Expanding the regulatory network that controls nitrogen fixation in *Sinorhizobium meliloti*: elucidating the role of the two-component system hFixL-FxkR

Alma Reyes-González,1,2 Chouhra Talbi,1 Susana Rodríguez,1 Patricia Rivera,1 David Zamorano-Sánchez3 and Lourdes Girard1

1Programa de Dinámica Genómica, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico
2Instituto de Investigaciones Básicas y Aplicadas, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, Mexico
3Department of Microbiology and Environmental Toxicology, University of California, Santa Cruz, USA

In *Sinorhizobium meliloti*, nitrogen fixation is regulated in response to oxygen concentration through the FixL-FixJ two-component system (TCS). Besides this conserved TCS, the field isolate SM11 also encodes the hFixL-FxkR TCS, which is responsible for the microoxic response in *Rhizobium etli*. Through genetic and physiological assays, we evaluated the role of the hFixL-FxkR TCS in *S. meliloti* SM11. Our results revealed that this regulatory system activates the expression of a *fixK* orthologue (*fixKα*), in response to low oxygen concentration. Null mutations in either hFixL or FxkR promote upregulation of *fixK1*, a direct target of FixJ. Furthermore, the absence of this TCS translates into higher nitrogen fixation values as well as higher expression of *fixN1* in nodules. Individual mutations in each of the *fixK*-like regulators encoded in the *S. meliloti* SM11 genome do not completely restrict *fixN1* or *fixN2* expression, pointing towards redundancy among these regulators. Both copies of *fixN* are necessary to achieve optimal levels of nitrogen fixation. This work provides evidence that the hFixL-FxkR TCS is activated in response to low oxygen concentration in *S. meliloti* SM11 and that it negatively regulates the expression of *fixK1*, *fixN1* and nitrogen fixation.

INTRODUCTION

*Rhizobium* and *Sinorhizobium* are part of the select group of micro-organisms able to symbiotically fix nitrogen in association with legumes. The establishment of an effective association between rhizobia and legumes requires the coordinated expression of many bacterial and plant genes. Many regulatory circuits operate inside the nodule to accomplish both bacterial differentiation into nitrogen-fixing bacteroids and the expression of *nif* and *fix* genes. Due to the sensitivity of nitrogenase to oxygen, low oxygen conditions are required for nitrogen fixation to take place. In consequence, low oxygen concentration is the most important regulatory signal for *nif* and *fix* gene regulation. The requirement for a low oxygen environment and the demand for respiration to produce ATP seems paradoxical in nitrogen fixing bacteria (Dixon & Kahn, 2004; Fischer, 1994; Udvardi & Poole, 2013). Leghaemoglobin, a plant-produced oxygen-carrying nodulin (Downie, 2005), and the bacterial high oxygen affinity *cbb*3-type oxidase (Preisig et al., 1993; Mandon et al., 1994) make possible the metabolic adjustment necessary for optimal oxygen consumption under this condition. To regulate the expression of proteins specifically required for microaerobic metabolism, rhizobia use the detector–effector system described below.

