUS2 IDM | + |
| JS05              | ΔnifV2: Δkan, gene replacement deletion | - |
| JS06              | prnS::lacZ, pFUS2 IDM | - |
| JS07              | prnS::lacZ, pFUS2 CMD | + |
| JS08              | fix::lacZ, pFUS2 IDM | + |
| JS09              | fix::lacZ, pFUS2 IDM | + |
| JS10              | regR::lacZ, pFUS2 IDM | + |
| JS11              | regS::lacZ, pFUS2 IDM | + |
| JS12              | ΔregR, markerless deletion mutant | + |
| JS13              | JS12 fix::lacZ, pFUS2 IDM | + |
| JS14              | JS12 fix::lacZ, pFUS2 IDM | + |
| JS15              | JS119 nifA2::lacZ, pFUS2 CMD, 336 bp of nifA2 promoter preceding lacZ | + |
| JS16              | nifA2::lacZ, pFUS2 CMD, 426 bp of nifA2 promoter preceding lacZ | - |
| JS17              | nifA2::lacZ, pFUS2 CMD, 293 bp of nifA2 promoter preceding lacZ | - |
| JS18              | ΔfixV: Δkan, gene replacement deletion of msi360, renamed fixV in this work | - |
| JS19              | fix::lacZ, pFUS2 IDM | - |
| JS20              | SB01 nifA2::lacZ, pFUS2 CMD | - |
| JS21              | JS11 JS211 nifH::lacZ, pFUS2 IDM | - |
| JS22              | JS116 nifH::lacZ, pFUS2 IDM | - |
| JS23              | JS118 nifH::lacZ, pFUS2 IDM | - |
| JS24              | JS103 JS203 msi158::lacZ, pFUS2 IDM | P |
| JS25              | JS101 JS201 msi036::lacZ, pFUS2 IDM | + |
| JS26              | Δmsi036::Δkan msi158::lacZ, gene replacement deletion of msi036, pFUS2 IDM of msi158 | P |
| JS27              | Δ [msi262-263]: Δkan, gene replacement deletion of msi262-msi263 | P |
| JS28              | Δ [fdxN-fixU]: Δkan, gene replacement deletion of fdxN-nifZ-fixU | - |
| JS29              | Δ [nifZ-fixU]: Δkan, gene replacement | - |
| JS30              | ΔfixU: Δkan, gene replacement deletion | + |
| JS31              | msi31::lacZ, pFUS2 IDM | + |
| JS32              | JS120 JS220 ccpR::lacZ, pFUS2 IDM | + |
| JS33              | JS07 CCPR::lacZ, pFUS2 IDM | + |
| JS34              | JS102 JS202 msi071::lacZ, pFUS2 IDM | + |
| JS35              | JS124 JS224 msi083::lacZ, pFUS2 IDM | + |
| JS36              | JS123 JS223 metE::lacZ, pFUS2 IDM | + |
| JS37              | JS122 JS222 metE::lacZ, pFUS2 IDM | + |
| JS38              | JS126 JS226 pepM::lacZ, pFUS2 IDM | + |
| JS39              | JS104 JS204 msi260::lacZ, pFUS2 IDM | + |
| JS40              | JS105 JS205 msi262::lacZ, pFUS2 CMD | P |
| JS41              | JS106 JS206 acdS::lacZ, pFUS2 IDM | + |
| JS42              | JS128 JS228 aatA::lacZ, pFUS2 IDM | + |
| JS43              | JS127 JS227 aatB::lacZ, pFUS2 IDM | + |
| JS44              | JS125 JS225 exS::lacZ, pFUS2 IDM | + |
A novel regulatory protein FixV activates nifA2 expression

nifA2 is preceded by msi360 which encodes a regulator of the LacI/GalR family. BLAST searches revealed that the most closely related Msi360 orthologs (approx. 60–76% amino-acid identity) are found within other rhizobial species including Mesorhizobium ciceri, R. etli, R. leguminosarum and non-symbiotic Mesorhizobium strain CJ1. In many cases the genes encoding these regulators preceded genes encoding sugar epimerases homologous to Msi119. In several rhizobia, the msi360 homolog was also divergently transcribed from the mocD operon required for catabolism of the rhizopine L-3-O-methyl-scyllo-inosamine (Fig. 2). Mutants in msi360 were constructed by marker replacement (strain SB01; Δmsi360::Vkan) and insertion duplication (strain JS18; msi360::lacZ). The mutants were symbiotically defective and wet weights of

