



# Role and Regulation of ACC Deaminase Gene in *Sinorhizobium meliloti*: Is It a Symbiotic, Rhizospheric or Endophytic Gene?

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Plant-associated bacteria exhibit a number of different strategies and specific genes allow bacteria to communicate and metabolically interact with plant tissues. Among the genes found in the genomes of plant-associated bacteria, the gene encoding the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (*acdS*) is one of the most diffused. This gene is supposed to be involved in the cleaving of plant-produced ACC, the precursor of the plant stress-hormone ethylene toning down the plant response to infection. However, few reports are present on the actual role in rhizobia, one of the most investigated groups of plant-associated bacteria. In particular, still unclear is the origin and the role of *acdS* in symbiotic competitiveness and on the selective benefit it may confer to plant symbiotic rhizobia. Here we present a phylogenetic and functional analysis of *acdS* orthologs in the rhizobium model-species *Sinorhizobium meliloti*. Results showed that *acdS* orthologs present in *S. meliloti* pangenome have polyphyletic origin and likely spread through horizontal gene transfer, mediated by mobile genetic elements. When *acdS* ortholog from AK83 strain was cloned and assayed in *S. meliloti* 1021 (lacking *acdS*), no modulation of plant ethylene levels was detected, as well as no increase in fitness for nodule occupancy was found in the *acdS*-derivative strain compared to the parental one. Surprisingly, *AcdS* was shown to confer the ability to utilize formamide and some dipeptides as sole nitrogen source. Finally, *acdS* was shown to be negatively regulated by a putative leucine-responsive regulator (LrpL) located upstream to *acdS* sequence (*acdR*). *acdS* expression was induced by root exudates of both legumes and non-leguminous plants. We conclude that *acdS* in *S. meliloti* is not directly related to symbiotic interaction, but it could likely be involved in the rhizospheric colonization or in the endophytic behavior.

**Keywords:** *Sinorhizobium meliloti*, ACC deaminase, ethylene, *acdS*, nitrogen sources, endophytic colonization, rhizosphere

## INTRODUCTION

Plant-bacteria interactions have been studied since long time as reciprocal beneficial association (symbiosis), as neutral interaction (commensalism), and as pathogenic interaction. Despite many details are known on the molecular bases of all the above-mentioned interactions (Lugtenberg et al., 2002) a number of genes present in the genome of plant-associated bacteria is still under debate and a complete explanation of the various mechanisms used by plant-associated bacteria is still lacking. Recent analyses suggested the presence of a core set of genes in plant-associated bacterial genomes (Pini et al., 2011), which include genes related to transport, regulation, sugar metabolism, etc. However, many plant-associated bacteria exhibit several additional genes, related to the different type of interaction they have (e.g., nitrogenase for symbiotic rhizobia). One of the mostly diffused additional genes among rhizospheric and endophytic bacteria is that encoding the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, referred to as *acdS* (Nascimento et al., 2014; Singh et al., 2015). Biochemically, ACC deaminase is able to degrade the precursor of ethylene biosynthesis, ACC, into ammonium and  $\alpha$ -ketobutyrate (Honma and Shimomura, 1978). Ethylene is working as a plant hormone and affects all stages of plant development and growth (Deikman, 1997), mainly in relation with biotic and abiotic stresses (Abeles and Heggstad, 1973). The ACC deaminase structural gene (*acdS*) has been found in many rhizosphere bacteria, in symbiotic rhizobia, in bacterial endophytes, in fungi and in the genomes of several plants, as *Arabidopsis* (Singh et al., 2015).

The presence of ACC deaminase in plant-associated bacteria, has been interpreted as a way to use the additional nitrogen source (represented by ACC), consequently decreasing the amount of ACC available by the plant for the production of the phytohormone ethylene (Glick et al., 1998; Holguin and Glick, 2003; Prigent-Combaret et al., 2008; Gamalero and Glick, 2015). The reduction of ethylene production by the plant may have positive effect over colonization of plant tissue by bacteria. Indeed, ethylene is known to have an inhibitory effect on rhizobial infection, limiting formation and number of nodule for plants and the root growth (Nukui et al., 2006). The decrease of ethylene emission may increase root system development (Penrose and Glick, 2003) and enhance nutrients and water uptake (Reid and Renquist, 1997), thus allowing a higher number of symbiotic nodules to be formed on host plant root system. Moreover, endophytic plant growth promoting bacteria (as *Burkholderia phytofirmans* PsJN, *Pseudomonas fluorescens* YsS6, and *Pseudomonas migulae* 8R6) are less effective when their *acdS* orthologs are deleted (Sun et al., 2009; Ali et al., 2012; Nascimento et al., 2014). In the nitrogen-fixing symbiont *Mesorhizobium loti* *acdS* gene has been shown to be transcribed inside root nodules, suggesting an involvement in the symbiotic process.

Phylogenetic analyses suggested that horizontal gene transfer (HGT) has played a strong role in *acdS* spreading within taxonomic groups (Blaha et al., 2006; Nascimento et al., 2012; Lemaire et al., 2015). On the other hand, recently (Nascimento et al., 2014), a detailed phylogenetic reconstruction has been

performed, showing that *acdS* orthologs are preferentially vertically inherited along the bacterial phylogeny. However, due to the scattered occurrence in the same species, HGT events can still be supposed, at least at the species or genus level, and selective advantages conferred to strains has to be clarified. In particular, comparative genomic analyses have shown that *acdS* orthologs are part of the dispensable genome fraction in species as the model symbiotic rhizobium *Sinorhizobium meliloti*. In *S. meliloti*, a previous genome analysis suggested *acdS* as one of the genes which may explain different symbiotic phenotypes among strains (Galardini et al., 2011). However, no experimental indication of its role in the symbiotic performance was reported.

Previous works demonstrate the presence and the correlation of a regulatory region upstream to *acdS* gene belonging to the *lrp* family (leucine-responsive regulatory gene like), called *acdR* (Grichko and Glick, 2000; Ma et al., 2003b) in *Pseudomonas putida* UW4, *Rhizobium leguminosarum* bv.viciae 128C53K (Grichko and Glick, 2000), *Azospirillum lipoferum* 4B and most other *acdS*+ *Proteobacteria* (Prigent-Combaret et al., 2008), confirming that usually in *Proteobacteria* the regulatory genes are close to the genes they regulate. However, its mode of regulation (in relation with *acdS* and its promoter) is not totally clarified, especially in relation with the symbiotic partner plants.

In this work, we aimed at define the evolution and the functional profile of *acdS* and its regulatory gene *acdR* in the model plant symbiont *S. meliloti*. Our study showed that HGT has played a strong role in shaping *acdS* phylogeny in *S. meliloti*, suggested additional roles, not related with ethylene modulation and symbiosis, which may have selected its presence in the dispensable genome fraction of *S. meliloti* (Nascimento et al., 2014) and allowed to confirm common trends on the evolution of modules of regulatory interactions in bacteria (Babu et al., 2006).

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

The strains and plasmids used in this work are listed in **Table 1** and Supplementary Table S1. In particular a collection of *S. meliloti* strains from different geographical areas was used (Carelli et al., 2000; Roumiantseva et al., 2002, 2014; Talebi Bedaf et al., 2008; Trabelsi et al., 2009, 2010). *S. meliloti* strains were cultured on solid or liquid TY medium with 0.2 g/liter  $\text{CaCO}_3$ , or Vincent Minimal Medium (VMM, or Rhizobium Defined Medium, RDM), while *Escherichia coli* strains were grown in Luria Bertani (LB) medium, supplemented with antibiotics when necessary.