In most rhizobia, the FixL-FixJ two-component system (TCS) regulates the expression of *fix* genes (Dixon & Kahn, 2004; Fischer, 1994; Udvardi & Poole, 2013). In *Sinorhizobium meliloti* 1021, *fixA* and *fixD* are encoded in an operon within the symbiotic plasmid *pSymA*. *fixD* encodes a canonical histidine kinase that anchors to the cell membrane. The autophosphorylation and phosphatase activity of FixD are regulated by the concentration of oxygen and by the activity of the anti-kinase regulator FixT (Lois et al., 1993; Foussard...
et al., 1997). FixL senses oxygen through a haem moiety bound to a conserved PAS domain (Gong et al., 1998). When oxygen concentration is low, the phosphate group in FixL can be transferred to the response regulator FixJ. In this condition, Fix-P is then responsible for the activation of nifA and fixK (David et al., 1988; Batut et al., 1989). NifA and FixK are master regulators of nitrogen fixation genes. NifA activates expression of the structural genes encoding the nitrogenase. FixK induces the operon fixNOQP that encodes the cbuB cytochrome oxidase. In other rhizobia such as Bradyrhizobium japonicum the regulation of nifA and fixK is split in two separate pathways, the RegS-RegR TCS activates nifA, and the FixL-FixJ TCS is responsible for the activation of fixK (Dixon & Kahn, 2004). Interestingly, not all rhizobia employ the FixL-FixJ TCS to activate expression of fixK and the FixK regulator. In Rhizobium etli CFN42 and Rhizobium leguminosarum bv. viciae VF39, FixL is an orphan hybrid histidine kinase (hFixL) without predicted transmembrane segments. This gene is located on plasmid pCFN42f in a region that contains copies of the fixK, fixNOQP and fixG genes, which are reiterated in the symbiotic plasmid (Patschkowski et al., 1996; Girard et al., 2000). R. etli hFixL initiates the regulatory circuit of fix genes in coordination with FxkR, a novel response regulator that belongs to the OmpR/PhoB family, and without the participation of FixJ (Zamorano-Sánchez et al., 2012). In R. etli CFN42, FxkR directly activates fixK expression in response to low oxygen concentration by binding to a specific sequence, the K-box (GTTCACA-N6-GTTCACA), located in the regulatory region of fixK. The hFixL protein of R. etli CFN42 contains, in addition to the haem-binding N-terminal PAS domain, a second PAS haem-less domain and a C-terminal receiver domain (Girard et al., 2000; Sousa et al., 2013). Biochemical studies indicate that this protein possesses very low O2 affinity. Mutants without the haem-less PAS or the putative phosphorylation site (Asp) in the C-terminal receiver domain increased the O2 affinity of the protein (Sousa et al., 2013). The presence of this TCS does not seem to be restricted to R. etli and R. leguminosarum. In a previous bioinformatic analysis, hFixL and FxkR-related proteins encoded in close proximity to a fixK-like gene were found in several alpha-proteobacteria, including S. meliloti (Zamorano-Sánchez et al., 2012). In the S. meliloti RM1021 reference genome, hfxL (Sma1142), fixK (Sma1138) and a fixK-like gene (Sma1141) are located on pSymA (Galbert et al., 2001). However, the fxeK gene has a frameshift mutation that generates a truncated non-functional response regulator. This frame shift mutation is absent in the genome of S. meliloti SM11. S. meliloti SM11 belongs to the dominant indigenous strains isolated from alfalfa nodules during a long-term field release experiment, with bioluminescent S. meliloti strain 2011 performed in Germany (Selbitschka et al., 2006). Its genome consists of a chromosome, two megaplasmids (pSmeSM11c and pSmeSM11d) and two smaller plasmids (pSmeSM11a and pSmeSM11b) (Schneiker-Bekel et al., 2011). The gene content of the SM11 chromosome and pSmeSM11d are very similar to the chromosome and pSymB plasmid of Rm1021, respectively. Although the megaplasmid pSmeSM11c and the symbiotic plasmid pSymA of Rm1021 share 55% of their CDSs, including syntenic regions where the nod, nif and fix genes are located, pSmeSM11c also possesses additional gene clusters not present on pSymA (Stiens et al., 2006, 2007; Schneiker-Bekel et al., 2011). pSmeSM11a and pSmeSM11b are part of the group called accessory plasmids that are present in many S. meliloti field isolates. A 42 kb region of pSmeSM11a (orf107 to orf149) that is homologous to pSymA (Sma1076 to Sma1169) contains the genes encoding hFixL, FxkR and FixK proteins (pSmeSM11ap135, 133 and 134, respectively) (Stiens et al., 2006, 2007; Zamorano-Sánchez et al., 2012). We refer to the FixK homologue of S. meliloti SM11 as FixKa because it is encoded in plasmid pSmeSM11a.

S. meliloti SM11 encodes both the FixL-FixJ-FixK regulatory system that controls expression of the fixNOQP and fixGHIS operons, and the hFixL-FxkR-FixKa proteins (see Fig. S1, available in the online Supplementary Material). The involvement of this secondary regulatory cascade in the microoxic response remains to be tested. This regulatory organization represents an excellent model to analyse how signaling pathways have evolved by adapting different elements from a ‘core’ system to fulfill the needs of a specific organism. By a genetic and functional characterization of the low oxygen-sensing regulatory module composed of hFixL and FxkR in S. meliloti SM11, we clearly demonstrate that both proteins are functional in this strain. Interestingly, this regulatory circuitry limits nitrogen fixation and its absence results in an increase in expression of the fix and nif genes that are regulated by the TCS FixL-FixJ.

**METHODS**

**Bacterial strains, plasmids and culture growth conditions.** The bacterial strains and plasmids used are listed in Table 1. Rhizobium strains were grown at 30 °C in PY rich medium (Noel et al., 1984) or in Y minimal medium (MMY) with succinate (10 mM) and ammonium chloride (10 mM) as carbon and nitrogen sources, respectively (Bravo & Mora, 1988). Escherichia coli strains were grown at 37 °C in Luria-Bertani medium. Antibiotics were added at the following concentrations: carbenicillin (Cb), 100 µg ml\(^{-1}\) (E. coli); fosfomycin (Fm), 100 µg ml\(^{-1}\) (Rhizobium); gentamicin (Gm), 15 µg ml\(^{-1}\) (E. coli and Rhizobium); kanamycin (Km), 30 µg ml\(^{-1}\) (E. coli and Rhizobium); nalidixic acid (NaI), 20 µg ml\(^{-1}\) (Rhizobium); neomycin (Neo), 60 µg ml\(^{-1}\) (Rhizobium); spectinomycin (Sp), 100 µg ml\(^{-1}\) (E. coli or Rhizobium); streptomycin (Sm), 200 µg ml\(^{-1}\) (Rhizobium); tetracycline (Tc), 10 µg ml\(^{-1}\) (E. coli and Rhizobium). When required sucrose was added at 12.5% (w/v). β-Galactosidase activity was routinely used for selection of recombinant plasmids. 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was used in plates at 20 µg ml\(^{-1}\). For β-gluconuronidase activity 5-bromo-4-chloro-3-indolyl-β-D-galacturonic acid (X-gluc) was used in plates or tubes at 20 µg ml\(^{-1}\). For expression analysis, cultures were grown until mid-exponential phase in MMY minimal medium. Micro-oxic cultures were grown in 150 ml bottles closed with an airtight stopper and flushed with several volumes of a 1% oxygen/99% argon mixture as previously described (Girard et al., 1996).

