The Two-Component System AcJK is Involved in Acid Stress Tolerance and Symbiosis in Sinorhizobium meliloti

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The Two-Component System ActJK is Involved in Acid Stress Tolerance and Symbiosis in *Sinorhizobium meliloti*

**Running Head:** The role of *Sinorhizobium meliloti* ActJK

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Graphical Abstract
HIGHLIGHTS

- ActJK contributes to *Sinorhizobium meliloti* growth at low pH.
- ActJK is involved in rhizobium acid-tolerance response (ATR).
- ActJK-controlled functions are crucial for optimal symbiosis development.

ABSTRACT

The nitrogen-fixing α-proteobacterium *Sinorhizobium meliloti* genome codifies at least 50 response regulator (RR) proteins mediating different and, in many cases, unknown processes. RR-mutant library screening allowed us to identify genes potentially implicated in survival to acid conditions. *actJ* mutation resulted in a strain with reduced growth rate under mildly acidic conditions as well as a lower capacity to tolerate a sudden shift to lethal acidic conditions compared with the parental strain. Mutation of the downstream gene *actK*, which encodes for a histidine kinase, showed a similar
phenotype in acidic environments suggesting a functional two-component system. Interestingly, even though nodulation kinetics, quantity, and macroscopic morphology of *Medicago sativa* nodules were not affected in actJ and actK mutants, ActK was required to express the wild-type nitrogen fixation phenotype and ActJK was necessary for full bacteroid development and nodule occupancy. The actJK regulatory system presented here provides insights into an evolutionary process in rhizobium adaptation to acidic environments and suggests that actJK-controlled functions are crucial for optimal symbiosis development.

**Keywords:** MEDICAGO, SINORHIZOBIUM, STRESS, TCS, SYMBIOSIS
1. INTRODUCTION

Rhizobia are Gram-negative soil-dwelling bacteria that can eventually establish a symbiotic association with leguminous plants. Through a complex molecular dialogue, rhizobia induce root nodules to fix atmospheric nitrogen; in turn, they receive carbon compounds derived from plant photosynthates. The rhizobium-legume association presents different degrees of specificity with respect to the associated species. *Medicago sativa* (alfalfa) nodulation is particularly restricted to *Sinorhizobium (Ensifer) meliloti* and a few other rhizobia (Del Papa et al., 1999; Torres Tejerizo et al., 2016; Villegas et al., 2006). Several key events of rhizobium-legume interaction can be affected by acidity, such as nodulation factor production (Caetano-Anollés et al., 1989), rhizobium-root attachment (Caetano-Anollés et al., 1989), nodule quantity (Munns, 1968) and nitrogenase complex activity (Vassileva et al., 1997). In this context, the isolation of efficient and more acid-tolerant rhizobium strains was one of the experimental strategies used to improve the symbiotic association (Del Papa et al., 2003; Glenn et al., 1999). From the empirical point of view, rhizobium acid tolerance has long been considered a phenotypic trait that would positively affect the establishment of symbiosis at low pH (Howieson et al., 1988). Thus, the identification and rational manipulation of acid tolerance genetic markers is a high-priority task to improve rhizobial inoculants.

The ability of most bacteria to sense and adapt to changing environments is frequently mediated by the so-called two-component signal transduction systems (TCS) (Hoch, 2000). They generally consist of a sensor histidine kinase (HK) and a response regulator (RR). The HK senses environmental changes and triggers the adaptive response by transferring a phosphoryl group to the RR, which modulates gene
expression by acting as a transcriptional regulator (Hoch and Silhavy, 1995). Hagiwara and co-workers identified 40 HKs on the genome of S. meliloti 1021, one phosphotransfer intermediate (HPt) and 58 RRs (Hagiwara et al., 2004).

To date, the molecular basis of rhizobial acid tolerance associated with TCS has not been systematically studied. To our knowledge, S. medicae WSM419 and S. meliloti 1021 ActR/ActS and ExoS/Chvl were the only TCS reported so far as proteins necessary for acid adaptation (Tiwari et al., 1996; Wang et al., 2010). However, the underlying biochemical mechanisms behind them remain largely unexplored.

To better understand the role of TCS in acid tolerance, we used a set of RR mutants constructed in S. meliloti 2011 (wt) background, a strain closely related to S. meliloti 1021 (Galibert and Others, 2001; Sallet et al., 2013). Screening under sub-lethal acidic conditions allowed us to identify an uncharacterized RR-encoding gene (locus_tag: SMc02366, here referred to as actJ) whose mutation produced an acid-sensitive phenotype, showing its importance in acid stress adaptation. Furthermore, we demonstrate that ActJ and its cognate histidine kinase ActK is also important in S. meliloti symbiotic biology.

2. MATERIALS AND METHODS

2.1. Media, bacterial strains and DNA manipulations

The bacterial strains and plasmids used in this work are listed in Table 1. Rhizobia were grown at 28 °C in tryptone-yeast medium (TY) (Beringer, 1974). When required, TY media were supplemented with 120 μg ml⁻¹ neomycin (Nm), 400 μg ml⁻¹ streptomycin (Sm) or 50 μg ml⁻¹ gentamicin (Gm). Escherichia coli strains were grown at 37 °C in
Lysogeny Broth medium (LB) (Sambrook et al., 1989). When required, LB media were supplemented with 50 μg ml⁻¹ kanamycin (Km) and 10 μg ml⁻¹ Gm. Screening of acid-sensitive *S. meliloti* mutants and most phenotypic studies were performed in glutamate-sucrose (GS) minimal medium (27.45mM sucrose, 2.67 mM Na-glutamate, 0.15 mM K₂HPO₄·3H₂O, 0.15 mM KH₂PO₄, 0.7 mM Na₂SO₄, 1.0 mM MgSO₄·7H₂O, 1.0 mM CaCl₂·2H₂O, 2.95 μM thiamine-HCl, 4.2 μM Ca-pantothenate, 0.08 μM biotin, 48.0 μM H₃BO₃, 10.0 μM MnSO₄, 10.0 μM ZnSO₄, 48.0 μM CuSO₄, 0.5 μM CoCl₂, 1.0 μM Na₂MoO₄·2H₂O, 1.0 μM FeCl₃·6H₂O (Del Papa et al., 1999). GS minimal medium was supplemented with 20 mM MES buffer (2-[N-morpholino]ethanesulfonic acid) or 20 mM PIPES [(piperazine N,N´-bis(2-ethanesulfonic acid)] to maintain pH at 5.6 or 7.0, respectively. pH was adjusted with KOH prior sterilization in autoclave and then completed with sterile micronutrients and vitamins. Small changes in pH values were readjusted using KOH or HCl.