### Detection of *acdS* Gene, Genomic Context, Analysis, and Phylogenetic Reconstruction

The presence of *acdS* orthologs in a collection of 133 *S. meliloti* strains was performed by PCR amplification on crude lysates using the two sets of primers and the PCR conditions described in Duan et al. (2009). *S. meliloti* 1021 was used as negative control, while *S. meliloti* AK83 was used as positive controls. Agarose gel electrophoresis on 1.5% TAE buffer and ethidium bromide staining (10 mg/l) was used for visualization of amplification products on an UV transilluminator. Positive amplification

**TABLE 1 | Strains and plasmids used in the work.**

Strains (or plasmids)	Description	References
<i>S. meliloti</i> 1021, BM 678	Str <sup>r</sup> derivative from strain 2011	Meade et al., 1982
<i>S. meliloti</i> AK83, BM 493	Lacks part of the microaerophilic gene set on pSymA-homolog megaplasmid	Galardini et al., 2011
<i>E. coli</i> S17-1 $\lambda$ pir	Tp <sup>r</sup> , Sm <sup>r</sup> , <i>recA</i> , <i>thi</i> , <i>hsdR</i> <sup>−</sup> M <sup>+</sup> , RP4::2-Tc::Mu::Km::Tn7, $\lambda$ pir lysogen	Simon et al., 1983
BM 193	<i>E. coli</i> S17-1 $\lambda$ pir + expression vector lac promoter regulation pSRK-Km (Km <sup>r</sup> )	L. Ferri, unpublished
BM 637	<i>E. coli</i> S17-1 $\lambda$ pir + pSRK-Km (Km <sup>r</sup> ) with <i>acdS</i> gene from AK83 strain	This work
BM 261	<i>S. meliloti</i> 1021 pSRK- Km (Km <sup>r</sup> )	Pini et al., 2014
BM 641	<i>S. meliloti</i> 1021 + pSRK- <i>acdS</i> AK83 (Km <sup>r</sup> )	This work
BM 634	<i>E. coli</i> S17-1 $\lambda$ pir +promoterless vector GFP pOT2 (Gm <sup>r</sup> )	This work
BM 668	<i>S. meliloti</i> 1021 pOT2 (Gm <sup>r</sup> )	This work
BM 689	<i>E. coli</i> S17-1 $\lambda$ pir pOT2 + <i>acdS</i> promoter (144 bp) (Gm <sup>r</sup> )	This work
BM 690-144 bp	<i>S. meliloti</i> 1021 pOT2 + <i>acdS</i> promoter (144 bp) (Gm <sup>r</sup> )	This work
BM 674	<i>E. coli</i> S17-1 $\lambda$ pir pOT2 + <i>acdS</i> promoter + regulator (620 bp) (Gm <sup>r</sup> )	This work
BM 697-620 bp	<i>S. meliloti</i> 1021 pOT2 + <i>acdS</i> promoter+ regulator (620 bp) (Gm <sup>r</sup> )	This work

BM; Laboratory ID code, stands for "Bazzicalupo Marco"

products from two strains, representative of the collection (BO21CC, 2B13) were cloned into pGEM<sup>®</sup>-T Easy Vector Systems (Promega) following manufacturer's instructions and sequenced for confirmation of *acdS* presence.

Orthologs of *acdS* and *acdR* were retrieved from GenBank database running a blast search over Rhizobiaceae (taxid:82115) non-redundant nucleotide database on 2016-05-16 by using *acdS* gene from AK83 (Sinme\_5642) and *acdR* from AK83 (Sinme\_5643) as query sequence. The alignment of aminoacidic sequences was performed with ClustalW (Goujon et al., 2010). For phylogenetic reconstruction, a Model Test was run on aligned sequences to choose the best substitution model (Supplementary Data 1). The model with the lowest Bayesian Information Criterion (Schwarz, 1978; Nei and Kumar, 2000) was chosen for running Maximum Likelihood phylogenetic reconstruction (Anisimova and Gascuel, 2006). Robustness of dendrograms topology was inferred by running 500 bootstrap replicates. All steps of the phylogenetic reconstruction were performed with MEGA 7 software (Kumar et al., 2016). Alignments and nexus files of the phylogenetic reconstructions are included as Supplementary Data 1.

## Preparation of Cloning Vectors and Transformation of Strains

The *acdS* gene from AK83 (Sinme\_5642) strain was cloned into pSRK-Km vector under lac-promoter (Khan et al., 2008) and firstly used for transformation of *E. coli* S17-1 cells. Transformant cells were selected for resistance to Km (10  $\mu$ g/ml), and positive clones were used for biparental conjugation to *S. meliloti* 1021 (resistant to streptomycin, 200  $\mu$ g/ml). Conjugal transfer was performed as previously described (Pini et al., 2014). Gene expression was induced by treating *in vitro* plantlets inoculated with IPTG (at concentration of 0.23 mM). The promoter (144 bp fragment) and the promoter in association with transcriptional regulator (620 bp fragment) from BL225C strain was cloned into the promoter-less vector pOT2 containing GFP-uv (green fluorescent protein) as reporter gene (Karunakaran et al., 2005).

Recombinant vectors were used for transformation of *E. coli* S17-1 cells selected for resistance to Tc (10  $\mu$ g/ml), then the positive clones were used for biparental conjugation to *S. meliloti* 1021.

## ACC Deaminase Assay

Permeabilized cells were obtained from 5 ml overnight liquid cultures after harvesting cells by centrifugation, washing the pellet with 0.9% NaCl solution. Cell permeabilization was performed by adding by 600  $\mu$ l of 100 mM Tris HCl pH 8.5 and 30  $\mu$ l of toluene and vortexing for 30 s. After 1 h incubation at 4°C, lysed cells were centrifuged at 12000 rpm for 10 min and toluene was removed. The permeabilized cell suspensions were used for total protein content determination with Bradford reagent (Sigma-Aldrich) and enzymatic assays. ACC deaminase activity was quantified on crude cell extracts by measuring the amount of  $\alpha$ -ketobutyrate produced by the deamination of ACC, as previously described by Honma and Shimomura (1978) and Penrose and Glick (2003).

## In vitro Symbiosis Assays

Seedlings of *Medicago sativa* (cv. Pomposa) were sterilized in HgCl<sub>2</sub>, repeatedly washed, and germinated in sterile plastic Petri dishes for 72 h in the dark and 48 h in the light at room temperature. For *in vitro* assays, seedlings were transferred in Petri dishes containing Buffered Nod Medium (Ehrhardt et al., 1992) and 16 g/l of type A agar (Sigma-Aldrich). Plantlets were grown for an additional 3 to 5 days before inoculation with *S. meliloti* 1021, *acdS*-derivative and the parental strains. For nodulation assays, strains were grown in liquid TY medium at 30°C for 48 h with antibiotics if necessary, then washed three times in 0.9% NaCl solution and resuspended to an OD<sub>600</sub> nm of 1.0. Then, aliquots of  $1 \times 10^7$  cells were used, as previously described (Pini et al., 2013, 2014). Cells were directly spread over the seedling root. Plates were kept in a growth chamber maintained at 26°C with a 16-h photoperiod (100 microeinstein m<sup>−2</sup> s<sup>−1</sup>) for 40 days.

## Ethylene Measurement

Ten *M. sativa* (cv. Pomposa) germinated seeds (treated as previously described) were singularly sown in 120 ml glass vials containing 30 ml of Buffered Nod Medium (Biondi et al., 2009) and 16 g/l of type A agar (Sigma-Aldrich). Seedlings were grown for additional 2 days before inoculation with *S. meliloti* strains (1021 with the empty pSRK vector, 1021 *acdS*-derivative and the parental strain). The strains were grown in liquid TY medium at 30°C for 48 h, washed in 0.9% NaCl solution and resuspended to an OD 600 nm of 0.5 as previously described. Each vial was then inoculated with 500 µl of bacterial suspension (corresponding to  $\sim 5 \times 10^4$  cells).