**Measurement of β-glucuronidase activity.** Aerobic and micro-aerobic cultures of Rhizobium strains harbouring transcriptional fusions were grown as described previously (Girard et al., 2000). Quantitative β-glucuronidase activity was determined in 1 ml culture samples using 4-nitrophenyl β-D-glucuronide as substrate as described previously.
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
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<tr>
<td>DH5α</td>
<td>supE44ΔlacU169(p80lacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td>pCR2.1 TOPO</td>
<td>Cloning vector for PCR products, Ap’Km’</td>
<td>Invitrogen</td>
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<tr>
<td>pK* mobSacB</td>
<td>Suicide cloning vector, Kmr</td>
<td>Schäfer et al. (1994)</td>
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<tr>
<td>pRK415</td>
<td>Broad host range plasmid, Tc’</td>
<td>Keen et al. (1988)</td>
</tr>
<tr>
<td>pBBMCS53</td>
<td>ΔplacZ pBBR1MCS-3 derivative, carrying the promoterless aidA, Gmr’</td>
<td>Girard et al. (2000)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Conjugation helper plasmid, Km’</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pfxkR-Ret</td>
<td>pRK415 derivative carrying the R. etli fskR gene under the lacZ promoter</td>
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<tr>
<td>pARG4</td>
<td>pBBMCS3 derivative, SM11fixKα::uidA</td>
<td>This study</td>
</tr>
<tr>
<td>pARG5</td>
<td>pBBMCS3 derivative, SM11fixK1::uidA</td>
<td>This study</td>
</tr>
<tr>
<td>pARG6</td>
<td>pK* mobSacB derivative, fixLΔSM11::loxPsp</td>
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<td>pARG7</td>
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</tr>
<tr>
<td>pARG14</td>
<td>pK* mobSacB with fixNΔSM11 289 bp</td>
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<tr>
<td>CE3</td>
<td>Sm’ derivative of CFN42</td>
<td>Noel et al. (1984)</td>
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<td>S. meliloti SM11</td>
<td>Wild type</td>
<td>Selbitschka et al. (2006)</td>
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<tr>
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<td>SM11 fixKa::pK* mobSacB</td>
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<tr>
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<td>SM11 fix::pK* mobSacB</td>
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<td>SM11 fixK1::pK* mobSacB</td>
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<tr>
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<tr>
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<td>SM11 fixN1::pK* mobSacB</td>
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<tr>
<td>SM11FixN2-</td>
<td>SM11 fixN2::pK* mobSacB</td>
<td>This study</td>
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For Southern hybridizations, DNA was digested with appropriate restriction enzymes, electrophoresed in 1% (w/v) agarose gels and blotted onto nylon (Hybond N+). Hybridization was carried out under high stringency conditions using Amersham’s Rapid-hyb buffer. Specific probes were normally obtained by PCR and labelled with α32P-CTP by random priming using Amersham’s Rediprime II Labelling System.

**PCR amplification.** Specific PCR primers were designed using the Oligo 6.0 software and were purchased from Unidad de Síntesis Química IBT-UNAM. PCR amplifications were done in a Veriti 96-well Thermal Cycler (Applied Biosystems). Accuprime Taq DNA Polymerase High Fidelity (Invitrogen) was used in PCR reactions with a cycling regime that included a denaturing step at 94°C for 1 min followed by 30 cycles of 94°C for 30 s, 45–60°C (according to the primers’ melting temperature) for 30 s and 72°C for 90 s. A final elongation step was

(Girard et al., 2000). Data were normalized to total cell protein concentration determined by the Lowry method on a second set of 1 ml samples. Specific activities are reported in nanomoles of product per minute per milligram of protein (nmol min⁻¹ mg of protein⁻¹).

**Microbiological and recombinant DNA methods.** Total and plasmid DNA isolation, digestion with restriction enzymes, ligations, agarose gel electrophoresis and *E. coli* transformation were performed using standard protocols (Sambrook et al., 1989). Enzymes used for DNA restriction and modification were purchased from either Invitrogen or Fermentas and used according to the manufacturer’s instructions. Conjugative transfer of plasmids from *E. coli* to *Rhizobium* was done by triparental crosses using pRK2013 as conjugation helper. For determination of plasmid profiles, a modified Eckhardt procedure was used (Hynes & McGregor, 1990).
made at 72 °C for 5–10 min, according to the length of the DNA frag-
ment to be amplified. Table S1 shows the primers used in this work.