For acid tolerance (ATR) assays Evans minimal media was used (Evans, 1970) (glucose 10 g/l, NH₄Cl 0.7 g/l, KCl 0.4 g/l, NaH₂PO₄ 0.6 g/l, Na₂SO₄ 0.14 g/l, citric acid 0.2 g/l, MgCl₂ 6H₂O 0.13 g/l, CaCl₂ 2H₂O 3 mg/l, FeCl₃ 6H₂O 27 mg/l, MnCl₂ 4H₂O 10 mg/l, ZnO 2 mg/l, CuSO₄ 5H₂O 1 mg/l, CoCl₂ 6H₂O 2 mg/l, H₃BO₃ 3 mg/l, NaMoO₄ 2H₂O 20 ng/l, FeSO₄ 7H₂O 247 μg/l, rivoflavin 20 μg/l, 4-aminobenzoic acid 20 μg/l, thiamine-HCl 20 μg/l, pyridoxine-HCl 20 μg/l, biotin 20 μg/l. MES or PIPES [piperazine-N,N9-bis(2-ethanesulfonic acid)] buffers were used at 20 mM to adjust pH to 6.1 or 7.0 respectively.

We used standard techniques for bacteria mating, cloning and PCR amplification.

2.2. Mutant screening for the identification of *S. meliloti* 2011 response regulators involved in acid tolerance
Starter cultures were grown overnight in GS minimal medium at 28 °C, pH 7.0 and 180 rpm shaking until exponential growth phase and then inoculated into fresh GS medium at the different pH values to obtain an initial concentration of 10^6 CFU ml⁻¹. Bacterial culture kinetics was studied monitoring optical density at 600 nm in a microplate reader (BMG Labtech, Germany) set at 28 °C with continuous agitation. Mutants that poorly grew on GS medium at pH 5.6 but grew well at pH 7.0 were selected for further analysis.

2.3. Total RNA extraction, purification and cDNA synthesis.

Total RNA from bacteria cells was extracted with acid-phenol-guanidinium isothiocyanate (TRIzol, Life Sciences) and chloroform, following the manufacturer’s instructions. To purify the RNA isopropanol precipitation was conducted. Before reverse transcription, the RNA was treated with DNase I for 1h at 37°C (Thermo Scientific; 1 U DNase I per µg RNA). DNase I was inactivated by incubation at 65°C after the addition of 0.1 volume of 50 mM EDTA. The purified RNA was then quantified by UV absorbance (NanoDrop; Thermo Scientific, USA) and the quality of the preparation further assessed by denaturing agarose gel electrophoresis (Sambrook et al., 1989). Total RNA was retrotranscribed using a M-MLV Retrotranscriptase (Embiotec Inc, Quilmes, Argentina) and random hexamers as primers.

2.4. Amplicon sequencing and sequence analysis

PCR products were purified using ADN PuriPrep-GP Kit (HighWay Tandil, Argentina) and then sequenced at Macrogen Inc. (Korea) following standard procedures. The resulting sequences were compared with those found in the GenBank database for identity assignment. Function predictions were computed using standard bioinformatic
tools such as BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). Protein identity values were determined using BLASTp. Sequence comparisons and alignments were performed by means of BLAST (www.ncbi.nlm.nih.gov/blast) and Clustal Ω (Sievers et al., 2011).

2.5. Construction of TCS double-deletion mutants

The loss-of-function mutants were constructed by crossover PCRs (Sukdeo and Charles, 2003) using the suicide vector pK18mobsacB (Schafer et al., 1994), which cannot replicate in S. meliloti. In brief, PCR on S. meliloti 2011 genomic DNA was conducted to amplify N- or C-terminal fragments of the target gene using primer pairs A-B and C-D (Table S1). The resulting PCR fragments were gel-purified and combined as templates for a third PCR reaction using primer pairs A-D (third PCR). The success of this PCR relied on 21-bp complementary sequences designed on 5′ ends of primers B and C (Table S1, sequences in bold). The product of the third PCR was ligated to pK19mobsacB plasmid, using EcoRI and HindIII restriction sites located on 5′ ends of primers A and D. Gene replacement in S. meliloti 2011 was carried out by introducing pK18mobsacB derivatives by E. coli S-17 and S. meliloti 2011 conjugation and selecting for Sm and Nm-resistant single-crossover cointegrants. Subsequent selection for double-crossover events was carried out on TY plates containing 15% sucrose, followed by screening for Nm sensitivity. The precise gene deletion was confirmed by PCR amplification using primer pairs A-D (Table S2) and DNA sequencing.

In order to complement S. meliloti mutants, a fragment containing each gene was amplified by PCR using primers listed on Table S1 and S. meliloti 2011 genomic DNA as template. For amino acid substitutions, actKH250A and actJD55A coding sequences
were constructed by crossover PCR using primers SMc02367-Fw/SMc02367_Rv_H250A and SMc02367-RV/SMc02367_Fw_H250A for actKH250A; and SMc02366-Fw/SMc02366_Rv_D55A and SMc02366-RV/SMc02366_Fw_D55A for actJD55A. The fragments were digested with KpnI and Ndel and ligated into a Ndel- KpnI-digested pSRKGm, a S. meliloti pBBR-derived expression vector containing a lacIβ promoter (Khan et al., 2008). After confirmation by PCR and DNA sequencing, the resulting plasmids (Table S1) were introduced to S. meliloti 2011 mutants by conjugation.

2.6. EGFP transcriptional fusions and fluorescence assays.

To evaluate degP1 and actJ promoter activities, 350bp upstream sequences of the transcription start sites were PCR-amplified and cloned in pPHU231 (McIntosh et al., 2008) using XbaI / PstI cloning sites. Primers used are shown in Table S2.

For promoter-EGFP assays, cultures were grown in GS medium at pH 7.0 or 5.6 with shaking at 180 rpm. EGFP fluorescence (485 nm excitation / 485 nm emission) was plotted as fluorescence units divided by cell growth measured at 600nm (F/OD600). F/OD600 background of wt and mutant strains carrying pPHU231 empty plasmid were subtracted.