| Strain Background | Description* | R7A mutant Fix phenotypeb |
|-------------------|--------------|--------------------------|
| JS45 JS115 JS215  | nifQ::lacZ, pFUS2 IDM | P                        |
| JS46              | msi338::lacZ, pFUS2 CMD | +                       |
| JS47              | Δmsi337::Δkan, gene replacement deletion | P |
| JS48              | Δmsi338::Δkan, gene replacement deletion | P |
| JS49 JS108 JS208  | msi280::lacZ, pFUS2 IDM | +                       |
| JS50              | Δ[msi274-276]::Δkan, gene replacement deletion of msi274-msi275-msi276 | + |
| JS51 JS107 JS207  | msi276::lacZ, pFUS2 CMD | +                       |
| JS52 JS109 JS209  | msi321::lacZ, pFUS2 IDM | +                       |
| JS53 JS110 JS210  | msi332::lacZ, pFUS2 CMD | +                       |
| JS54 JS117 JS217  | fixQ::lacZ, pFUS2 CMD | +                       |

*IDM = insertion duplication mutant in which coding sequence disrupted; CMD = cis-merodiploid insertion mutant in which gene is not inactivated as mutant retains wild-type copy of gene including entire promoter region downstream of lacZ fusion (except for JS16 and JS17 in which promoter is truncated).

Symbiotic effectiveness of mutants determined by measuring the wet weights of 15 L. corniculatus seedlings at 6 weeks post-inoculation. Data were compared with those obtained for seedlings inoculated with the wild-type and uninoculated controls. + = Fix+ (fully effective); − = Fix− (ineffective); P = partially effective (see Table 5).

doi:10.1371/journal.pone.0053762.t002

Table 3. Plasmids used in this study.

| Plasmid       | Description                                      | Reference |
|---------------|--------------------------------------------------|-----------|
| pFAJ1700      | Broad-host-range IncP plasmid, TcR               | [69]      |
| pFAJ1708      | pFAJ1700 containing nptII promoter              | [69]      |
| pUF52         | oriC<sup>AB</sup> ori<sup>IMK</sup> lacZ transcriptional reporter; suicide vector, GmR | [62]      |
| pJ3200        | Broad-host-range IncP plasmid, TcR               | [70]      |
| pPH1J1        | IncP plasmid, GmR                               | [71]      |
| pQ200SK       | Suicide vector containing sacB gene, GmR        | [65]      |
| pJS100        | pFAJ1700 containing nifA2 and preceding 626 bp  | This study|
| pJS101        | pFAJ1700 containing the 626 bp that precedes nifA2 fused at the start codon to the complete nifA1 gene | This study|
| pJS102        | pFAJ1700 containing rpoN1 and preceding 118 bp  | This study|
| pJS103        | pFAJ1700 containing the 570 bp that precedes prxS fused at the start codon to the complete rpoN2 gene | This study|
| pJS104        | pFAJ1700 containing fixV and preceding 295 bp   | This study|
| pJS105        | pFAJ1700 containing msi158 and preceding 392 bp | This study|
| pJS106        | pFAJ1700 containing msi262 and preceding 739 bp | This study|
| pJS107        | pFAJ1700 containing 279 bp upstream of nifB, with in-frame deletion of nifB and complete fdxN, nifZ and fixU genes | This study|
| pJS108        | pFAJ1700 containing the nifB promoter region, with the 5’ end of nifB fused in-frame to the 3’ end of fdxN, and complete nifZ and fixU genes. | This study|
| pJS109        | pFAJ1700 containing the nifB promoter region, with the 5’ end of nifB fused in-frame to the 3’ end of nifZ, and complete fixU gene. | This study|
| pJS110        | pFAJ1700 containing nifQ cloned behind nptII promoter | This study|

doi:10.1371/journal.pone.0053762.t003
Table 4. Symbiotic expression of various genes in wild-type, ΔnifA1 and ΔnifA2 backgrounds.