The vials, sealed with Teflon septum and crimped with aluminum caps, were kept in a growth chamber at 23°C  $\pm$  1, under a 16 h photoperiod and 60 µmol m<sup>-2</sup> s<sup>-1</sup> photosynthetically active radiation provided by cool-white fluorescent lamps. The ethylene accumulation was detected at 30 or 60 days post inoculation (dpi).

Ethylene concentrations in the headspace were determined using an ultra-sensitive ETD-300 photo-acoustic laser spectrophotometer (Sensor Sense B.V., Nijmegen, The Netherlands. <http://www.sensor-sense.nl>) in combination with a gas handling system. In brief, the detector consists of a CO<sub>2</sub> laser and a photo-acoustic cell, in which the gas is detected. The detector is able to detect on-line about 300 parts per trillion by volume of ethylene within a 5-s time scale. The gas handling was performed by a valve control box (type VC-6, Sensor Sense B.V., Nijmegen, the Netherlands), designed for measuring up to six sampling cuvettes per experiment. In this experiment, the valve control box allowed automated sampling of ethylene accumulated into vials at a flow rate of 3 l h<sup>-1</sup> and its transport to the ETD-300 alternately, in succession for 15 min for each cuvette.

The air control was sourced completely from the compressed air source and was measured to contain less than 0.001 µl l<sup>-1</sup> ethylene. Statistical analysis of data has been performed with one-way ANOVA and Tukey *post-hoc* comparison by using Past software (Hammer et al., 2001).

## Nodule Colonization Measurement

Estimation of bacterial loads in nodules in single and mixed inocula has been performed with a Real Time PCR method (Checcucci et al., 2016). In brief, single nodules of the same size ( $\sim$ 1 mm in length) were excised from plants, surface sterilized with 0.1% NaHClO and crushed for the DNA extraction. Real Time PCR was performed with the *acdS* specific primers and *S. meliloti* specific primers (Trabelsi et al., 2009) *acdS* specific primers (fw-5'- TGAATTGTGTCGTCATCCAG -3', rv-5'- CTGTCGGCGCCCATCAGTTT-3') were designed with Primer3 software (<http://primer3.sourceforge.net/>) on the basis of *acdS* gene from AK83 strain (Sinme\_5642) from position 371379 nt to position 371479 nt

## Phenotype Microarray Experiments

Phenotype microarray (PM) experiments were performed to investigate the metabolic functions carried out by *AcidS*. *S. meliloti* 1021 pSRK- Km (BM261) and *S. meliloti* 1021 + pSRK-

*acdS* AK83 (BM641) strains were assayed by PM technology (Biolog) using microplates PM3, PM6, PM7, and PM8, which test different nitrogen and peptides compounds sources. PM data were analyzed to compare the activities of 1021 wild type strain with those of its derivative expressing *acdS*. Strains were grown at 30°C on TY agar plates for 2 days and then, colonies were picked with a sterile cotton swab from the agar surface and suspended in 15 ml of NaCl 0.8% until a cell density of 81% transmittance (OD<sub>600</sub> = 0.1) was reached on a Biolog turbidimeter. The inoculation fluids for PM panels was prepared adding 2 ml of each cell suspension and 240 µl of dye MixA 100x (Biolog) to 22 ml of M9 media depleted from carbon source and enriched with IPTG 1 mM (necessary for the activation of the promoter of pSRK vector and the expression of the gene). Then the inoculation fluid was transferred to the microplates (100 µl per well). All PM microplates were incubated at 28°C in an OmniLog reader, and changes of color in the wells were monitored automatically every 15 min. Readings were recorded for 96 h, and data were analyzed using OminoLog PM software which generated a time course curve for tetrazolium color development.

Positive PM results were then confirmed by inspecting the growth of *S. meliloti* 1021 pSRK- Km (BM261) and *S. meliloti* 1021 + pSRK- *acdS* AK83 (BM641) strains on VVM medium containing formamide as sole nitrogen source after 24 h at 30°C.

## Formamidase Activity Assay

Formamidase activity present in crude permeabilized cells was performed by using the Berthelot reaction with a colorimetric determination of ammonium (Anderson and Ingram, 1993) as described in Skouloubris et al. (1997) with minor modifications. Aliquots of 50 µl of cell extracts in 100 mM Tris-HCl pH 8.5 buffer were mixed with 100 µl of 100 mM formamide solution in the same buffer. After 30 min incubation at room temperature 400 µl of salicylate-citrate-nitroprusside solution was added and incubated for 15 min followed by the addition of 400 µl of the alkaline hypochlorite reagent. After 1 h incubation sample absorbance at 655 nm was read. Blank samples were prepared by boiling cell extracts 20 min prior to the addition of formamide. Ammonia released was determined from a standard curve. Formamidase activity was expressed in units corresponding to the degradation of 1 µmol of formamide min<sup>-1</sup> mg<sup>-1</sup> protein.

## Promoter Activation and Regulation Patterns

To investigate *acdS* gene promoter activation patterns, putative *acdS* promoter region (144 bp) and the upstream region including also its putative transcriptional regulator (620 bp) were cloned in the promoter-less vector pOT2 (Karunakaran et al., 2005). Firstly, the plasmids were used for transformation of *E. coli* S17-1 cells, then transformant cells were selected for resistance to Gentamicin (10 µg/ml), and positive clones were used for biparental conjugation to *S. meliloti* 1021. Conjugal transfer was performed as previously described (Pini et al., 2014). Recombinant *S. meliloti* 1021 strains [*S. meliloti* 1021 pOT2 + *acdS* promoter (144 bp) (BM 690), and *S. meliloti* 1021 pOT2 + *acdS* promoter+ regulator (620 bp) (BM 697)] were grown on TY plates and the induction tests were performed by



suspending a single colony in tubes with M9, M9 supplemented with  $\text{NH}_4\text{Cl}$  (10 g/l) and M9 supplemented with ACC (5 mM), and in tubes with 300  $\mu\text{l}$  0.9% NaCl solution and 200  $\mu\text{l}$  of root exudates (Ogawa and Long, 1995). After incubation for 3 h, cultures were placed in a microtiter plate and GFP-uv gene expression was measured on a microplates reader (Tecan Infinite 200 PRO, Tecan, Switzerland).

## Root Exudates Production

Seedlings of eight leguminous and not leguminous plant species (*M. sativa* cv. Pomposa, *Phaseolus vulgaris*, *Lens culinaris*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *Daucus carota*, *Rafanus sativus*, and *Lepidium sativum*) were used for the production of root exudates. *M. sativa* seeds were sterilized in  $\text{HgCl}_2$ , repeatedly washed in sterilized water, and germinated in sterile plastic Petri dishes for 72 h in the dark and 48 h in the light at room temperature with 2–3 ml of sterile  $\text{ddH}_2\text{O}$ . *A. thaliana* seeds were sterilized for 1' in EtOH 70% and in a solution of Bleach 10%,  $\text{ddH}_2\text{O}$  90%, and Triton X-100 0.1% for 10'. The seeds were then repeatedly washed and germinated in Magenta waving containing M&S based Medium. The other seeds were sterilized similarly to *A. thaliana*, but germinated in sterile plastic Petri dishes for 5–6 days in the light in a growth chamber maintained at 26°C with a 16-h photoperiod (100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). All the plantlets were then grown for 6–7 days, and then transferred over a filter of Whatman paper, in 13 ml tubes with 10 ml of sterile  $\text{ddH}_2\text{O}$ . Each tube contained approximately the same amount of root biomass. After 2 weeks of incubation in the growth chamber, the exudates were extracted, filtered and stored at  $-80^\circ\text{C}$ .