2.7. Stress tolerance assays

The role of actJ in triggering an ATR+ phenotype was investigated as described by Draghi et al (2010). Briefly, 1 ml of exponential S. meliloti 2011 wt or deletion strains cultures grown at pH 7.0 or pH 6.1 in Evans minimal media (Evans, 1970) were centrifuged at 14,000 x g for 5 min at room temperature and resuspended in 1 ml of fresh Evans medium at pH 4.0 at a final density of about 2x10⁸ CFU ml⁻¹ (beginning of the acid shock). During the acid shock, cells were incubated at 28 °C and 180 rpm. Aliquots were
removed and plated on agarized Evans medium pH 7.0 to register cells that survived the acid shock. Results were taken from a representative experiment among a set of three. Data were plotted on a semi-log scale and the decimal reduction time (D_{10}, time required for the number of cells to be reduced by a factor of 10) was obtained from the slope.

For abiotic stress tolerance assays, cultures grown until mid-exponential phase were diluted and incubated at 42°C (GS pH 7.0), challenged with 55mM H₂O₂ (TY, 28°C), or challenged with 500mM NaCl (GS pH 7.0, 28°C). Growth kinetics were analysed monitoring OD₆₀₀ (180 rpm) in a microplate reader (BMG Labtech, Germany).

For sodium dodecyl sulfate (SDS) tolerance assays, 100-μl aliquots of cells adjusted to an OD₆₀₀ of 0.2 were plated on TY. After 30 min, sterile filter paper disks (6-mm diameter) were placed on plates. SDS (Gibco BRL) was diluted to 10% (w/v) in water, and disks were spotted with 5 μl.

### 2.8. Plant nodulation and root colonization assays

For nodulation assays, *Medicago sativa* seeds (alfalfa, cv. Super Monarca, obtained from Instituto Nacional de Tecnología Agropecuaria, Argentina) were surface-sterilized for 10 min using commercial bleach (NaClO, concentration equivalent to 55 g active Cl₂ per litre) in a 20% v v⁻¹ water solution followed by ten washes with sterile distilled water. The sterilized seeds were germinated on water-agar (1.5%, w v⁻¹). One-day seedlings were placed into vermiculite-filled plastic pots (10 plants per pot). After 5 days, plants were inoculated with 10⁷ rhizobia per pot. Plants were irrigated with distilled water and nitrogen-free Fåhraeus-modified mineral solution (Lodeiro et al., 2000), pH 7.0, and grown in a plant-growth chamber (Binder™, KBW400) with a 22 °C-16-h-light and 16 °C-8-h-dark photoperiod. 35 days after inoculation, plants were collected, and the
extent of nitrogen fixation was assessed by measurement of the aerial dry weight. Error bars indicate the standard error of the mean (\(\sigma/\sqrt{n}\)). Results are taken from a representative experiment among a set of three.

To study nodulation kinetics, 40 individual plants were grown in sterile plastic growth pouches (Mega Minneapolis International, Minneapolis, Minn.) containing 10 ml of nitrogen-free Fåhraeus-modified mineral solution, pH 7.0 (Lodeiro et al., 2000). Four days later, primary roots were inoculated by dripping 100 µl of a nitrogen-free solution containing \(10^5\) CFU rhizobia on the root surface, from the tip toward the base. Nodule appearance was recorded over time.

Root colonization assays were performed according to Salas et al (2017). Briefly, one-day-old alfalfa seedlings were placed in plastic pots with vermiculite following inoculation with \(S.\ meliloti\) 2011 wt and mutant strains. The input inoculum was \(10^7\) CFU/pot. The double inoculation studies of mutant strains and \(S.\ meliloti\) 2011 20MP6 GFP\(^+\) (Pistorio et al., 2002) were performed in a 1:1 ratio. After 3 days, plants were removed, and root-adhered bacteria were recovered by vortexing in sterile physiologic solution. Differences between the input and output proportions of mutant/\(S.\ meliloti\) GFP\(^+\) ratio were determined. As control, single inoculation experiments were performed.

### 2.9. Microscopy of plant nodules

\(Medicago\ sativa\) plants were grown and infected as described above, and nodules were collected 28 days post-inoculation.

For optic microscopy, nodules were fixed in buffer containing 4% paraformaldehyde, 0.25% glutaraldehyde and 2.5% sucrose in 50 mM potassium phosphate buffer, pH 7.4, and incubated overnight at 4 °C with gentle agitation
(Rodriguez-Haas et al., 2013). Dehydration was carried out using a graded ethanol series (0, 30, 50, 70, 85, 90, 95, 100%) at 4 °C. Subsequently, nodules were infiltrated with LR-White Resin (London Resin) overnight at 4 °C. Embedded nodules were transferred to gelatine capsules and polymerized in fresh LR-White resin for 16 h at 60 °C. The material was sectioned in a SuperNova Reichert-J ultramicrotome. For light microscopy, 2-µm-thick dried sections were stained with saturated solution of Toluidine blue. Photographs were taken using a Nikon E200 photomicroscope and processed with the software provided by the manufacturer.

For electron microscopy, 70-nm ultrathin sections were placed on 200-mesh copper collodion-treated grids. The sections were stained with 0.5 to 1% (wt vol⁻¹) uranyl acetate and 1% (wt vol⁻¹) lead citrate for 10 and 5 min, respectively, washed in distilled water, and air dried. The grids were viewed in a JEM 1200 EX II (Jeol) transmission electron microscope at 80 kV, and photographs were taken on Kodak electron image film. The observations were carried out at the Microscopy Service, Faculty of Veterinary Sciences, UNLP.

For live/dead staining and confocal imaging, 28-day nodules were harvested, embedded in 6% (w v⁻¹) low melting agarose and dissected in 70 µm sections using a Leica VT1200S vibratome. Sections were treated 20 min in live/dead staining solution (5 mM SYTO9 and 30 mM PI in 50 mM Tris pH 7.0 buffer; Live/Dead BacLight, Invitrogen), washed with deionized water and mounted for microscopy observation. Images were acquired with a Leica confocal laser scanning microscope TCS SP2 and processed with LasX Software (Leica).
3. RESULTS

3.1. Identification of novel response regulator genes involved in S. meliloti 2011 acid tolerance

Using a Tn5-mutant library (Pobigaylo et al., 2006), we performed a phenotypic screening of RR mutants to identify novel RR involved in S. meliloti acid tolerance (37 mutants, Table 1). Out of a set of 37 RR mutants analyzed, three displayed growth defects under acid stress conditions compared to the parental strain, namely SMc01043, SMc04044 and SMc02366.

Disruption of locus SMc01043 codifying for NtrC, a member of the well-studied TCS NtrBC (Marcela et al., 2004; Patriarca et al., 1993), led to a mutant with significantly reduced ability to grow in GS at pH 7.0 and with complete growth depletion under acidic conditions (pH 5.6) (Fig. 1A).