Plasmids and S. melloti mutant construction. To generate plas-
mids for expression analysis, the corresponding regulatory regions were
obtained by PCR using total DNA from S. melloti SM11. The amplifica-
tion products were cloned by T-A annealing into pCR 2.1-TOPO (Invi-
trogen) and sequenced. The appropriate restriction fragments were
cloned in the broad-host plasmid pBBRMCS53 carrying a promoterless
uidA gene (Girard et al., 2000). To generate transcriptional fusion of the
SM11 fixxa gene, a PCR product was obtained using primers Up-
SM11Ka-gus and Lw-SM11Ka-gus. The XbaI and BamHI sites intro-
duced by the primers were used to clone into pBBMCS53 to obtain
pARG4. Plasmid pARGs carrying fixx1::uidA transcriptional fusion was
constructed by cloning the XbaI-KpnI 474 bp fragment synthesized
by PCR using primers Up-SM11K1-gus and Lw-SM11K1-gus, with
the restriction sites being introduced by the primers.

The hfixL (SmeSM11ap135), fxkR (SmeSM11ap133) and fixl
(SM11_pC0961) genes were amplified by PCR from the total DNA from
strain SM11 using primers Up-SM11hfixL-DR and Lw-SM11hfixL-DR,
Lw-SM11Ka-gus and Lw-SM11fixKR-DR and Up-SM11fixKL-DR and
Lw-SM11fixL-DR, respectively. The amplification products were cloned
into pCR 2.1-TOPO and sequenced. The inserts were then subcloned
into the suicide plasmid pK7mobsacB (hfixL and fxkR) (SCHAFFER et
al., 1994) or pQ200mp18 (fxl) (QUANDT & Hynes, 1993). To introduce an
insertion mutation into hfixL, a losP interposon (Martinez-Salazar
& Romero, 2000) was cloned in the SalI site at position 691 in the hfxL
coding region (plasmid pARG6). The fxkR gene was interrupted by
inserting an fSmsf/ps cassette in the EcoRI site of the gene, located 60
nt downstream of the ATG (plasmid pARG7). An fSmsf/ps cassette
was inserted in the XhoI site at position 778 of fixl coding region (plasmid
pARG8).

Replacement of S. melloti SM11 hfixL, fxkR and fixl wild-type alleles by
the hfixL::losP, fixkR::losP and fixl::osp mutant alleles in plasmids
pARG6, pARG7 and pARG8, respectively, was carried out by homoge-
neous notication using the sacB marker present in the donor plasmids.
Double recombinants were selected as SacI, FosI, SmI, NeoI or SacI, FosI,
SmI, GmI transconjugants. To verify that the desired gene replacements
had occurred, the total DNA blots of all the derivatives were analysed by
Southern hybridization (see Fig. S2).

SM11FixKa, SM11FixK, SM11FixK1, SM11FixK2, SM11FixN and
SM11FixN2 mutants were obtained by vector-insertion mutagenesis
(VIM) (Landeta et al., 2011). An intragenic segment, specific for each
gene, was synthesized by PCR using specific primers and analyzed
by sequencing. Each intragenic region was cloned into the pK7mobsacB
vector resulting in plasmids pARG9 to pARG14. Plasmids pARG9
through pARG14 were individually transferred to SM11 by conjugation
and co-integrated by a single recombination with the homologous tar-
get, producing a knockout of the gene of interest (Landeta et al., 2011).
Mutants were selected as FosI, SmI transconjugants. The correct integration
of the plasmids was verified by PCR using one specific primer of the corre-
sponding gene and a vector primer (Up-SRpK18). Primers used in all
constructions are described in Table S1.

RNA purification and semi-quantitative RT-PCR analysis. Total
RNA was purified from 200 mg of frozen alfalfa nodules using Trizol
reagent (Life Technologies) following the manufacturer’s instructions.
The samples were preserved at –80 °C until tested. RNA was quanti-
ﬁed using a spectrophotometer (Nanodrop, ThermoScientific) and
electrophoresed under denaturing conditions in agarose-formaldehyde
gels. Fifteen µg of total RNA was digested with DNase I (Fermentas)
in the presence of ribonuclease inhibitor (RiboLock, Fermentas) follow-
ing the manufacturer’s instructions. Absence of genomic DNA contamina-
tion was conﬁrmed by PCR ampliﬁcation, using primers designed for
the rpoA reference gene. The level of expression of the SM11 genes was
quantified by two-step RT-PCR. The first-stranded cDNA was synthe-
ized from 2.5 µg of DNA-free RNA by the random priming method
with the RevertAidH Minus First Strand cDNA Synthesis Kit (Fer-
mentas). Accuprime high-fidelity Taq DNA polymerase (Invitrogen)
was used in PCR reactions with a cycling regime that included a dena-
urring step at 95 °C for 1 min, followed by 30 cycles of 94 °C for 1 min,
60 °C for 1 min and 72 °C for 1 min. A final elongation step was made
at 72 °C for 10 min. Speciﬁc primers were designed and used to obtain RT-
PCR products. The PCR products were analysed by electrophoresis in
2 % (w/v) agarose gels. The rpoA gene was used to normalize gene
expression and the intensity of bands from RT-PCR ampliﬁcations was
quantified by densitometry using Photoshop software. Experiments
were done using two independent biological samples. Primers and intra-
genic regions are shown in Table S1.