| Gene fusion       | *β-galactosidase activity (Miller units) in: |
|-------------------|-------------------------------------------|
|                   | R7A | JS01 (ΔnifA1) | JS02 (ΔnifA2) |
| No lacZ fusion    | 6.4±1.5 | 5.0±0.7 | 10.0±3.2 |
| nifA+ genes       | 297.9±191.2 | ND | 10.6±1.6* |
| nifA2CMD::lacZ    | 307.5±96.3 | 293.1±134.2 | ND |
| Genes with NifA/RpoN promoters | | |
| nifD::lacZ        | 369.8±167.7 | 547.2±111.9 | 7.5±4.4** |
| nifB::lacZ        | 347.7±70.6 | 268.8±73.3 | 7.6±1.8*** |
| nifQ::lacZ        | 527.8±145.4 | 355.3±74.8 | 7.8±2.4** |
| msi036::lacZ      | 275.8±39.9 | 75.6±16.6* | 4.3±1.6** |
| msi071::lacZ      | 618.4±59.6 | 646.0±157.9 | 6.3±2.2* |
| msi158::lacZ      | 1281.7±251.8 | 1150.0±211.1 | 5.5±9.9*** |
| msi360::lacZ      | 1339.3±72.5 | 685.9±374.5 | 5.1±2.1*** |
| msi262::lacZ      | 613.2±120.7 | 442.5±139.3 | 7.4±1.3** |
| msi276CMD::lacZ   | 552.9±108.0 | 301.7±106.8 | 14.2±7.8** |
| msi520::lacZ      | 371.6±125.7 | 268.9±101.4 | 7.3±1.3** |
| msi521::lacZ      | 319.8±84.7 | 205.4±63.8 | 11.7±2.1** |
| msi332::lacZ      | 446.6±71.4 | 253.7±89.9 | 14.6±10.3** |
| msi360::lacZ      | 684.9±79.7 | 535.7±89.8 | 28.6±23.8*** |
| nifH::lacZ        | 854.8±126.1 | 610.6±86.6 | 9.4±7.2*** |
| nifS::lacZ        | 492.5±144.3 | 413.0±125.7 | 5.9±3.7* |
| prx5::lacZ        | 204.1±31.1 | 188.8±36.1 | 17.5±12.9** |
| prxS::CMD::lacZ   | 779.8±183.1 | 707.3±174.8 | 6.1±3.4** |
| rpoN2::lacZ       | 138.7±50.8 | 118.0±25.1 | 19.4±2.0** |
| Genes without NifA/RpoN promoters | | |
| nifD::lacZ        | 2455.8±110.6 | 2289.8±314.0 | 16.2±7.4*** |
| nifB::lacZ        | 861.7±107.8 | 809.2±174.9 | 22.0±6.7*** |
| nifQ::lacZ        | 495.5±223.2 | 524.0±166.5 | 8.7±0.7** |
| msi276::lacZ      | 223.6±91.9 | 171.5±43.4 | 28.1±17.3* |
| msi520::lacZ      | 730.2±227.2 | 467.1±157.2 | 11.1±7.3*** |
| msi521::lacZ      | 280.0±85.4 | 259.6±117.0 | 217.8±98.9 |
| msi521::lacZ      | 206.0±112.2 | 149.3±108.3 | 9.7±10.0** |
| pepM::lacZ        | 827.1±107.4 | 625.1±168.8 | 7.7±2.0*** |
| rpoN1::lacZ       | 29.7±6.0 | 25.1±7.2 | 24.9±8.5 |

*β-galactosidase assays were performed on bacteroid suspensions from nodules harvested 14 days post-inoculation. All activity values are the average of at least two assays ± Standard Deviation. Significant differences in expression observed between R7A and R7AΔnifA1: Δkan ** = P<0.005, between R7A and R7AΔnifA2: Δkan ** = P<0.005, between R7AΔnifA1: Δkan and R7AΔnifA1::Δikan Δkan and R7AΔnifA1::Δikan Δikan P<0.005 (as determined by unpaired t test). doi:10.1371/journal.pone.0033762.t004

To examine nifA2 expression in the msi360 mutant background, strain JS19 (Δmsi360: Δkan nifA2::lacZ::CMD) was constructed. Expression of nifA2 in 2-week-old nodules formed by this strain was largely abolished (13.1±6.6 Miller Units compared to 307.5±53.6 Miller Units for strain JS15 (nifA2::lacZ::CMD)), indicating that Msi360 either directly or indirectly activates nifA2 expression. We also introduced a fixA::lacZ::CMD fusion into SB01, creating JS20 (Δmsi360: Δkan fixA::lacZ::CMD). Analysis of lacZ expression in bacteroids from nodules formed by this strain showed that fixA expression was abolished in the msi360 mutant background (9.6±7.6 Miller Units compared to 527.8±145.4 Miller Units for strain JS54 (R7A fixA::lacZ::CMD), consistent with a lack of nifA2 expression. These results led us to rename msi360 as fixV in recognition of its contribution to the regulation of nifA2 expression.