The SMc04044 mutation also resulted in a strain with differential performance compared with the wt strain. Its growth rate was similar to that of the wt at neutral pH, but this trait was significantly reduced when challenged at acid conditions (Fig. 1A). SMc04044 gene codifies for the orphan CpdR1. Considering that the role of CpdR1 has been well-studied in S. meliloti (Biondi et al., 2006; Iniesta et al., 2006; Kobayashi et al., 2009), we did not characterize this mutant any further.

The strain lacking SMc02366 locus showed a remarkable growth defect in GS medium at low pH, while its growth rate was not affected at neutral pH (Figs. 1A and 1B). At mid-exponential phase (OD 0.5±0.05), wt cultures maintained the buffered medium at pH 5.6, while mutant strain cultures reduced the acidity in 0.2 units (data not shown). The NCBI and EMBL-EBI repositories name locus tag SMc02366 and its
cognate HK SMc02367 as RR ragA and HK ragB. This annotation comes from the \textit{Bradyrhizobium japonicum} ragAB genes for rpoH3- associated genes (Narberhaus et al., 1996). However, to our knowledge there is no solid evidence in the literature to support such denomination. We renamed SMc02366 and SMc02367 as actJ (for \textit{acid} tolerance gene \textit{J}) and actK (for \textit{acid} tolerance \textit{kinase}) respectively and studied their function in \textit{S. meliloti}.

\textbf{3.2. In silico analysis of actJ gene context}

RR-HK gene pairs often form operons in prokaryotic genomes. This arrangement allows for the coordinated expression of the two genes and provide robustness to the signaling pathway (Løvdok et al., 2009). The contiguous location of actJ and actK, together with BLASTp predictions, supported a functional ActJK TCS.

The actJK gene context is thoroughly conserved within Rhizobiales order (Fig. S1). Upstream of actJ, we found the cycHJKL operon (Fig. 2), which encodes for proteins involved in cytochrome c-type biosynthesis required for respiratory nitrate reduction \textit{ex planta} and for symbiotic nitrogen fixation in root nodules (Kereszt et al., 1995). We also found \textit{degP}1 (Fig. 2), which encodes a protease that degrades aggregated or denatured proteins from the inner-membrane and periplasmic space in \textit{E. coli} (Jones et al., 2007). Downstream of actJ, we found actK and SMc02368, which code for an uncharacterized HK protein and a putative glutamate-ammonia-ligase/adenylyltransferase protein (GlnE) respectively (Fig. 2).

Transcriptomic studies found promoters regulated by RpoD ($\sigma^{70}$) and RpoE2 ($\sigma^{E2}$) upstream of \textit{degP}1 and actJ transcription start sites (Schlüter et al., 2013). Our studies of cappable-seq (Ettwiller et al. 2016), conducted on a \textit{S. meliloti} 1021 derivative strain
lacking all RpoE-type sigma factors but the housekeeping sigma factor (\(\sigma^{70}\)) (Lang et al., 2018), suggested that the transcription of \(\text{degP1}\) and \(\text{actJ}\) are \(\sigma^{E2}\)-independent (Meier and Müller, unpublished results) (Fig. S2).

### 3.3. \text{actJ} and \text{actK} are co-transcribed and both genes are relevant for \textit{S. meliloti} 2011 acid growth

Schlüter and collaborators (Schlüter et al., 2013) mapped transcription start sites all over the genome of \textit{S. meliloti} strain 1021 and suggested that \(\text{actJ}\), \(\text{actK}\) and \(\text{glnE}\) were part of an operon. To experimentally confirm this prediction, we performed intergenic PCR reactions on randomly synthetized cDNA in order to analyze which genes were co-transcribed in a unique mRNA. We confirmed that at pH 7.0 and pH 5.6 \(\text{actJ}\), \(\text{actK}\) and \(\text{glnE}\) were co-transcribed in a unique mRNA (Fig. 3, B and C). \(\text{degP1}\), however, was co-transcribed with \(\text{actJ}\), \(\text{actK}\) and \(\text{glnE}\) mainly at pH 5.6 (Fig. 3A). It may be likely that \(\text{degP1}\) and \(\text{actJ}\) promoter regions possess distinct activities at each pH values. In such scenario, \(\text{rpoD}\) promoter predicted before \(\text{actJ}\) may function at pH 7.0 and pH 5.6, while \(\text{rpoE2/rpoD}\) predicted promoters controlling \(\text{degP1}\) may gain activity at pH 5.6. Next, we asked whether the native expression of \(\text{degP1}\) and \(\text{actJ}\) are regulated by pH. Using promoter-EGFP fusions we found that \(\text{degP1}\) and \(\text{actJ}\) transcription augments in low pH in an ActJ-dependent manner (Fig. 4).

The operon structure and prompted us to determine whether \(\text{actJ}\), \(\text{actK}\) or \(\text{glnE}\) mutations had any effect on acid stress tolerance. Since \(\text{actJ}\) was the first Open Reading Frame (ORF) of the operon and the plasmid insertion in \(\text{actJ}\) strain might also create a polar mutation, in-frame deletion mutants were constructed to further evaluate their associated phenotypes (\(\Delta\text{actJ}\), \(\Delta\text{actK}^1\), \(\Delta\text{actK}^2\), \(\Delta\text{actJ}\Delta\text{actK}^1\) and \(\Delta\text{glnE}\)). \(\Delta\text{actK}^2\) was
constructed to maintain the predicted asRNA_1142 and asRNA_1143 coding sequences, since asRNA_1142 was disrupted in ∆actK'. Results showed a significantly reduced growth rate at low pH for single- and double-deleted TCS strains, whose phenotypes were restored when the wt version of the genes were expressed in trans (Fig. 5). However, ∆glnE growth in acidic conditions, however, did not differ from the wt control, showing that GlnE is not involved in such stress tolerance.

The altered growth condition of TCS mutant strains suggested an active role of the system in defense against acid pH and seems to do not take part in the so-called General Stress Response (Francez-Charlot et al., 2015) since we did not find any growth difference between ∆actJ and wt strain when exposed to other abiotic stresses (Fig. S3A). Moreover, we could not associate this putative TCS with mechanisms related with envelope stress tolerance, since wt and ∆actJ strains exhibited similar growth when where plated in TY media supplemented with SDS (Fig. S3B).

Protein domain analysis predicted a conserved aspartate (Asp55) in ActJ and a conserved histidine (His250) in ActK. We hypothesized that these two residues were necessary for the phosphorylation cascade. Indeed, while wt ActJ and ActK rescued mutant acid growth, ActJD55A and ActKH250A variants did not (Fig. S4).