## Confocal Imaging

Plantlets were germinated and grown on BNM medium plates as previously described (see Symbiotic and nodulation assays). One-week-old plantlets were placed on a sterilized microscope slide spread with a thin layer of BNM Agar Medium and inoculated with 50  $\mu\text{l}$  (corresponding to  $4 \times 10^4$  cells/ $\text{cm}^2$  on the glass slide surface) from an overnight culture of 1021 pOT2 + *acdS* promoter (144 bp) (BM 690), and 1021 pOT2 + *acdS* promoter + regulator (620 bp) (BM 697) strains grown in TY medium and washed three times with 0.9% NaCl solution. Images were taken using an upright Leica laser-scanning confocal microscope SP5 (Leica Microsystems Wetzlar GmbH, Germany).

## RESULTS

### Occurrence and Phylogeny of *acdS* in *S. meliloti*

We inspected the presence of *acdS* genes in a collection of 133 *S. meliloti* strains from different geographical areas (Supplementary Table S1). Thirty-one strains (22.6% of total) gave positive amplification. The percentages of positive strains varied from 0% (Tunisia) to 44% (Italy). Kazakhstan and Iran strains collections both showed 13% of positive strains. In the Kazakhstan collection, the six positive strains were distributed among different host plants, viz. *M. lupulina*, *M. falcata*, *M. trautvetteri*, *Melilotus* sp.

The phylogenetic analysis, based upon *acdS* orthologs found among Rhizobiaceae, highlighted the presence of two main clades of the *acdS* orthologs in *S. meliloti* (Figure 1A), distributed within *R. leguminosarum* strains. The genomic context analysis of the *acdS* orthologs from the two clades, results showed the presence of Mobile Genetic Element (MGE), as transposases and integrases in the close proximity of *acdS* gene, in every *S. meliloti* strain (Figure 1B).

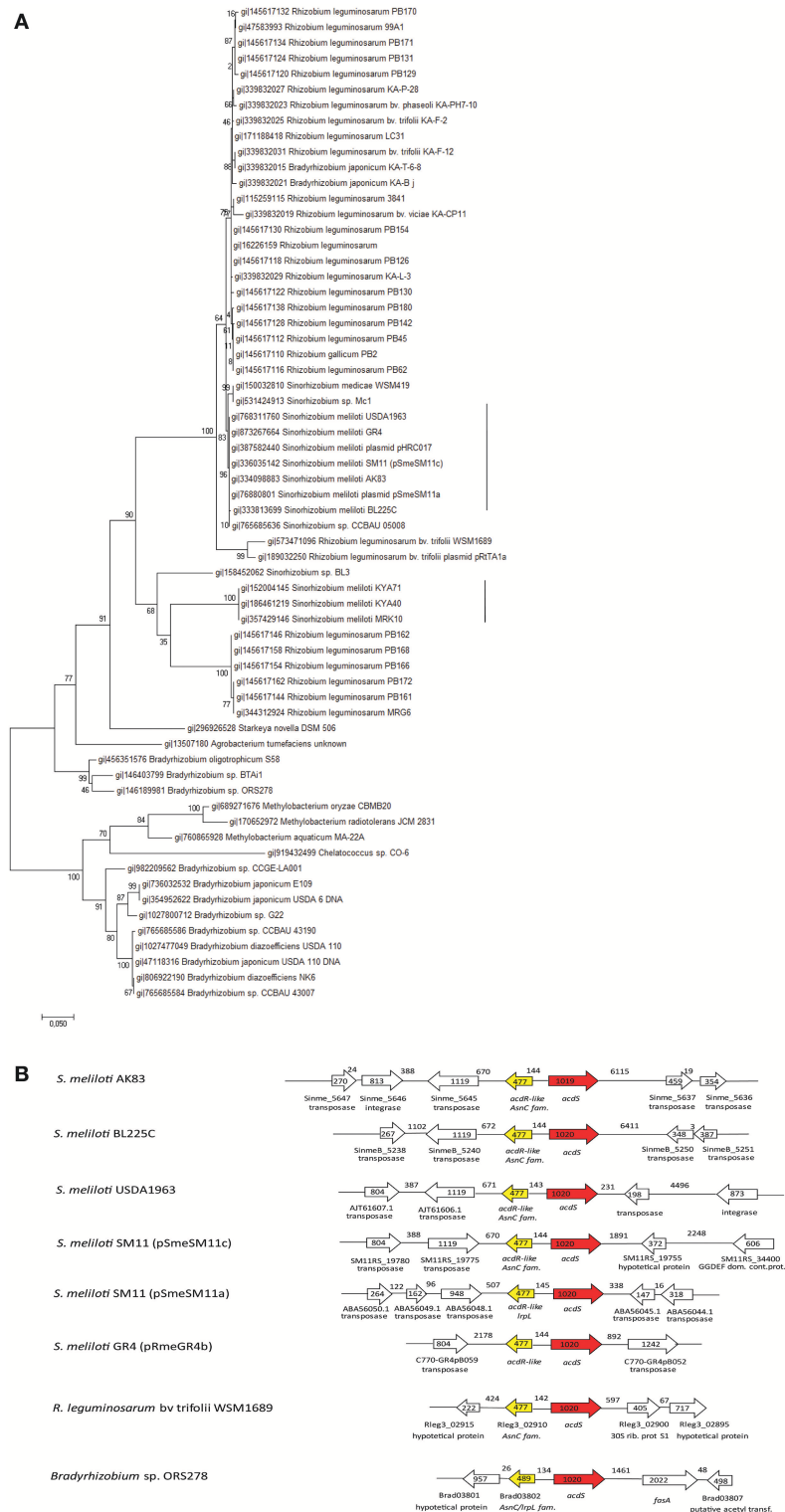
### Function and Control of Acids in *S. meliloti*

To shed light on the functional roles of *acdS* in *S. meliloti*, *acdS* gene from AK83 (Sinme\_5642) was cloned under *lac* promoter and introduced into *S. meliloti* 1021 strain [producing 1021 + pSRK- *acdS* AK83 (BM641), see Table 1]. This strain showed a positive ACC deaminase activity under IPTG induction (Supplementary Table S2). Then, to investigate the functional role of *acdS* in *S. meliloti* and its putative involvement in the reduction of plant ethylene production, the ethylene accumulation of the host plants infected by the recombinant and wild type strains was measured (Figure 2). No statistically significant difference in ethylene produced by the host plants was detected and all the samples tested showed values similar to the control (not infected plant) ( $0.5 < p\text{-values} > 0.005$ ).