Expression analysis of genes preceded by NifA-regulated promoters

Expression studies were carried out to confirm that the 15 putative nifA-regulated promoters present on ICEMSymR7A that preceded intact genes were subject to NifA-mediated regulation, and to determine whether NifA1 influenced expression from any of these promoters. In most cases, IDM mutants of the first gene downstream of each promoter were constructed using the suicide vector pFUS2 in the wild-type (R7A), ΔnifA1 (JS01) and ΔnifA2 (JS02) strain backgrounds, although in a few cases CMD recombinant strains that did not inactivate the gene were also constructed. The resultant strains contained transcriptional fusions of the mutated gene to lacZ, enabling both the symbiotic phenotype and the expression of the gene to be determined. Mutants in the JS01 background were designated JS101 through to JS128, and those in the JS02 background JS201 through to JS228 (Table 2).

All putative NifA-regulated genes examined were strongly expressed in bacteroids harvested from nodules at two weeks post-inoculation in both the R7A and JS01 backgrounds and expression was abolished in the JS02 background, indicating nifA2 was an absolute requirement for their expression under symbiotic conditions (Table 4). In most cases, expression of the fusion in the JS01 background was less than in the R7A background, but with the exception of msi036::lacZ the differences were not statistically significant.

Symbiotic phenotypes of NifA-regulated genes

All IDM and CMD mutant strains were assessed for nitrogen-fixing ability on L. cornutus to determine whether the mutated gene had a symbiotic role, and to confirm that the CMD strains remained Fix−. Visual observations of plant growth and colour were made and wet weights were measured at six weeks post-inoculation. The fixation phenotypes of all recombinant strains are summarised in Table 2. Only strains JS02, JS05A, JS05B, JS06A, JS06B, JS21, JS22, JS23, JS28, and JS29 containing mutations within nifA2, rpoN2, prxS, nifH, nifB, fdxN and nifY respectively were completely Fix− (Table 2), producing nodulated seedlings that were otherwise indistinguishable from uninoculated seedlings that displayed severe signs of nitrogen deficiency. However a strain carrying a mutation in msi158 (JS24), a marker exchange mutant in which msi262 and msi263 were deleted (JS27), and JS30, a marker exchange fixU mutant, were partially effective, as plants inoculated with these strains showed growth intermediate between fully Fix− and Fix+ (Tables 2 and 5). The other mutants and all CMD strains tested (with the exception of the nifA2::pFUS2 CMD strains JS16 and JS17, see above) formed fully effective nodules (Table 2).
msi158 encodes an outer membrane porin with strong similarity to members of the ompW family (COG3047). While only moderate differences in wet weights were observed between seedlings inoculated with the wild-type and strain JS24 (msi158::lacZ) (Table 5), plants infected with the mutant were pale yellow-green in appearance at six weeks post-inoculation in comparison to the wild-type. The plasmid pJS105, containing msi158 and the preceding 392 bp, restored JS24 to a wild-type phenotype.

C. BlastX output showing amino-acid similarity between the two fragments in the fixV-nifA2 intergenic region and Msi109.

\[ \text{doi:10.1371/journal.pone.0053762.g001} \]
functionally redundant, a double mutant JS26 (\(\text{msi036}::\text{Vkan}\ msi158::\text{lacZ}\)) was constructed and showed a symbiotic phenotype indistinguishable from that observed for the \(\text{msi158}\) mutant.

The \(\text{msi262}\) (\(\text{iscN}; \text{COG0316}\)) and \(\text{msi263}\) (\(\text{iscU}; \text{COG0822}\)) genes were deleted by marker exchange, producing the double mutant JS27 (\(\text{D}[\text{msi262-msi263}]::\text{Vkan}\)). The mutant showed a partial defect in nitrogen fixation (Table 5). The plasmid \(\text{pJS106}\), which contained only \(\text{msi262}\) and the preceding 739-bp non-coding region, restored JS27 to the wild-type symbiotic phenotype, indicating that only \(\text{msi262}\) was required for a fully effective symbiosis.

fdxN, nifZ, fixU, and \(\text{msi351}\) are located within the \(\text{nifB-fdxN-nifZ-fixU-msi351}\) cluster. To determine whether these genes have a symbiotic role, three marker exchange mutants, designated JS28