3.4 - ActJ triggers an adaptative acid-tolerance response (ATR) in S. meliloti 2011

Mildly acidic pre-incubation increases the ability of some bacteria to survive to a subsequent exposure to lethal acidic conditions. This adaptative mechanism is known as Acid-Tolerance Response (ATR) (Foster and Hall, 1990) and is potentially present in S. meliloti and many bacteria (Chorianopoulos et al., 2011; Draghi et al., 2010; Fingermann and Hozbor, 2015; Ryan et al., 2016).
We sought to determine whether the RR ActJ had an active role in triggering the ATR phenotype. To this aim, we employed a condition that induces *S. meliloti* ATR+ in batch cultures (Draghi et al., 2010). While pre-incubating ∆actJ or wt strains in neutral condition showed no significant differences on their decimal reduction rate (D10, time required for the number of cells to be reduced by a factor of 10) during the ulterior acid shock, pre-incubation of both strains in mildly acidic conditions (pH 6.1) followed by an acid shock resulted in an almost two-times decrease of ∆actJ D10 compared with *S. meliloti* 2011 (Fig. 6). In conclusion, these results demonstrated that ActJ is actively involved in developing an ATR response.

3.5 - ActJ and ActK are not essential for root colonization or nodulation

To further examine ActJK function in *S. meliloti* biology, we tested the ability of both mutants to colonize alfalfa roots. Based on a previously developed strategy to evaluate root colonization (Salas et al., 2017), we carried out double inoculation studies of mutant strains and *S. meliloti* 2011 20MP6 GFP+ (Pistorio et al., 2002) in a 1:1 ratio. The results showed no significant differences between the input and output proportions of mutant/S. *meliloti* GFP+ ratio (Fig. 7A). In order to rule out the possibility of extracellular complementation of the mutants by the wt, comparisons of the root colonization abilities of wt and mutant strains were performed in single inoculation experiments. No differences were detected by comparisons of the *Medicago sativa* root colonization among *S. meliloti* 2011 wt or mutant strains (Fig. 7B) suggesting that the ActJK TCS would not participate in pre-infective root colonization stages.

In order to evaluate the involvement of ActJ and ActK in plant tissue colonization and nodulation, we carried out nodulation kinetics assays. The mutants behaved in a
similar way to the parental strain, and alfalfa plants yielded a comparable number of
root nodules when inoculated with wt or mutant strains (Fig. S6). Moreover, the
macroscopic morphology of the nodules showed no differences in size and shape (data
not shown).

Altogether, these data show clear evidence that ActJK do not participate in early
stages of the symbiotic association.

3.6 - ActK is required for proper biological nitrogen fixation

The efficiency of symbiotic association was analyzed by indirect evaluation of
biological nitrogen fixation (BNF) activity in the host plant *M. sativa*. Alfalfa plants were
inoculated with *S. meliloti* 2011 wt or its isogenic mutants. Compared with wt-inoculated
plants, Δ*actK*1-, Δ*actK*2-, or Δ*actJΔactK*1-inoculated plants displayed lower aerial
biomass; while Δ*actJ* did not show statistically significant differences (Fig. 8). These
results demonstrate that ActJ and ActK contributed distinctly in *S. meliloti* symbiotic
processes and raise the possibility that ActK would regulate additional proteins apart
from ActJ when bacteria are associated with the host. The complemented strain (Δ*actK*1
+ p::*actK*) did not restore levels of the shoot dry weight (Fig. 8). A possible explanation
for this is that the expression and activity of this protein could be dosage or
phosphorylation dependent. We could not use IPTG for inducing *actK* in planta and then
the ActK temporal expression or phosphorylation may not be optimal in this study.

In addition, given that there were no differences between Δ*actK*1- or Δ*actK*2-
inoculated plants (Fig. 8), we also propose that asRNA_1142 did not have a relevant
role in the BNF process.

3.7. ActJ and ActK are required for proper nodule occupancy
Mature indeterminate nodules have four zones which define tissue organization (Vasse et al., 1990). Zone I corresponds to the apical meristem in the distal portion of the nodule. Zone II is characterized by recently infected plant cells where rhizobia are released into host cells and begin to differentiate to bacteroids. Zone III is an intermediate-large N₂-fixing zone constituted mostly by infected cells, containing a dense population of symbiosomes; i.e., compartments containing a single nitrogen-fixing bacteroid engulfed by the peribacteroid membrane (PBM). Zone IV, known as a senescent zone, appears in mature nodules and is characterized by a loss of symbiotic partnership (Franssen et al., 1992; Puppo et al., 2005).

We hypothesized that ActJK function may be important in symbiosis since bacteroids encounter a naturally acid environment in the peribacteroid space (PBS, the space between the PBM and the bacteroid) (Mellor, 1989). To investigate whether ActJ and ActK proteins could potentially play a role in any stage of nodulation such as tissue organization, bacteroid differentiation, or nodule maturation, we analyzed longitudinal histological sections of wt- or mutant-occupied nodules by light microscopy. Micrographs of *M. sativa* nodules induced by actJK mutants did not present obvious differences in tissue organization compared with those generated by wt rhizobia (data not shown). However, mutant-derived nodules showed a lower occupation by bacteroids in zone III (Fig. 9A). In nodules infected with ∆actJ or ∆actK¹, certain irregular granules could also be observed within the plant cells in zone III (Fig. 9A, panels b and c). According to size, morphology and coloration obtained by light reflection, they might correspond to starch granules (Vasse et al., 1990).
These pieces of evidences suggest that both ActJ and ActK are necessary for full bacteroid nodule occupation.

3.8. ActJ and ActK are critical for nodule structure progression and terminal bacteroid differentiation

Transmission electron microscopy (TEM) was employed to evaluate the ultrastructure of wt and mutant bacteroids and their associated symbiosomes (Fig. 9B). In all cases, differentiated bacteroids were clearly identified. In plant cells occupied by actJK mutants, an increased amount of white structures was observed in the cellular periphery, probably comprising reserve granules, consistent with that observed by light microscopy. When bacteroids ultrastructure was analyzed, marked differences were also observed. Wt bacteroids presented uniform cellular interior, while mutant bacteroids showed intercalated regions of high and low contrast, probably corresponding to different nucleic acid condensation degrees (Vasse et al., 1990) (Fig. 9B, right column). In addition, mutant bacteroids exhibited an elongated morphology compared to wt bacteroids (Fig. 9B, right column).