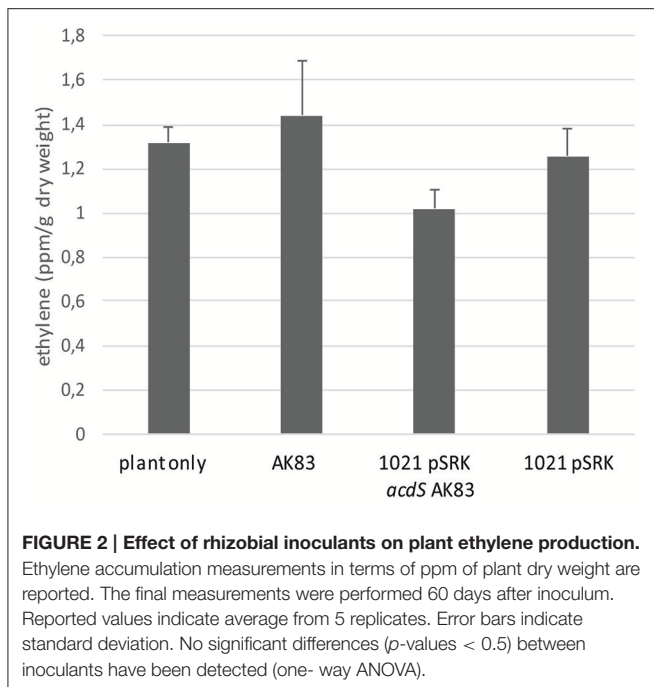
We then tested the possible role of *acdS* expression in the symbiotic performances and competitiveness of *S. meliloti*. In the symbiotic test, both single and mixed inocula did not show significant differences in the percentage of nodulated plants (Figure 3A) as well as in the overall rhizobial colonization of root nodules ( $p\text{-value} < 0.5$ ) (Figure 3B). Concerning the competitiveness inside nodule and the capability of colonization, though both strains were detected, the empty vector 1021 strain [1021 pSRK- Km (BM261)] showed for most of the nodules a higher titre than its *acdS*-expressing derivative 1021 + pSRK- *acdS* AK83 (BM641) (Figure 3C) (mean  $7.14 \times 10^2$ , standard deviation  $1.05 \times 10^3$ , median  $1.74 \times 10^2$ ). This result suggested that *acdS* expression does not allow to better compete in nodule colonization, but that in our experimental setup the expression of *acdS* under *lac* promoter possibly reduced the growth and or the differentiation abilities inside root nodule tissue.

To evaluate additional metabolic abilities conferred by *acdS*, Phenotype Microarrays were performed. We tested a total of 384 different nitrogen and peptide sources using plates PM3, PM6, PM7, and PM8, on BM261 and its *acdS*-expressing derivative 1021 + pSRK- *acdS* AK83. Results showed almost complete identical behavior for the two strains but, surprisingly, 1021 + pSRK- *acdS* AK83 strain induced with IPTG displayed higher metabolic activity in formamide than the control 1021 pSRK-Km (BM261). 1021 + pSRK- *acdS* AK83 strain was indeed able to grow better on formamide as sole nitrogen source, then its parental BM261 strain (in Figure 4, the results obtained in Biolog Plate PM3), as confirmed by the growth on VVM medium (Supplementary Table S3). However, 1021 + pSRK- *acdS* AK83 strain induced with IPTG did not show higher formamidase activity than BM261 strain (data not shown), hampering to evaluate the biochemical basis of the detected growth difference.

As shown in Figure 1B, in the genomic context analysis, a quite conserved region upstream to *acdS* gene in every *S. meliloti*



**FIGURE 1 | Phylogeny of the ACC deaminase gene cassette. (A)** Maximum Likelihood phylogenetic reconstruction of *acdS* gene sequences. The phylogenetic analysis is based upon *acdS* orthologs found in Rhizobiaceae. The dendrogram highlights the presence of two clades of the gene in *S. meliloti*, vertical lines **(B)** Genomic context of the *acdS* orthologs from the two clades of *acdS* orthologs in *S. meliloti* strains and from *Bradyrhizobium* and *Rhizobium leguminosarum* strains. *acdS* region map pointed out the presence of MGE (Mobile Genetic Elements) close to *acdS*. The length (bp) of genes and intergenic regions is indicated, as well as ORF orientation (using arrows). The GTR+G model has been chosen for the reconstructions after model test evaluation (Supplementary Data 1).



strains is present. The region is composed by an intergenic spacer (the putative promoter, 144 bp long), downstream to an open reading frame, in opposite orientation with respect to *acdS*, annotated as a putative leucine-responsive regulator (IrpL/AsnC family) (476 long, here called *acdR* in agreement with previous naming; Grichko and Glick, 2000; Prigent-Combaret et al., 2008). We considered as putative promoter the DNA fragment between the translation start codons of *acdS* gene and that of the putative regulator (Ma et al., 2003a). Previous works have described that in other species, as *R. leguminosarum* bv. *viciae* 128C53K, the *acdR* gene (a putative leucine-responsive regulator, named also as *lrpL*) is required for the expression of *acdS* (Ma et al., 2003a, 2004) and that the only presence of both *acdS* and *lrpL* genes can allow the expression of ACC deaminase (Stiens et al., 2006). Since a putative LRP box is present in the 144 fragment (5'-AAGCAAAATTAGAGA-3' at 62 nt from the *AcdS* start codon) we wanted to investigate *acdS* regulation in relation to the presence of the putative *acdR* gene. Consequently, we cloned the sole putative promoter (1021 pOT2 + *acdS* promoter (144 bp), BM690 in Table 1 and the entire region, 620 bp long, [putative promoter and the *acdR* gene, 1021 pOT2 + *acdS* promoter+regulator (620 bp), BM697 strain in Table 1] from strain AK83 into the promoter-less vector pOT2 and tested reported gene activation (GFP-uv) in *S. meliloti* 1021. The strain with the sole promoter (1021 pOT2 + *acdS* promoter (144 bp) (BM690) showed transcriptional activity higher ( $p$ -values  $< 0.0001$ ) than the strain with the putative leucine-responsive regulator [1021 pOT2 + *acdS* promoter+ regulator (620 bp), BM697] in M9 medium supplemented with both ACC and ammonium as sole nitrogen source (Figure 5), suggesting a negative control of the transcriptional regulator *AcdR* toward *acdS*.

We then investigated which conditions may allow the release of the repression by *AcdR*. considering that M9 mineral medium

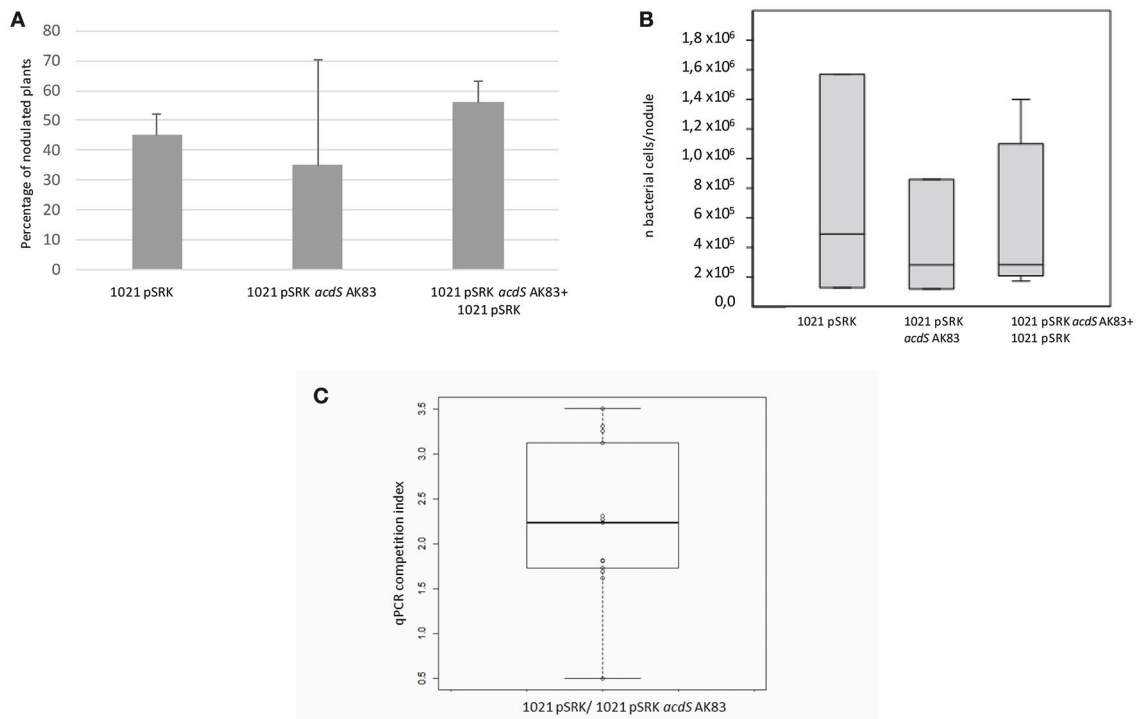
supplemented with ACC as sole nitrogen source was tested but no activity was detected. (Figure 5). Then, to observe if plant proximity may be a factor allowing to induce gene expression, 1021 pOT2 + *acdS* promoter + regulator (620 bp) strain was spread close to the roots of *M. sativa* plantlets and visualized by fluorescence microscopy. Results showed that *M. sativa* roots are able to induce promoter activation (Figure 6). No fluorescence was observed with the 1021 pOT2 (BM668, empty vector) (data not shown). Finally, to further quantitatively evaluate the level of promoter activation and understand if the activation may be specific of the symbiotic host plant (*Medicago* spp.), we incubated BM697 and BM690 strains in presence of root exudates (Ogawa and Long, 1995). The results are showed in terms of ratio between the level of activation of 1021 pOT2 + *acdS* promoter + regulator (620 bp) (BM697) and of 1021 pOT2 + *acdS* promoter (144 bp) (BM690) (Figure 7). The results showed that most of all tested root exudates induced promoter activation (ratio  $> 0.4$ ), in particular those of *M. sativa*, *L. culinaris*, *Rafanus sativum*, *P. vulgaris*, and *Lepidium sativum*, allowing BM697 to restore the level of GFPuv expression of the strain with the sole promoter 1021 pOT2 + *acdS* promoter (144 bp) (BM690). Interestingly, the data highlight root exudate from *Daucus carota* showed statistically significant differences with all the other tested root exudates (ratio  $> 1$ ).