**Figure 2. Comparison of the genetic organization of gene clusters associated with \(\text{fixV}\) homologs in a range of rhizobial species.**

Genes are shown as arrow symbols and are to scale; colours specific for each gene are used to indicate genes that encode similar proteins in other clusters. Black indicates genes lacking homology to any other genes within the clusters. Fr notes gene fragment, IS denotes insertion sequence. doi:10.1371/journal.pone.0053762.g002

**Table 5.** Symbiotic properties of partially effective and ineffective mutants.

| Inoculum strain | Genotype | Mean wet foliage weight in mg/\(\pm\)SD | % effectiveness based on wet weight | Acetylene reduction $b$ |
|-----------------|----------|----------------------------------------|------------------------------------|--------------------------|
| none            |          | 19.1$\pm$ 3.0**                      | 22.2                               | 0                        |
| R7A             | Wild-type| 86.0$\pm$ 32.2                        | 100                                | 100$\pm$ 35              |
| JS24            | \(\text{msi158::lacZ}\) | 59.2$\pm$ 9.8$\times$2         | 68.8                               | 51.5$\pm$ 18.2$\times$2  |
| JS27            | \(\Delta [\text{msi262-msi263}]::\text{Vkan}\) | 64.2$\pm$ 22.7                      | 74.6                               | 72.6$\pm$ 33.5           |
| JS42            | \(\text{nifQ::lacZ}\) | 61.9$\pm$ 12$\times$2          | 71.9                               | 64.0$\pm$ 15.4$\times$2  |
| JS28            | \(\Delta [\text{fdxN-fixU}]::\text{Vkan}\) | 17.4$\pm$ 11.6$\times$2        | 20.3                               | 0                        |
| JS29            | \(\Delta [\text{nifZ-fixU}]::\text{Vkan}\) | 23.6$\pm$ 8.3$\times$2         | 27.4                               | 0                        |
| JS30            | \(\Delta \text{fixU}::\text{Vkan}\) | 67.5$\pm$ 21.7                      | 78.4                               | 103$\pm$ 22.2            |

$^a$Mean wet foliage weight of 30 plants $\pm$ Standard Deviation. Data were analysed using the Students T-test. $^b$Percentage of acetylene reduction relative to R7A. Nitrogen fixation activity was measured as the amount of C$_2$H$_2$ reduced (\(\mu\text{mol h}^{-1}\)) per plant root for 10 plants $\pm$ standard deviation. Data were analysed using the Students T-test. A single asterisk represents $P<0.05$ and two asterisks $P<0.005$ when compared to R7A.

doi:10.1371/journal.pone.0053762.t005
Expression and symbiotic phenotypes of genes not preceded by NifA-regulated promoters

In addition to the NifA-regulated operons, the expression of a selection of ICEAM/SymR7A genes encoding metabolic functions and an ABC transporter was examined. The genes were chosen because preliminary screening of lacZ expression from IDM mutants showed the genes were expressed at higher levels in 14-day-old nodules than in G/RDM broth cultures (β-galactosidase activity of less than 20 Miller Units in broth culture for all mutants). This suggested these genes may be subject to symbiosis-specific regulation. The genes selected included msi083, which encodes the beta subunit of transketolase, metE (msi160; 5-methyltetrahydropteroylglutamylhomocysteine methyltransferase), metK (msi166; S-adenosylmethionine synthetase) pepM (msi327; phosphoenolpyruvate phosphomutase), msi260 (putative diaminobutyrate-2-oxoglutarate transaminase), aatA (msi326; aspartate aminotransferase), asnB (msi323; asparagine synthetase) and exsA (msi339). exsA encodes a MsbA-like saccharide-exporting ABC transporter similar to S. meliloti ExsA (71% amino acid identity) that is involved in the export of the exopolysaccharide succinoglycan [27]. The nifQ gene (msi336) was also selected. nifQ is located with the msi337-nsi337-nifQ cluster. Homologs of msi337 (dfxB) and msi338 are associated with nif and fix gene clusters preceded by NifA-regulated promoters in some diazotrophs, but a NifA-regulated promoter does not precede the ICEAM/SymR7A cluster. The IDM mutants of msi083, metE, pepM, msi260, aatA, asnB, exsA and nifQ were designated JS33, JS36, JS37, JS38, JS39, JS42, JS44 and JS45 respectively. msi337 and msi338 were inactivated by marker exchange mutagenesis producing strains JS47 and JS48.