Fig. 9 also depicts mutant-associated PBM with irregular or disrupted phenotype contrasting with what is shown in wt-occupied nodules (Fig. 9B, center and right columns). In addition, irregular PBM correlated with the occurrence of more evident PBSs. This latter finding was more noticeable in ΔactK^1 and ΔactK^2 nodules (Fig. 9B, panels 9 and 12), suggesting an impaired physical PBM-bacteroid connection that would lead to an altered transport activity.

To evaluate whether mutant bacteroids progressed to premature death due to an inappropriate interaction with PBM, we carried out live/dead staining assay on freshly
dissected nodules, followed by confocal microscopy analysis. Generally, all ActJK mutants exhibited a depleted phenotype regarding nodule occupation in zone III (Fig. 10, left and center panels). Furthermore, bacterial death in ΔactJ-nodule zone III indicated a possible alteration in bacteroid membrane integrity (Fig. 10, Panel 5). Impaired nodule occupation was undoubtedly noticeable in zone IV, where far more empty cells in mutant- vs wt-induced nodules could be seen. This finding could reflect the arrest of bacteroid differentiation or the loss of bacteroid persistence (Horváth et al., 2015).

Altogether, we demonstrated that ActJK is critical for terminal bacteroid differentiation and for bacteroid-PBM physical interaction.

4. DISCUSSION

It is well established that low environmental pH strongly affects viability and symbiosis in *S. meliloti* and other rhizobia (Caetano-Anollés et al., 1989; Munns, 1968; Vassileva et al., 1997; Zahran, 1999). In view of this fact, and of the global distribution of acid soils, the understanding of rhizobium acid adaptation is extremely important to improve symbiosis efficiency under such detrimental conditions. Bacteria use TCSs to sense changes in the surroundings and consequently adapt their physiology to cope with stressful environments. As two-component signal transduction modulates RR activities leading to differential gene expression, focusing the study on the roles of RR in developing cell adaptation becomes a useful approach.

In this work, a collection of 37 Tn5 transposon mutants and plasmid insertional mutants was screened to identify RR-encoding genes potentially involved in low pH
adaptation. The analysis of mutant growth rate under normal and stress conditions (Fig. 1) allowed us to identify mutants with impaired ability to cope with acid stress (ntrC\textsuperscript{−}, cpoR1\textsuperscript{−} and SMc02366). Here we focused on the role of SMc02366 (renamed as actJ).

Interestingly, although the majority of TCS exerts its regulatory function by phosphotransfer reactions without changing its expression, this work shows that actJ and actK transcription increase in acid conditions (Fig. 4) in agreement on previous reported transcriptomic data (Draghi et al., 2016; Hellweg et al., 2009). The conservation of the ActJK system within Rhizobiales (Fig. S1) suggests that the ActJK signaling pathway may be operating in other organisms in addition to S. meliloti. Indeed, the ActJK orthologue RS05450/RS05455 in Rhizobium tropici CIAT 899 (89% and 69% identity, respectively, at amino acid level) was recently found to be up-regulated in acid stress response (Guerrero-Castro et al., 2018).

When we evaluated actJ operon and degP1 transcription at distinct pHs, we found that while actJ operon is equally expressed at pH 7.0 and pH 5.6, degP1 expression is increased at pH 5.6 (Fig. 3). This suggests that σ\textsuperscript{E2} function on degP1 becomes more important under acid stress. In unstressed conditions, our cappable-seq data showed that σ\textsuperscript{70} controls the expression of both degP1 and actJ operon (Meier and Müller, unpublished results) (Fig. S2). Accordingly, previous studies showed that whereas σ\textsuperscript{70} is responsible for the transcription of most genes expressed in exponentially growing cells (Gruber and Bryant, 1997), σ\textsuperscript{E2} emerge as important for expressing genes related to stress situations (Flechard et al., 2010; Rouvière et al., 1995; Sauviac et al., 2007)
Using in-frame deletion mutants, we demonstrated that individual- or double-mutation of \textit{actJ} and \textit{actK} negatively impacted on \textit{S. meliloti} acid growth (Fig. 5). However, \textit{glnE} mutant strain was insensitive to such stress condition. It has been reported that organisms like \textit{E. coli} can harness glutamine to cope with acid stress (Djoko et al., 2017), indicating a physiologic connection between nitrogen metabolism and acid stress tolerance. Although was found highly conserved in Rhizobiales order (Fig. S1), our experimental approach suggests that the yet unexplored GlnE protein would not act in \textit{S. meliloti} acid adaptation.

Proteins ActJ and ActK contain typical RR and HK domain architectures and amino acid residue conservation. We tested whether the absence of phosphorylatable residues in ActJK would impact in \textit{S. meliloti} phenotype. Consistent with the bioinformatic prediction, neither ActJD55A nor ActKH250A restored wt levels of growth under acid conditions (Fig. 5), providing solid evidence that a TCS is operating within these proteins. The possibility that H$^+$ ions directly interact with ActK sensing domain and activate the TCS signaling will deserve special attention. In many proton-sensing HKs, such mechanism relies on histidine and glutamate residue protonation occurring in mildly acidic conditions -nearly at pH 6.0 and pH 4.0, respectively- (Perez and Groisman, 2007; Soncini and Groisman, 1996). In this context, we hypothesized that ActK proton sensing might be occurring in the sole and conserved histidine located in the periplasmic sensing domain (Fig. S5).

The naturally acidic environment of the infection thread and the symbiosome (Mellor, 1989; Pierre et al., 2013), together with the increase of \textit{actJ} and \textit{actK} transcription found in \textit{Medicago truncatula} nodules (Roux et al., 2014) encouraged us
to hypothesize that ActJK would be actively operating in plant-rhizobium interaction as well. Unexpectedly, we found that ActK was relevant for the establishment of BNF while ActJ was not (Fig. 8) suggesting that ActJ and ActK might contribute distinctly on key processes taking place in *Medicago sativa* nodules. In this regard, we hypothesized that the difference observed between ∆actK1 and ∆actJ inoculated plants would be associated with a phosphorylation crosstalk (Laub and Goulian, 2007; Skerker et al., 2008) between ActK and another cytosolic factor when ActJ is absent that would lead to the expression of important genes for symbiotic interaction. In addition, in many cases a sensor kinase can also acts as a phosphatase, and crosstalk from small molecules such as acetyl phosphate can drive phospho-response regulator levels to significant levels causing spurious activation. Further studies are needed to determine whether this is possible.