Plant growth and evaluation of nitrogen ﬁxation capacity. Med-
icago sativa L., cv. Aragon seeds were surface-sterilized in 96 % (v/v)
ethanol for 30 s, and incubated for 2 h in a 6 % sodium hypochlorite
solution with shaking. After that, the seeds were thoroughly washed
with sterilized water and placed on plates containing 1 % water-agar to
germinate at 30 °C for 60 h in the dark. Selected uniform seedlings were
planted in sterile 1 kg pots (fifteen/pot) with vermiculate as rooting sub-
strate. Each seedling was inoculated with 1 ml of the desired strain (10⁸
cells ml⁻¹) and grown in a controlled environment chamber (14/10 h
light/dark cycle, 22/16 °C, photosynthetic photon flux 400 µmol m⁻²
s⁻¹ and relative humidity 60 to 70 %). Plants were alternately watered
with free-nitrogen nutrient solution (Fahraeus, 1957) and water.
Nitrogen-activity (ARA) was determined by acetylene-reduction assay
in detached nodulated roots as described by Hardy et al. (1968). Plant
and nodule dry weight were determined after drying fresh plant material
at 60 °C for 48 h.

Leghaemoglobin content. Leghaemoglobin content was measured as
previously described by LAURE & Child (1979). Essentially, nodules
(0.3 g) were ground with 4 ml LB extraction buffer (40 mM
Na₂HPO₄–2H₂O, pH 7.4; 10 mM NaH₂PO₄·H₂O, pH 7.4; 0.02 % K₂Fe
(CN)₆·0.1 % NaHCO₃) supplemented with 0.1 g polyvinylpolypyrrolidone
(PVPP). The homogenate was centrifuged at 12 000 g at 4 °C for 20 min
to obtain the supernatant. Clear supernatant (50 µl) and saturated oxalic
acid (3 ml) were mixed in screw-capped tubes, which were sealed and
autoclaved for 30 min at 120 °C and then allowed to cool to room tem-
perature. The fluorescence of the solutions was measured using a
spectrophotofluorometer. The excitation wavelength was 405 nm and
the emission monochromator setting was 650 nm. The difference in
fluorescence between heated and unheated samples was proportional to
haem protein content.

Statistical analysis. All results were analysed by one-way ANOVA,
and means were separated using Tukey at P=0.05 with SPSS software.

RESULTS

Plasmids pSmeSM11c and pSmeSM11a encode multiple fix genes in S. melloti SM11

The genome of S. melloti SM11 encodes unique as well as reiterated fix genes. The fixL–fixT operon, as well as three fix-
NOQP operons, two fixT and fixK genes and one fixGHIS
operon are encoded on the pSmeSM11c megaplasmid. This
replicon also contains a truncated copy of fixI located
upstream of the fixNOQP-2 operon and fixH fixI and fixS
genes located downstream of fixNOQP-3 (Fig. S1). The
absence of the anaerobox (TTG-N8-CAA) in the regulatory
region of the fixNOQP-3 operon suggests that it is not
under the control of an Fnr-like regulator such as the ones analysed in this study (data not shown). As mentioned, the homologues of hFixL, FxkR and FixKf are located on pSmeSM11a (Fig. S1) in the region homologous to pSymA of *S. meliloti* 1021. Despite the high degree of similarity (>87%) exhibited among the open reading frames (ORFs) located in these two regions, single base pair deletions/insertions occur. For example, the SM11 FxkR homologue (pSmeSM11a133) is divided into two ORFs, sma1138 and sma1139, in pSymA (Stiens et al., 2006, 2007; Schneikert-Bekel et al., 2011). The expression and functionality of the pSmeSM11a-encoded hFixL-FxkR TCS as well as the FixKa regulator has not been reported, and is the focus of this work as detailed in the following text.

**fixKa gene is expressed in microaerobiosis under the control of hFixL and FxkR**

In a previous study we identified a *fixKf* homologue in *S. meliloti* SM11, hereafter referred as *fixKa*. This gene is expressed divergently from *fxkR* and might be in an operon with *fxl*F (Fig. S1). The regulatory region of *fixKa* has a conserved K-box, and a transcriptional fusion containing the *fixKa* promoter fused to the *uidA* gene was found to be induced under microaerobic conditions in the heterologous host *R. etli* CE3 (Table 2, line 1). Expression of *fixKa* in this background depended on the presence of hFixL and FxkR (Table 2, lines 2 and 3). These results demonstrated that *fixKa* has a functional promoter that can be activated by an hFixL-FxkR TCS. To define the functionality of the hFixL-FxkR TCS encoded in *S. meliloti* SM11 we analysed the expression of the *PfixKa-uidA* transcriptional fusion in the wild-type strain and isogenic strains with mutations in either hfixL or fxkR under low oxygen condition. Measurements of GUS activity in the wild-type strain (243 ± 37) revealed that *S. meliloti* SM11 contains the regulatory elements necessary to activate the expression of *fixKa*. In contrast, expression of this gene was not detected in the hFixL (3 ± 4) or FxkR (5 ± 5) mutant strains. These results indicate that hFixL and FxkR proteins are functional in *S. meliloti* SM11. Furthermore, levels of *fixKa* expression remained unaltered in a *fixL* mutant (240 ± 30), indicating that its expression depends on the presence of hFixL and FxkR but not on FixL.