With the exception of the nifQ (msi339), msi337 and msi338 mutants which formed partially effective nodules compared to R7A (Table 5), all of the mutants formed a fully effective symbiosis. The nifQ gene was amplified by PCR and cloned adjacent to the nptII promoter in pFAJ1708 producing plasmid pJS110. This plasmid complemented JS45, JS47 and JS48 to a fully Fix+ phenotype, indicating that nifQ was the only gene required within the msii337-nsii337-nifQ cluster for an effective symbiosis.

β-galactosidase assays performed on extracts from 14-day-old root nodules formed by IDM mutants revealed that, with the exception of msi083, all of the genes were poorly expressed in the msi42 mutant background, but were strongly expressed in the wild-type and nifA1 mutant backgrounds. msi083 was strongly expressed in all three backgrounds (Table 4).

Discussion

Our results show that the regulators FixJ, FixK and RegR that initiate symbiotic gene expression in other rhizobia are not required for symbiotic nitrogen fixation in M. loti. Instead M. loti has evolved a different mechanism for the activation of nifA expression. Although nifA1 encodes a functional NifA protein and is in an identical genomic context to the sole nifA gene in several other rhizobial species, it is under the regulation of the product of the second nifA gene, nifA2. The nifA2 gene in turn is under...
the regulation of a novel regulator FixV that is a member of the LacI/GalR family. A model for the regulatory network governing symbiotic nitrogen fixation in *M. loti* is shown in Fig. 3.

The *fixV* gene is located upstream of *psx2* and the 530-bp intergenic region was found to contain gene fragments homologous to an ICE*E*Sym*R* strain *msi119* that encodes a sugar epimerase. Homology was detectable at both the nucleotide and amino acid levels. Analysis of a series of mutants with a nested set of promoter deletions showed that sequences required for *psx2* expression were located downstream of *fixV* but upstream of the gene fragments. Bioinformatic analysis revealed that homologs of *msi119* were located downstream of *fixV* homology in *M. ciceri*, *R. etli*, *R. leguminosarum* and *Mesorhizobium* sp. strain CJ1 (Fig. 2). Taken together, these results suggest that expression of *psx2* was placed under FixV control by a translational event involving *fixV* and a downstream promoter that it stimulates. Furthermore it seems likely that FixV responds to a carbohydrate signal to initiate *psx2* transcription. The LacI/GalR family of transcriptional regulators consists of an N-terminal helix-turn-helix DNA-binding domain and a C-terminal ligand-binding domain that is structurally homologous to periplasmic sugar-binding proteins [28,29,30]. While most family members are repressors, a few members are activators. The most closely related ortholog outside of the analogous copy on the *M. ciceri* and *R. etli* *msi119* intergenic region was found to contain gene fragments homologous to *psx2* under symbiotic conditions [31]. None of these genes corresponded to those found to be down-regulated in *Lotus* nodules in the current study.
In common with studies of other rhizobia, we observed that many genes that were strongly expressed in nodules did not produce an overt symbiotic phenotype when mutated. However, of the genes not previously shown to be required for symbiosis, msi158 and nifZ that are regulated by NifA and nifQ that lacks a NifA-regulated promoter were found to be required for a fully effective symbiosis. The msi158 mutant formed partially effective nodules and the plants were yellowish, indicating nitrogen deficiency. The msi158 gene encodes an outer membrane protein of the OmpW family (COG3047) that shares strong similarity with the gene products of yAMB present on pNGR234a of Rhizobium sp. NGR234 and bll1766 from the symbiotic region of B. japonicum. NifA-regulated promoters are also located upstream of these two orthologs [5,11,37]. A bll1766 mutant formed normal nitrogen-fixing nodules on soybean [37]. However, a strongly conserved bll1766 ortholog (bl1311) is located elsewhere on the B. japonicum USDA110 genome [38]. No orthologs are present in the S. meliloti 1021, R. leguminosarum bv. viciae strain 3841, R. leguminosarum bv. trifolii WSM1325, or R. etli CFE42 genomes, or in M. loti MAFF303099 outside of the symbiosis island [3,39,40,41,42,43,44]. The E. coli OmpW protein forms an eight-stranded β-barrel with a hydrophilic channel and may be involved in the transport of small hydrophilic molecules across the bacterial outer membrane [45]. In Salsola entérica serovar Typhimurium, the ompW gene is part of the SoxRS regulon that protects against oxidative stress and it has been suggested that the porin functions as an efflux channel for toxic compounds generated during oxidative stress [46]. A similar role in M. loti would make msi158 the third member of the NifA-RpoN regulon together with ccpS and cprR likely involved in protection against reactive oxygen species.