Since initial steps of mutant-plant association – i.e., root colonization (Fig. 7) – were not impaired and because mutant-induced nodules explored by microscopy exhibited less occupation (Figs. 9 and 10), we were tempted to speculate that the acidic environment within the infection thread or the mature nodule (Geddes et al., 2014; Pierre et al., 2013) was detrimental for actJK mutants survival. However, our microscopic studies demonstrate that ActJK is not an important TCS for bacteroid survival in symbiosomes (Fig. 10) since PBM disintegration in mutant-occupied symbiosomes did not mirror an eventual bacteroid exposure to plant reactive oxygen species or proteases (Puppo et al., 2005). Of note, the impaired interaction of mutant bacteroids with symbiosomal PBM constitutes a key aspect that will need future consideration. The plant-derived PBM is not only a barrier between the bacterium and
the host. It has a crucial role in nutrient exchange between the symbiotic partners (Catalano et al., 2004; Saalbach et al., 2002; White et al., 2007) and it has been proposed that a physical interaction is needed (Bolanos et al., 2004). In this context, the impaired PBM-bacteroid interaction found in actK-occupied nodules might contribute to the altered BNF reported.

5. CONCLUSIONS

This work characterizes the TCS ActJK in the biology of S. meliloti and achieves an important step in comprehending the genetic basis of acid stress response in S. meliloti 2011. ActJK constitutes a TCS whose transcription is triggered in acid condition and is important for mild-acid growth and for establishing an ATR phenotype. In addition, our results show that ActJK-controlled functions are crucial for optimal symbiosis development, presenting ActJK as a system with a broad function in S. meliloti dual-life. Although our analysis does not provide a complete picture of the role of this TCS, it represents a missing piece of the puzzle in previous studies. Future proteomic and transcriptomic approaches will help to identify the regulon under ActJ control at low pH conditions.

AUTHOR CONTRIBUTIONS

Francisco J. Albicoro: Conceptualization, Methodology, Investigation, Writing, Walter O. Draghi: Investigation, Visualization, María C. Martini: Investigation, María E. Salas:
Investigation, **Gonzalo A. Torres Tejerizo:** Investigation, **Mauricio J. Lozano:** Investigation, **José L. López:** Investigation, **Carolina Vacca:** Investigation, **Juan H. Cafiero:** Investigation, **Mariano Pistorio:** Investigation, **Hanna Bednarz:** Resources, **Doreen Meier:** Visualization, Investigation, **Antonio Lagares:** Conceptualization, **Karsten Niehaus:** Conceptualization, Resources, **Anke Becker:** Conceptualization, Resources, **María F. Del Papa:** Writing- Reviewing and Editing, Supervision.

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**FIGURE LEGENDS**

**Figure 1**

**Fig. 1.** Identification of response regulator genes involved in acid stress tolerance. A. Growth rate analysis (μ) of *S. meliloti* 2011 wt and SMc01043 (ntrC), SMc04044 (cpdR1) or SMc02366 (actJ) mutants, grown in batch GS media at indicated pH. 34 additional *S. meliloti* RR mutants also tested (Table 1) are not shown since their growth rate did not differ from the wt. Bars represent standard deviation of the mean of three experiments. t-test analysis indicates lower growth rates with 99% of confidence. B. Colony forming units (CFU) registered over time of *S. meliloti* 2011 wt or SMc02366 (actJ) insertion mutant grown in GS media pH 7.0 or pH 5.6. Bars represent standard deviation of the mean of three experiments. ND: non-detectable.

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**Fig. 2.** Genetic elements located on actJ boundaries. Schematic representation of a 14 kb region of *S. meliloti* 2011 chromosome surrounding response regulator gene actJ (locus tags SMc02361-9). Open reading frames are indicated with colored arrows. Gene denominations, *in silico* predicted promoters, and cis-encoded antisense RNAs (asRNA), are also displayed. The dotted triangles indicate actJ, actK, and glnE coding regions that were in-frame deleted to yield ∆ActJ, ∆ActK, and ∆GlnE mutant strains. Numbers in parentheses indicate the amino acids deleted.
Fig. 3. actJ, actK and glnE are co-transcribed in a unique mRNA. Gel electrophoresis assays showing end-point PCRs conducted on cDNA samples using primers that amplify the boxed intergenic regions degP1-actJ (A), actJ-actK (B), or actK-glnE (C). To evaluate transcriptional differences related to the acid condition, we randomly retrotranscribed cDNA using RNA samples from neutral (pH 7.0) or acid cultures (pH 5.6). As internal controls S. meliloti 2011 wt genomic DNA (+) or distilled water (-) were used. Previous to retrotranscription, PCRs on DNAse-treated RNA were performed as controls.
**Fig. 4.** *S. meliloti* ActJ is required for the expression of *degP1* and *actJ* in acid conditions. *degP1* (A) and *actJ* (B) promoter activities estimated as fluorescence mediated by promoter-EGFP fusions in minimal medium GS at pH 7.0 and 5.6 in *S. meliloti* expressing or not expressing ActJ. Fluorescence measurements were performed at 20 h after inoculation (logarithmic growth phase). Error bars show SDs of three biological replicates, Tukey’s HSD test (*p<0.05*).

*Fig. 4.* *S. meliloti* ActJ is required for the expression of *degP1* and *actJ* in acid conditions. *degP1* (A) and *actJ* (B) promoter activities estimated as fluorescence mediated by promoter-EGFP fusions in minimal medium GS at pH 7.0 and 5.6 in *S. meliloti* expressing or not expressing ActJ. Fluorescence measurements were performed at 20 h after inoculation (logarithmic growth phase). Error bars show SDs of three biological replicates, Tukey’s HSD test (*p<0.05*).
Fig. 5. ActJ and ActK are necessary for *S. meliloti* acid stress tolerance. Exponential growth rates (μ) of *S. meliloti* 2011 wt or mutant strains (ΔactJ, ΔactK¹, ΔactK², ΔactJΔactK¹, ΔglnE) in cultures grown on GS medium at pH 5.6. Full restoration of the wt phenotype was observed when ActJ and ActK were complemented in trans (ΔactJ + p::actJ, ΔactK¹ + p::actK, ΔactK² + p::actK). As internal control, mutants harboring empty pSRKGm vector were also evaluated (ΔactJ + p, ΔactK¹ + p and ΔactK² + p). Bars represent standard deviation of the mean of three experiments. The statistical analysis were performed by comparing each strain with wt strain (t-test analysis, *p*<0.05).
Fig. 6. Survival rates of *S. meliloti* 2011 wt or actJ mutant (∆actJ) in lethal acid condition (pH 4.0). Cells were previously grown in neutral pH or pH 6.1 Evans minimal medium until early-log phase and then resuspended in equal volume of fresh medium pH 4.0 as indicated in Materials and Methods. The death rate was followed by plating dilutions of the bacterial suspension at different times in Evans agar plates (pH 7.0). The decimal reduction rate (D_{10}) corresponding to bacteria previously grown in neutral condition are not different, while bacteria previously grown in acid condition led to statistically different D_{10} values (Tukey’s HSD test (*p<0.05). Bars represent standard deviation of the mean of three independent experiments.