## DISCUSSION

The gene encoding ACC deaminase (*acdS*) is considered to be important for bacteria plant interaction mainly since it is considered to lower the level of ethylene produced by the plant (Gamalero and Glick, 2015; Singh et al., 2015). However, in relation to rhizobial plant symbiosis only few data were present. In particular, for *Mesorhizobium loti*-*Lotus japonicum* association *acdS* activity has been shown to be present inside mature root nodules, in relation to NifA2 control (Nukui et al., 2006). For *S. meliloti*, past works have shown an increase in competitiveness of an engineered strain containing *acdS* genes with respect to its wild-type counterparts (Ma et al., 2004) and an increase in host plant growth (*M. lupulina*) when infected with an ACC deaminase-overproducing *S. meliloti* strain (Kong et al., 2015). However, no details on the frequency of *acdS* in *S. meliloti* strains, as well as on its regulation and functional role were present. Moreover, in these works *acdS* from other species (*P. putida* and *R. leguminosarum* bv. *viciae*) were used, consequently no indications of the actual role of *S. meliloti* native *acdS* were reported.

## Distribution and Evolutionary Pattern of *acdS* Gene in *S. meliloti*

In this work, we have shown that *acdS* genes have undergone extensive horizontal transfer events in *S. meliloti*. In particular, the analysis of a collection of 133 strains coming from Iran, Kazakhstan, Tunisia, and Italy showed that *acdS* is present in ca. 22% only of strains, thus confirming earlier reports indicating that *acdS* is part of the dispensable genome fraction in *S. meliloti* (Galardini et al., 2011). Some differences in the



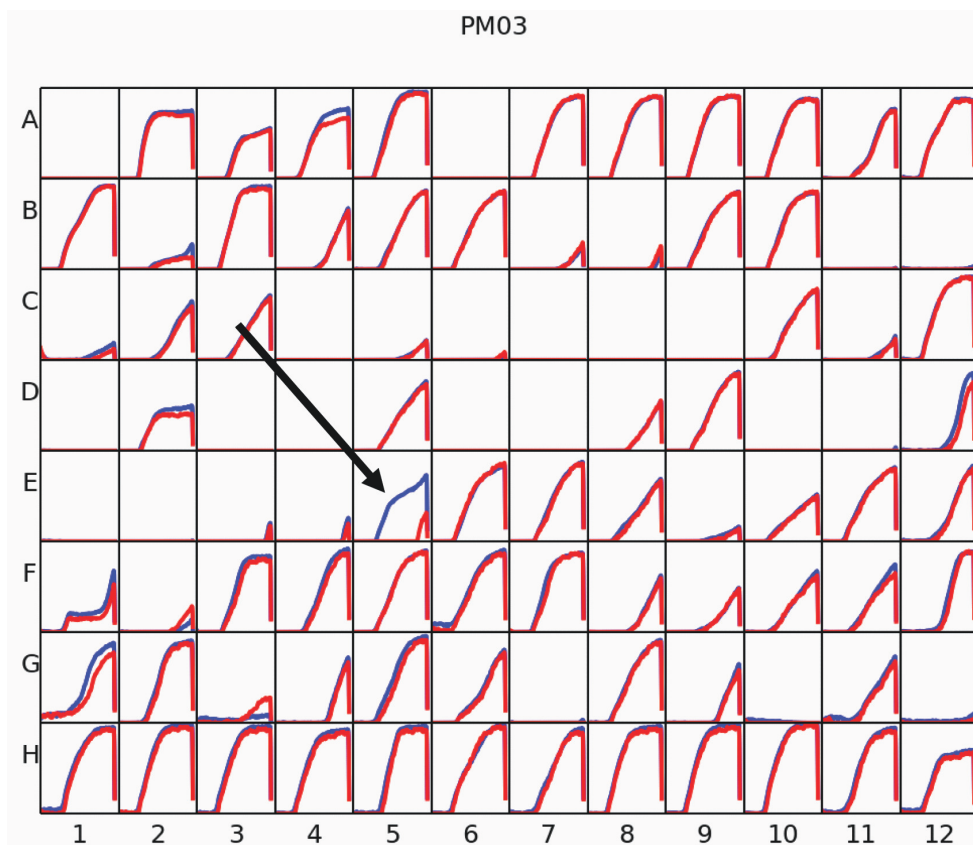
**FIGURE 3 | *acdS* expression does not confer competitive advantage in symbiosis.** (A) Nodulation efficiency and (B) competitiveness insides nodules of 1021 overexpressing *acdS* from AK83 strain compared to the parental one are reported. (A) The percentage of nodulated plants is evaluated on a set of 50 infected plants for each strain (20 for singular inoculum and 30 for the mixed inoculum). (B) Boxplots reports the rhizobial titres inside root nodules taken from 13 independent measures (different nodules from different plants) for each inoculant strain. (C) qPCR competition index (CI) to evaluate capability in the colonization of the nodules, reported as log of the ratio of qPCR estimates of rhizobial titres (number of rhizobial cell in the nodule, evaluated for each strain involved in the competition) between the two strains inoculated in the same individual plant (the parental strain 1021 with the empty vector, and strain 1021 overexpressing *acdS* from AK83 strain). The boxplot reports the data from 13 nodules from different co-inoculated plants.

in the percentage of *acdS* harboring strains from the different geographical areas were found. Even if a two-way PERMANOVA indicate a statistical significance of the geographical area (data not shown), on the basis of actual data related, we cannot indicate if such difference may be due to stochastic effects (linked to the composition of the collection) or to the host plants used for strain isolation. Indeed, Tunisian strains were isolated with *M. truncatula* only (Trabelsi et al., 2010), while Iran and Italian strains were isolated on *M. sativa* plants only, though from different cultivars (Carelli et al., 2000; Talebi Bedaf et al., 2008). The six Kazakhstan strains containing *acdS* were isolated from different hosts (either *Medicago* and *Melilotus*), but numbers are not adequate to provide a statistical evaluation of possible host plant preference. Sampling experiments performed with different host plant species in controlled conditions are needed to clarify if strains carrying *acdS* are advantaged during symbiosis with specific plant species.