The different rhizobial species vary considerably in the complement of nif genes that they share with the paradigm nitrogen-fixing microorganism, the free-living diazotroph Klebsiella pneumoniae, and it is apparent that the nitrogenase assembly machinery is to an extent species-specific in rhizobia (reviewed in [47]). For example, the msiB-fdxN-nifZ-fixU-msi351 cluster found in M. loti is present to varying extents in other rhizobia: it is complete in R. etli CFE42 and M. ciceri bv. biserrulae WSM1271, missing msi351 in Rhizobium sp. strain NGR234, missing nifZ and msi351 in S. meliloti 1021 and Bradyrhizobium species ORS278 and BTA11 (although nifZ is located elsewhere in the latter two species), while in R. leguminosarum only nifB is present [5,38,39,40,41,42,43,44,48,49]. We showed that mutations within the ICEMISymR7A and fdxN genes abolished nitrogen fixation. The nifZ gene is found in several diazotrophs and is involved in maturation of the Mo-Fe protein [50]. The FdxN protein is thought to serve in the pathway of electron transfer to nitrogenase. In S. meliloti mutations within fdxN also completely abolish nitrogen fixation [51]. The function of fixU (also called nifT in some diazotrophs) is unknown and inactivation of nifT in E. pneumoniae has no obvious effects on nitrogen fixation [52,53]. Our results show that active FixU is required for optimal N fixation in M. loti under the growth conditions used.

The msi338-msi337-nifQ gene cluster is not preceded by a NifA-regulated promoter although a NifA-regulated promoter is present upstream of a nifQ fragment that precedes msi329 on ICEMISymR7A. Homologs of msi337 (fdxB) and msi338 are located within or adjacent to nif and fix clusters preceded by NifA-regulated promoters in S. meliloti, R. etli and B. japonicum. R. leguminosarum possesses fdxB but not msi338 while nifQ is absent from S. meliloti and R. leguminosarum [38,39,40,41,42,43,44]. Homologs of Msi338 are also encoded within nitrogen fixation gene clusters of a wide range of bacteria [34]. NifQ participates as a molybdenum donor for FeMoCo biosynthesis [55]. Our results showed that nifQ was required for a fully effective symbiosis, in contrast to the situation in Rhizobium sp. strain NGR234 where mutation of nifQ had no effect on symbiotic nitrogen fixation [56]. The lack of a symbiotic defect in msi337 and msi338 mutants may reflect functional redundancy as probable orthologs of these genes are present in the msi276-msi275-msi274 gene cluster that is preceded by a NifA-regulated promoter. Consistent with this, a mutant strain JS00 (Δ [msi274-msi276]; OLNan) in which all these genes were deleted formed Fix+ nodules.

The msi262 and msi263 genes were renamed isN and isU respectively and are likely involved in the production of iron-sulfur clusters for nitrogenase. Msi262 shows 71% identity to the R. etli isN gene product that is thought to act as a scaffold protein for Fe-S biosynthesis. Mutants of R. etli defective in isN showed a 90% reduction in nitrogen fixation [57]. Msi263 is a member of the IscU protein family (COG0822). These proteins are similar to the N-terminal region of NifU and are also thought to play a scaffolding role in Fe-S cluster formation. As suggested for R. etli, it seems likely that the IscN and IscU homologs are partially functionally redundant. However the iscN-iscU double mutant was partially effective, suggesting that M. loti may harbor additional genes that can at least partially complement their function.

In summary, a novel regulator FixV together with NifA2 were identified as key regulators of genes required for nodule function in M. loti, with FixV activating nifE2 expression possibly in response to a plant-produced inositol derivative (Fig. 3). Many genes encoded on ICEMISymR7A were strongly expressed in nodules in a NifA2-dependent manner but not free-living rhizobia. Nevertheless most of these genes were not required for symbiotic nitrogen fixation. It seems likely that some of these genes have functional homologues elsewhere in the genome and that bacteroid metabolism may be sufficiently plastic to adapt to loss of various enzymatic functions.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The wild-type M. loti strain used in this study was R7A, a field reisolate of ICMP 3153 (NZP2238) [2]. Mutant strains constructed in the R7A, JS01 (R7AΔnafA1) and JS02 (R7AΔnafA2) backgrounds are described in Table 2. Plasmids are listed in Table 3. M. loti strains were grown at 28°C in TY [58] or in rhizobium defined medium with 10 mM glucose (S/RDM) or 10 mM succinate (S/RDM) as previously described [59]. Escherichia coli strain S17-1 [60] was used for cloning and as the donor for biparental matings. It was cultured in LB or TY medium. Antibiotics were used at the following concentrations: for E. coli, tetracycline 15 μg mL⁻¹, kanamycin 50 μg mL⁻¹ and gentamicin 25 μg mL⁻¹; and for M. loti tetracycline 2 μg mL⁻¹, neomycin 200 μg mL⁻¹, and gentamicin 50 μg mL⁻¹.