**Fig. 6.** Survival rates of *S. meliloti* 2011 wt or actJ mutant (∆actJ) in lethal acid condition (pH 4.0). Cells were previously grown in neutral pH or pH 6.1 Evans minimal medium until early-log phase and then resuspended in equal volume of fresh medium pH 4.0 as indicated in Materials and Methods. The death rate was followed by plating dilutions of the bacterial suspension at different times in Evans agar plates (pH 7.0). The decimal reduction rate (D_{10}) corresponding to bacteria previously grown in neutral condition are not different, while bacteria previously grown in acid condition led to statistically different D_{10} values (Tukey’s HSD test (*p<0.05). Bars represent the standard deviation of the mean of three independent experiments.
Fig. 7. ActJ and ActK are not involved in mechanisms related with root colonization. A. One-day-old alfalfa seedlings were placed in plastic pots with vermiculite following inoculation with a 1:1 mix of (input): wt S. meliloti 2011 / S. meliloti 2011 GFP+ (wt); S. meliloti 2011 actJ mutant / S. meliloti 2011 GFP+ (ΔactJ); or S. meliloti 2011 actK1 mutant / S. meliloti 2011 GFP+ (ΔactK1). After 3 days, plants were removed and root-adhered bacteria were recovered by vortexing in sterile physiologic solution. Bacteria recovery was registered by plating serial dilutions (output). Input (dashed bars) and output (filled bars) data were plotted proportionally to S. meliloti 2011 GFP+. B. Root colonization assays. One-day-old alfalfa seedlings were placed in plastic pots with vermiculite following inoculation with wt S. meliloti 2011 or mutant strains. After 3 days, plants were removed, and root-adhered bacteria were recovered by vortexing in sterile physiologic solution. In both A and B assays, the input inocula were 10⁷ CFU/pot. Bars represent the standard deviation of the mean of three independent experiments. Results of any given strain were compared with those of the wt strain in the same experiment by using a single-factor ANOVA test (p<0.05).
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Figure 8

Fig. 8. ActJK involvement in S. meliloti – M. sativa symbiosis. Symbiotic performance of S. meliloti 2011 wt or S. meliloti 2011 ActJK mutants (∆actJ, ∆actK1, ∆actK2, ∆actJ∆actK1), measured as aerial biomass of M. sativa plants after 35 days post-inoculation. The ability of ActK restoration in trans was also evaluated (∆actK1 + p::actK). The data shown in this figure is representative of three independent experiments. Error bars represent the standard error of the mean (SEM) of 30 plants. ANOVA statistical analysis were performed, and asterisks indicate significant differences related with wt strain (*, p<0.1; ****, p<0.0001). ∆actK1 + p, ∆actK1 strain transformed with pSRKGM empty vector; NI, not-inoculated plants; n.s., not statistically significant.

Fig. 8. ActJK involvement in S. meliloti – M. sativa symbiosis. Symbiotic performance of S. meliloti 2011 wt or S. meliloti 2011 ActJK mutants (∆actJ, ∆actK1, ∆actK2, ∆actJ∆actK1), measured as aerial biomass of M. sativa plants after 35 days post-inoculation. The ability of ActK restoration in trans was also evaluated (∆actK1 + p::actK). The data shown in the figure is representative of three independent experiments. Error bars represent the standard error of the mean (SEM) of 30 plants. ANOVA statistical analysis were performed, and asterisks indicate significant differences related with wt strain (*, p<0.1; ****, p<0.0001). ∆actK1 + p, ∆actK1 strain transformed with pSRKGM empty vector; NI, not-inoculated plants; n.s., not statistically significant.
Fig. 9. Lack of actJ and actK impact on nodule occupancy and bacteroid differentiation.

A. Light microscopy (LM) assays conducted on nitrogen-fixing regions (zone III) of 28-days-old nodules occupied by S. meliloti 2011 wt or actJK mutants. Plant cells seems to have lower bacteroid occupation in actJK mutants. At the periphery of plant cells possible starch granules can be distinguished, being more evident in ΔactJ and ΔactK1-occupied nodules (arrows). Head arrows depict bacteroids. IC, infected cell; nIC, non-infected cell; V, vacuole. B. Bacteroids ultrastructure were explored by transmission electron microscopy and three magnifications are shown (left, center and right column). Accordingly, with LM, white-refringents granules can be seen near plant cell membranes (arrows, left column). Bacteroids ΔactJ, ΔactK1, ΔactK2 and ΔactJΔactK2 manifest less physical contact with peribacteroids membranes than the wt (arrows, center and right columns), displaying bigger peribacteroid spaces (asteriks, center column). Finally, ultrastructure evaluation (right column) showed regions of different contrast in actJK mutants (exemplified with dashed arrows) that are not displayed in wt bacteroids (panel 3); B0, 2 μm (left column); 1 μm (center column); 0.5 μm (right column).
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**Fig. 10.** Live/dead staining performed on nodule sections visualized using confocal microscopy. 28-days-old nodules colonized by *S. meliloti* 2011 wt or *S. meliloti* deletion mutants were vibratome-dissected and stained with live/dead solution as indicated in Materials and Methods. Rows visualize Zone III (left and center columns) and Zone IV (right column) of the same nodule occupied by the indicated strain. Plant cells occupied by live or dead bacteroids are shown in green or red, respectively. Plant cells nucleus and meristems are red-stained, as well (big red dots and panel 7). White arrow in Panel 5 depicts a plant cell co-occupied with live and dead bacteroids. Bars: 100 μm in left and right columns, and 20 μm in center column.
Table 1. Bacterial strains used in this study

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<th>Strains</th>
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<th>Source/Reference</th>
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<td>wt, spontaneous Sm&lt;sup&gt;r&lt;/sup&gt; derived from strain SU47</td>
<td>J. Dénarié, France</td>
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**S. meliloti 2011 RR mutants**

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RR, response regulator; Sm, streptomycin; Nm, neomycin, Tc, tetracyclin.