The considerable level of horizontal spreading of *acdS* is present also in other rhizobia. A search over Integrated Microbial Genome Database (IMG, (Markowitz et al., 2013) showed that *acdS* is present in 94% of the completely sequenced *Bradyrhizobium* strains and in the 33% of *R. leguminosarum* strains, confirming that also in such genera/species is part of

the dispensable genome fraction (data not shown). Indeed such horizontal spreading has been highlighted in the whole Bacteria domain (Hontzeas et al., 2005; Blaha et al., 2006; Nascimento et al., 2014). In Nascimento et al. (2014) *S. meliloti* *acdS* sequences appeared split into two clades, the one containing strains AK83, BL225C and SM11, the other strains KYA40 and KYA71. We confirmed here the occurrence of these two clades for *acdS* in *S. meliloti*, suggesting that within *S. meliloti* pangenome *acdS* may have originated from different transfer events. The detected presence of Mobile Genetic Elements (MGE) close to *acdS* in *S. meliloti* may suggest recent HGT events of *acdS* in *S. meliloti*. This genome organization in *S. meliloti* appears to be quite similar to that of other bacterial species, as the well-investigated strain *P. putida* UW4 (Grichko and Glick, 2000; Li and Glick, 2001). Interestingly, MGE are not present at close distance in other *Rhizobiaceae* (as *B. japonicum* or *R. leguminosarum*), suggesting that the spreading of *acdS* in *S. meliloti* should have been more recent. This hypothesis is supported by the evidence that in the genomes of *S. meliloti* strains, *acdS* is present on the symbiotic megaplasmids (related to pSymA of strain 1021), which are known to be of relatively recent origin and have undergone large structural rearrangements, especially by MGE movements (Galardini et al., 2013). Moreover, it is quite relevant to notice





**FIGURE 4 | Phenotype Microarray (PM).** The results of PM analysis for nitrogen sources on Biolog Plate 3 in Rm1021 overexpressing *acdS* from AK83 strain compared to the parental one is reported. Measures were taken incubating strains with IPTG for inducing *acdS* gene expression. The red kinetic curves represent the strain 1021, while the blue one report the metabolic activity of 1021 pSRK *acdS* AK83. The black arrow underline the results obtained for the activity of the strains in presence of formamide.

that in *S. meliloti* SM11 strain, two *acdS* genes were found and they located into two different replicons, one in pSME11a and the other one in pSME11c (related to pSymA of the model strain 1021) (Schneiker-Bekel et al., 2011). Finally, we found upstream to several *acdS* orthologs the presence of the putative regulator *acdR*, highlighting a conservation of the gene cassette (Supplementary Figure S1).

## Functions and Regulation of *acdS*

Previous studies indicated that expression of ACC deaminase increases nodulation ability of *S. meliloti* toward *M. sativa* (Ma et al., 2004). Our results did not provide clear evidences of an effect on increase in competitiveness of the *acdS* expressing strain with respect to the parental one, neither as percentage of nodulated plants, nor as overall nodule colonization. Moreover, the competition index based on qPCR estimation in *M. sativa* nodules, showed on the contrary that the expression of *acdS* under lac promoter reduced the colonization of the nodules to the advantage of the parental strain. Of course, we cannot *a priori* exclude that other plant varieties and testing conditions may allow to detect differences. It is in fact known that symbiotic test may provide variable results, depending on the strain used

and on the plant genotypes (Crook et al., 2012). However, we can hypothesize that ACC deaminase expression did not provide a considerable advantage to the bacterium in the symbiotic interaction.

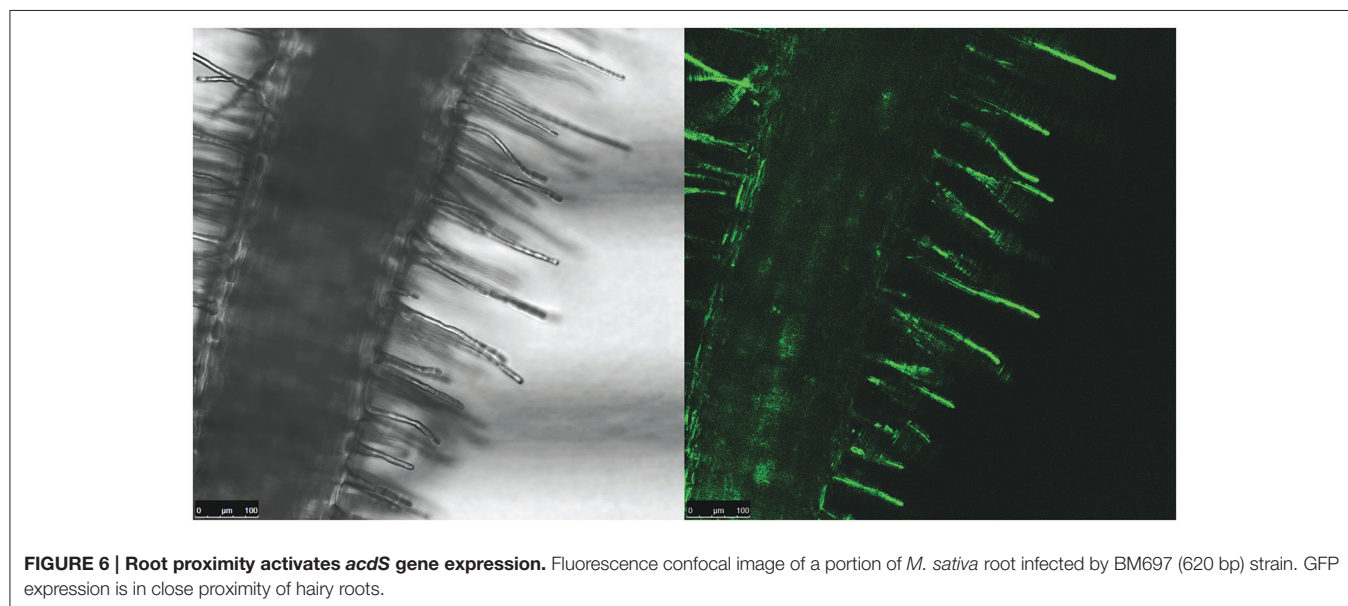
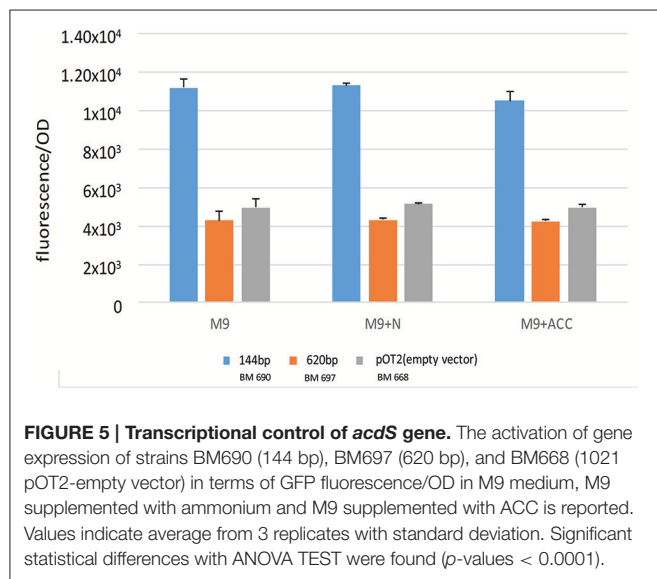
Concerning the potential ability of ACC deaminase in the reduction of plant ethylene production (Glick, 2005), our results did not support this conclusion. However, we cannot exclude that, because slightly less ethylene (differences were not statistically significant) was present in the plants inoculated with the *acdS* expressing strains, an effect could be detected by analyzing a higher biomass of plants or in different experimental conditions (e.g., with plants challenged with a stressing agent, as salt or heavy-metals). Indeed, in other systems (e.g., *M. loti*), ACC deaminase may lower plant ethylene levels, but only locally (Murset et al., 2012), suggesting then that on the overall plant (as in our conditions) effects could be minimized. Interestingly, Phenotype Microarray data showed a surprising phenotype of the *acdS* expressing strain, which was able to use formamide and Ile-Pro dipeptide as sole nitrogen source. This result led us to formulate a hypothesis of a role of ACC deaminase as scavenger of unusual nitrogen sources (in the rhizosphere and/or in the plant endosphere). Indeed, a