**hFixL-FxkR TCS negatively regulates the expression of fixKf**

The presence of functional FixL-FixJ and hFixL-FxkR regulatory systems in *S. meliloti* SM11 raises the question about how the micro-oxic response is coordinated in this organism. The microaerobic expression of *fixKf*, a direct target of the FixL-FixJ TCS, was analysed in the absence of hFixL, FxkR and FixL. Our results revealed that expression of this gene increased ~7-fold in the hFixL mutant and ~3.5-fold in the FxkR mutant when compared with the level of expression observed in the wild-type strain (Table 3, compare lines 2 and 3 with line 1). As expected, the expression of *fixKf* was null in the *fixL* mutant background (Table 3, line 4). These results strongly suggest that in *S. meliloti* SM11, the hFixL-FxkR TCS negatively regulates targets of the FixL-FixJ regulatory pathway by an unknown mechanism.

**Table 2. Expression pattern of the *S. meliloti* fixKa gene in the *R. etli* genetic background**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Strain genotype</th>
<th>Fusion</th>
<th>Expression level*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE3/pARG4</td>
<td>Wild type</td>
<td>SM11fixKa::uidA</td>
<td>102 ± 22</td>
</tr>
<tr>
<td>CFNX636/pARG4</td>
<td>hfxl::loxPsp</td>
<td>SM11fixKa::uidA</td>
<td>ND</td>
</tr>
<tr>
<td>CFN-LG1/pARG4</td>
<td>fxkR::loxPsp</td>
<td>SM11fixKa::uidA</td>
<td>2 ± 4</td>
</tr>
</tbody>
</table>

*G-1-glucuronidase-specific activity (nmol min⁻¹ mg protein⁻¹) was determined after 10 h of culture growth at 30 °C. Data are the mean of two replicas from three independent experiments.

**Table 3. Microaerobic expression analysis of fixKf gene in *S. meliloti* SM11**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Strain genotype</th>
<th>Fusion</th>
<th>Expression level*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM11/pARG5</td>
<td>Wild type</td>
<td>SM11fixKf::uidA</td>
<td>1016 ± 12</td>
</tr>
<tr>
<td>SM11hFixL-/pARG5</td>
<td>hfixL::loxPsp</td>
<td>SM11fixKf::uidA</td>
<td>7064 ± 44</td>
</tr>
<tr>
<td>SM11FxkR-/pARG5</td>
<td>fxkR::OsP</td>
<td>SM11fixKf::uidA</td>
<td>3696 ± 91</td>
</tr>
<tr>
<td>SM11FixL-/pARG5</td>
<td>fxl::OsP</td>
<td>SM11fixKf::uidA</td>
<td>3 ± 4</td>
</tr>
</tbody>
</table>

*G-1-glucuronidase-specific activity (nmol min⁻¹ mg protein⁻¹) was determined after 10 h of culture growth at 30 °C under microaerobic conditions. Data are the mean of two replicas from three independent experiments.
hFixL-FxkR TCS negatively regulates nitrogen fixation and the expression of fixK1 and fixN1 in nodules

Based on the results described in the preceding text we hypothesized that the hFixL-FxkR TCS would limit the ability of *S. meliloti* SM11 to fix nitrogen in symbiosis with *Medicago sativa*. To test this, alfalfa plants were inoculated with either the SM11 (WT), hFixL− (hfixL::loxPSp) or FxkR− (fxkR::OSp) mutant strains. Table 4 shows that the absence of hFixL or FxkR increased the nitrogenase-specific activity (ARA) and leghaemoglobin (Lb) content in alfalfa nodules. This increase was also reflected in plant and nodule dry weights (PDW and NDW, respectively). As expected, plants inoculated with either fixL or fixJ mutants had a Fix− phenotype (data not shown).