DNA manipulations

Plasmid DNA preparations DNA cloning, transformation of DNA into E. coli and Southern hybridisations were carried out using established techniques [61]. Genomic DNA was extracted as described previously [2]. PCR was performed using an Expand HiFi PCR kit (Roche).

Construction of mutants and lacZ promoter fusions

Insertion duplication mutants (IDM) and cis-merodiploid (CMD) lacZ fusions were constructed using the suicide vector.
pFUS2 [62]. Oligonucleotide primer pairs incorporating restriction sites were used to amplify 350–500 bp regions which comprised either intragenic regions of the target genes to create IMD mutants or the promoter region and 5′ end to create strains containing promoter-lacZ fusions, leaving the associated gene and its promoter region intact. PCR products were then cloned into pFUS2 adjacent to its promoterless lacZ gene and confirmed by sequencing using a lacZ-specific primer. pFUS2 constructs were transferred into M. loti by conjugation from E. coli strain S17-1 donors as described [7] and transconjugants were passaged three times on selective media and then confirmed by Southern hybridization.

Marker exchange mutants were constructed by replacing the gene of interest with the Q Kan interposon [63]. Oligonucleotide primer pairs were designed to amplify 1-kb regions that flanked the target gene and they contained restriction enzyme sites to facilitate cloning. The PCR products were digested with appropriate enzymes and ligated into pIJ3200 along with the Q Kan interposon from pHPl5Q Kan [63]. The resulting plasmid was confirmed by DNA sequencing and transferred into R7A by conjugation. Recombination was then forced by plasmid incompatibility using pPH11J [64] and the mutant confirmed by Southern hybridization. pPH11J was then removed from the strain by introducing pLAFR1 and an isolate that had lost pLAFR1 was selected as described previously [7].

Markerless deletion mutants of M. loti were constructed using the suicide vector pJQ200SK [65]. One-kilobase regions that flanked the gene were amplified by PCR using primers that included restriction endonuclease sites for cloning. The PCR products were digested and ligated into pJQ200SK. Clones were confirmed by DNA sequencing and then transferred to R7A by conjugation, followed by selection for gentamicin-resistant clones. Integration at the correct site was confirmed by Southern hybridization. Loss of sucrose sensitivity, caused by loss of the lacZ-encoded B-galactosidase activity, was confirmed by Plant assays.

Plant studies were performed using L. corniculatus cv. Goldie as previously described [66]. Surface-sterilized seeds were germinated on 0.8% water agar. Seedlings were planted on Jensen’s agar slopes in glass test-tubes. For testing mutants for symbiotic effectiveness, 15 plants were inoculated by addition of 100 μl of a cell suspension containing approximately 10^6 cells. Seedlings were cultivated under environmental conditions of 70% humidity, 25°C, 16 h light, 14°C, 8 h dark. Plants were harvested at six weeks post-inoculation and the effectiveness of the symbiosis determined by visual inspection and by measuring the wet weight of foliage above the first cotyledonary node [66]. Nitrogenase assays were performed on nodulated roots harvested from 15 seedlings as described previously [67]. B-galactosidase assays were performed on bacteroid suspensions as previously described [69] using bacteroid suspensions prepared from nodules harvested 14 days post-inoculation from six plants per inoculum.

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Acknowledgments

We thank Rochelle Enright, Natalie Schlegel, Htin Aung, Kien Ly, Regan Murney, Patsarin Rodthong, Fiona Clow, Damien Fleetwood and Duncan Lasz for their contributions to mutant construction and analysis, and nitrogense assays.

Author Contributions

Conceived and designed the experiments: JTS SDB CWR. Performed the experiments: JTS SDB. Analyzed the data: JTS SDB. Wrote the paper: CWR JTS.
Nitrogen Fixation Gene Expression in M. loti

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