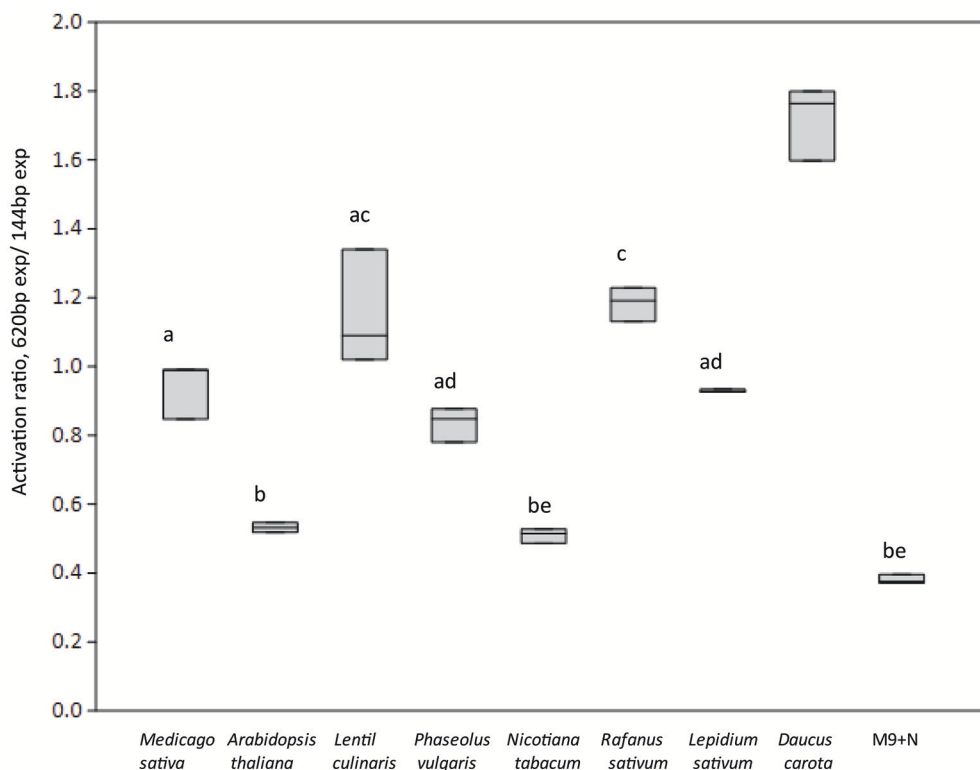
formamide concentration dependent growth was shown for the recombinant *acdS* expressing strain, though also the parental strain *S. meliloti* 1021 showed some ability to grow on formamide as sole nitrogen source. Such a metabolic hypothesis on ACC deaminase role in rhizobia in the scavenging of unusual nitrogen sources could allow to explain the presence of *acdS* in some non-mutualist rhizobial strains (Checcucci et al., 2016). In other words, ACC deaminase activity could allow some strains to better perform in rhizosphere and endosphere colonization because of increase nutrient availability, then also to explain the increased nodulation ability found in some rhizobial species (Ma et al., 2004). Indeed, *acdS* among rhizobia is not ubiquitous and different results in relation to the nodulation and symbiosis have been highlighted in different species, as *B. japonicum* (Murset

et al., 2012). However, the presence of *acdS* in the dispensable genome fraction and its polyphyletic pattern of evolution in *S. meliloti*, strongly suggest that the conferred advantage is only strain-specific (depending on the genomic background of the single strain) or that a balancing selection is acting on the *S. meliloti* population, reducing in some way the possible fitness advantages of *acdS*-harboring strains. Furthermore, the presence of the gene in a large number of non-symbiotic rhizospheric and endophytic bacterial species (Gamalero and Glick, 2015) support the hypothesis of its role in the colonization of the rhizosphere and endosphere.

The hypothesis of a main involvement of *acdS* in the broadening of rhizobial metabolic abilities (e.g., for the colonization of rhizosphere) and not in symbiosis was finally supported by the results of *acdS* gene regulation. We showed that the *acdR*-like gene is potentially acting as repressor of *acdS* expression. This result is in contrast with what was found for the orthologs present in *P. putida* (previously *Enterobacter cloacae* UW4), where a positive regulation mediated by *AcdR* was present (Grichko and Glick, 2000; Nukui et al., 2006), as well as with the data reported for *M. loti* where *NifA* is involved in promoting *acdS* expression (Nukui et al., 2006). Interestingly, in *S. meliloti* the repression operated by *AcdR* was not released by the presence of ACC in the medium, but by the presence of root and in particular by the incubation with root exudates from either *M. sativa* and other species (both leguminous and not leguminous plants), again in support of additional roles (not just the ACC degradation) of *acdS* in *S. meliloti*. In particular, these data let to hypothesize that most of the root exudates are able to activate the promoter and the transcription. Moreover, *D. carota* results suggest the presence of other molecules in root exudates, which act as positive modulators.

Finally, the different regulatory pattern of *acdS* in *S. meliloti* with respect to *P. putida* UW4, even in presence





**FIGURE 7 | Roots exudates from different plant species activates *acdS* gene expression.** Boxplots report the activation ratio between the expression (exp) of the entire cassette with respect to the sole promoter (BM697/BM690 strains (620 bp exp/144 bp exp) is reported. Different letters indicate significantly different values (one-way ANOVA,  $p < 0.05$ ).

of corresponding orthologs, is an interesting example of previous findings on the evolution of regulatory interactions in bacteria, where a higher conservation of effectors than of regulatory schemes is observed in different bacterial species (Babu et al., 2006; Galardini et al., 2015).

On the overall, the presented results strongly suggested that *acdS* spread in *S. meliloti* pangenome in relation to the colonization of plant roots more than to the symbiotic interaction. Consequently, we can hypothesize that *acdS* may be linked to an increase of fitness in non-symbiotic host plant species. The involvement of *acdS* in such non-symbiotic role may have contributed to the expansion of *S. meliloti* ecological niche. Indeed, pSymA megaplasmid is showing other non-symbiotic genes, as *nreB* (Pini et al., 2014, 2015), suggesting additional roles of pSymA, other than symbiosis and nitrogen-fixation. Indeed, comparative genomic analyses showed the pSymA megaplasmid to be a hot spot for structural variations (Galardini et al., 2015). Consequently, we can speculate that pSymA is undergoing evolutionary changes that partly can mirror those already occurred in the pSymB chromid (diCenzo et al., 2014), where additional genes integrated into an ancient dispensable plasmid, increasing the metabolic load and allowing to expand the ecological niche of *S. meliloti*, and ultimately leading of the formation of a non-dispensable element, the chromid.

## AUTHOR CONTRIBUTIONS

AC designed the work, performed most the analyses, provided interpretation of data, contributed in conceiving the work and drafted the manuscript. EA, SM, AD, GE, GS, and CV contributed analyses and provided interpretation of data. AM and MB conceived the work, provided interpretation of data and drafted the work. All authors contributed critically revised manuscript and gave the final approval for publication.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fgene.2017.00006/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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