The enhanced nitrogen fixation capacity of either hFixL or FxkR mutants indicates that these regulators play a repressive role in the *S. meliloti* SM11-alfalfa symbiosis. This also suggests that both regulatory systems (FixL-FixJ and hFixL-FxkR) are active in bacteroids. To determine if these regulators are expressed in nodules, we conducted an RT-PCR experiment using total RNA from the wild-type strain nodules. Our results revealed that hfixL, fixL, fixJ and fixK were expressed in nodules (Fig. 1a). We further evaluated the expression of the two copies of fixK (fixK1 and fixK2) by semi-quantitative RT-PCR (sRT-PCR) in SM11 WT and hFixL or FxkR mutants (Fig. 1b). The level of fixK1 expression increased significantly in the absence of hFixL or FxkR. This negative effect is only observed for the fixK1 reiteration. Interestingly, the absence of FixKa, the direct target of this TCS, did not seem to affect fixK1 or fixK2 expression. The increase in nitrogen fixation capacity observed in strains lacking hFixL or FxkR could be due to the up-regulation of the symbiotic cytochrome oxidase *cbb*. The expression of fixN1 and fixN2 was also analysed by sRT-PCR in the parental strain and isogenic strains with mutations in hfixL or fxkR (Fig. 1c). A differential expression pattern was obtained. While fixN1 is significantly induced in the hFixL or FxkR mutants, the expression of fixN2 is not affected by the absence of these regulators. We next analysed if single mutations in each of the *fixK* genes (fixK1, fixK2 and fixKa) had an effect on the expression of the predicted target *fixN1* and *fixN2*. As shown in Fig. 1c, no individual mutation in any FixK regulator significantly affected the expression of *fixN1* or *fixN2*. This result suggests a degree of redundancy in the activity of the FixK regulators in *S. meliloti* SM11.

**fixK genes have a differential effect on nitrogen fixation, whereas both fixN1 and fixN2 are required for optimal nitrogen fixation**

To evaluate the relative participation of *fixN* and *fixK* reiterated genes in nitrogen fixation in *S. meliloti* SM11, alfalfa plants were inoculated with the whole set of *fixN* and *fixK* mutants and evaluated afterward for different nitrogen
The absence of fixKa but not of fixK1 or fixK2 showed a slight decrease in NDW and ARA (Fig. 2a, b). Plants inoculated with the fixK1 or fixK2 mutants but not with the fixKa mutant, had a significantly increased plant growth reflected as PDW (Fig. 2c). The Lb content was not affected by the absence of individual FixK regulators (Fig. 2d).

The absence of either fixN1 or fixN2 resulted in a decrease of ~50% in NDW, ARA and PDW (Fig. 2a, b, c). These results are consistent with the significant decrease in Lb content occurring in the absence of fixN1 and fixN2; this effect was more pronounced in the fixN2 mutant (Fig. 2d). Our results showed that no individual FixK regulator is essential for nitrogen fixation and that both copies of fixN are necessary to achieve optimal levels of nitrogen fixation in alfalfa.

**DISCUSSION**

In this work, we looked into the role of the recently identified regulator FxkR and the hybrid histidine kinase hFixL in the field isolate *S. meliloti* SM11. The regulation of nitrogen fixation has been extensively studied in *S. meliloti*. Since the discovery of the FixL-FixJ TCS as the transducer module of the low oxygen signal, most research efforts have been aimed at characterizing the role of FixL as an oxygen sensor and in elucidating the complete regulon of the FixJ and FixK regulators that are controlled by FixL.

![Image](http://mic.microbiologyresearch.org)
Another interesting feature of this regulatory cascade was the presence of the small protein FixT, which acts as an inhibitor of the kinase activity of FixL. The FixK protein activates the fixT gene, encoding a stand-alone receiver-domain protein that was shown to inhibit FixL autophosphorylation (Foussard et al., 1997; Garnerone et al., 1999; Reutimann et al., 2010). Proteins such as FixT that modulate the phosphorelay in TCSs are known as TCS connectors. These proteins play key roles in signal integration and are widely distributed among Gram-positive and Gram-negative bacteria (Mitrophanov & Groisman, 2008). There are multiple mechanisms by which the TCS connectors exert their regulatory effect: they can modulate the phosphorylation state of the HK or of the RR or can act downstream by affecting the ability of the RR to bind to its DNA target or recruit the RNA polymerase (Mitrophanov & Groisman, 2008). The results presented here provide evidence that the previously neglected hFixL-FxkR TCS is active in S. meliloti SM11 and negatively regulates the FixL-FixJ cascade. We found that fixK1 is up-regulated in the absence of hFixL and FxkR under low oxygen conditions. fixK1 does not possess a conserved K-box or an anaerobox in its regulatory region, which would suggest that the negative regulation exerted by hFixL-FxkR is not direct. Based on this observation it is tempting to speculate that hFixL and FxkR inhibit FixL or FixJ activity. The ongoing work in our lab is aimed at determining if members of the FixL-FixJ and hFixL-FxkR TCS interact with one another. Another line of investigation will seek to identify TCS connectors between these two cascades. We are also exploring the possibility that hFixL-FxkR regulates the abundance or activity of the known connector FixT.

A very interesting observation was that the mutation of fixKa does not phenocopy the mutation of its regulators hFixL and FxkR. These results suggest that the hFixL-FxkR TCS might have additional targets in S. meliloti or, as mentioned above, they might have crosstalk interactions with FixL and FixJ. Both FixL and hFixL are oxygen sensors that act through a conserved haem-binding sensor domain, and it can be speculated that in the absence of hFixL more haem is available for binding to FixL. The results presented here suggest that hFixL has more repressive effect than FxkR on fixK1 expression both in free living and symbiotic conditions; nonetheless the absence of FxkR promotes a greater increase in nitrogen-fixation parameters. This evidence points to potential branching effects specific to hFixL and FxkR activity.

A striking observation was that the effect of the hFixL-FxkR TCS goes beyond the regulation of fix genes in low oxygen conditions. The absence of this TCS promotes a significant increase in the ability of S. meliloti SM11 to fix nitrogen in alfalfa nodules. This is yet another example of how the expensive process of nitrogen fixation is fine-tuned to preserve energy, perhaps to allow the maintenance of other fundamental physiological processes. We previously reported a mechanism, involving the FNR-like regulator StoRd that

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**Fig. 3.** Regulatory model of fix gene expression by FixL-FixJ and hFixL-FxkR TCS in S. meliloti SM11. Heavy grey lines indicate regulatory regions. Filled black squares indicate the presence of an anaerobox sequence. The open square with stripes represents a K-box. Arrows indicate activation, T-bars indicate repression. Dash lines represent the proposed model of regulation based on the genetic evidence presented and the presence of predicted regulatory binding sites.
limits nitrogen fixation in *R. etli* CFN42 (Granados-Baeza *et al.*, 2007). Now that we have found that the hFixL-FxkR TCS limits nitrogen fixation in the field isolate *S. meliloti* SM11, it is tempting to speculate that a variety of mechanisms have evolved to redirect the high energy investment that nitrogen fixation represents to processes that might increase reproductive fitness and in consequence lead to the dominance of specific strains in the field. In fact accessory plasmids such as pSmeSM11a have been proposed to confer not only selective advantages that enhance the growth and survival of their host functions but also contribute to bacterial evolution (Kuhn *et al.*, 2007; Mercado-Blanco & Toro, 1996; García-de Los Santos *et al.*, 1996).

The relevance of encoding multiple FNR-like regulators remains a mystery, as do the mechanisms that have evolved to accommodate multiple transcriptional regulators with similar DNA-target sequences. Here we show that the absence of the FixKa regulator in *S. meliloti* SM11 caused a slight decrease in its ability to fix nitrogen. However, the mutation of either *fxk1* or *fxk2* promoted plant growth even though it had no significant effect on nitrogen fixation (ARA and NDW) or *fixNOQP1-2* expression. It can be suggested that individual mutations in *fxk1* or *fxk2* have a positive effect on the ability of alfalfa to assimilate the fixed nitrogen more effectively. Future work should aim to characterize the response of the plant at both the transcriptional and post-transcriptional level as well as to identify bacterial genes that promote plant growth and that are under the control of the multiple FNR regulators encoded in rhizobial genomes. The degree of redundancy among these FixK-like regulators is still an open question. It is possible that the relative abundance of these regulators would determine their ability to occupy the anaerobox in the regulatory region of the *fixNOQP* operons and additional direct targets. *fixKa* is not a reiteration of the *fxk1* and *fxk2* genes, and perhaps FixKa has a different affinity for the anaerobox. As previously noted the mutation of *fixKa* produced a slight but significant decrease in nitrogen-fixation parameters, specifically in NDW and ARA. This suggests that FixKa regulates specific targets that are required for optimal levels of nitrogen fixation. Further analysis is required to unveil the mechanisms by which the expression of the *fixNOQP* operons and other genes important for nitrogen fixation are regulated by the FixK-like regulators.

We previously observed that only one of the *fixNOQP* reiterations encoded in the genome of *R. etli* CFN42 is required to sustain nitrogen fixation (Girard *et al.*, 2000). Interestingly, our results here showed that in *S. meliloti* SM11, similar to *R. leguminosarum* bv. viciae VF39 (Schlüter *et al.*, 1997), both copies are necessary to achieve optimal levels of nitrogen fixation, again showing that there are clear differences in the mechanisms used by different rhizobia to successfully fix nitrogen in symbiosis with legumes. Co-evolutionary adaptation has shaped the genetic circuits that control symbiosis and nitrogen fixation, leading to appreciable differences between unrelated symbiotic partners.

Based on the current knowledge of the regulation of *fix* genes in *S. meliloti* as well as the results of this study, we propose a model of regulation of *fix* gene expression in *S. meliloti* SM11 that includes the hFixL-FxkR TCS and the FixKa regulator (Fig. 3).

In summary, this work presents evidence supporting the role of the hFixL-FxkR TCS in the regulation of the micro-aerobic response and nitrogen fixation in *S. meliloti* SM11. In this bacterium, the hFixL-FxkR TCS seems to play a role as modulator of the response to low oxygen. Our results point to a mechanism of action characteristic of TCS connectors. The identity and mode of action of this connector await description. A better understanding of the role of TCS connectors in bacterial physiology will shed light on the evolution of TCS and will allow us to make more educated decisions about manipulating existing signalling modules or during the *de novo* design of signalling networks.

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