Specificity traits consistent with legume-rhizobia coevolution displayed by *Ensifer meliloti* rhizosphere colonization

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ORIGINAL SIGNIFICANCE STATEMENT

Over the last century rhizobia-legume symbioses served as central models of mutualistic plant-bacteria interactions. The early stages of these associations have been previously investigated with special interest since rhizobial competition for host space begins in the rhizosphere before any physical contact between both partners. Genetic studies on how rhizobia colonize rhizospheres, however, have been difficult experimentally. Here, using signature-tagged mutagenesis, we identified more than a hundred *Ensifer meliloti* genes that are relevant to rhizosphere colonization. Most remarkably, some of those genes were required for the rhizobia to express a preferential colonization of both host (*Medicago*, *Trigonella*) and phylogenetically related nonhost (*Trifolium*) plants (specificity). These results shed light on the genetic and evolutionary basis of the early interaction of rhizobia with roots.

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ABSTRACT

Rhizobia are α- and ß-Proteobacteria that associate with legumes in symbiosis to fix atmospheric nitrogen. The chemical communication between roots and rhizobia begins in the rhizosphere. Using signature-tagged-Tn5 mutagenesis (STM) we performed a genome-wide screening for *Ensifer meliloti* genes that participate in colonizing the rhizospheres of alfalfa and other legumes. The analysis of ca. 6,000 mutants indicated that genes relevant for rhizosphere colonization account for nearly 2% of the rhizobial genome and that most (*ca.* 80%) are chromosomally located, pointing to the relevance and ancestral origin of the bacterial ability to colonize plant roots. The identified genes were related to metabolic functions, transcription, signal transduction, and motility/chemotaxis among other categories; with several ORFs of yet-unknown function. Most remarkably, we identified a subset of genes that impacted more severely the colonization of the roots of alfalfa than of pea. Further analyses using other plant species revealed that such early differential phenotype could be extended to other members of the Trifoliae tribe (*Trigonella*, *Trifolium*), but not the Fabeae and Phaseoleae tribes. The results suggest that consolidation of *E. meliloti* into its current symbiotic state should have occurred in a rhizobacterium that had already been adapted to rhizospheres of the Trifoliae tribe.

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INTRODUCTION

As plant roots develop in the soil and microorganisms interact with those roots, a new environment with different physical, chemical, and biologic properties—the rhizosphere—is generated (Bruijn, 2013; Prashar et al., 2014; Rasmann and Turlings, 2016; York et al., 2016). The assimilable-carbon and -nitrogen sources of plant origin present in the rhizosphere make this environment a suitable niche for microorganisms to grow and communicate with each other. Such a circumstance makes rhizospheres well known active centers for horizontal gene transfer (Mølbak et al., 2007), the generation of biodiversity, and the potential generation of new adaptive phenotypes in relation to plants (Turner et al., 2013). The chemical communication and physical contact between roots and microorganisms in the rhizosphere constitute the initial instances in associative processes like that established between rhizobia and legumes (Cooper, 2007; Somers et al., 2008).

Rhizobia are Gram-negative soil bacteria that establish a nitrogen-fixing symbiosis with leguminous plants (Chen et al., 2003; MacLean et al., 2007). The establishment of a successful symbiosis is the result of a complex and tightly regulated sequence of coordinated interactions between both partners that concludes with the formation of new organs, the legume-root nodules, where the bacteria fix atmospheric nitrogen into ammonium ions and, in turn, receive photosynthetic carbon from the host plant [reviewed in (Jones et al., 2007; Gibson et al., 2008; Oldroyd and Downie, 2008)]. In addition to these clear benefits for both partners—and specifically to avoid unnecessary plant superinfections—a strict control exists in the number of root infections that will end up in mature root nodules (Bhuvaneswari et al., 1981; Caetano-Anollés and Bauer, 1988). Rhizobial accessibility to the symbiotic niche is naturally limited—with that capacity being regulated by the plant to meet the plant's N needs—and within the reach of only the most, so-called, competitive rhizobial strains present within the neighboring soil (Triplett, 1988; Triplett and Sadowsky, 1992).

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Current evidence indicates that rhizobial competitiveness for nodulation is a complex phenomenon in which processes taking place in the rhizosphere are of particular relevance (Triplett and Sadowsky, 1992; Savka et al., 2002). The key role of certain surface polysaccharides of alfalfa-, bean- and pea-nodulating rhizobia (Lagares et al., 1992; Bittinger et al., 1997; Williams et al., 2008) and of the type-III secretion system in the example of *Lotus*-nodulating rhizobia has been demonstrated (Sanchez et al., 2009). In addition to the synthesis of surface components, the rhizobial capacity to use specific metabolites such as inositol (Fry et al., 2001; Jiang et al., 2001), glycerol (Ding et al., 2012), proline (Jiménez-Zurdo et al., 1995; Jiménez Zurdo et al., 1997; van Dillewijn et al., 2002), arabinose, and protocatechuate (Garcia-Fraile et al., 2015) has been described as a positive attribute for competitiveness. The ability to generate specific forms of plant-nodulation factors (Lamrabet et al., 1999), to elaborate and tolerate toxins (Triplett and Barta, 1987; Triplett, 1988, 1990; Robleto et al., 1998), to lessen the rate of ethylene generation by the plant (Dominguez-Ferreras et al., 2009), and finally to tolerate abiotic stresses (Draghi et al., 2010) have also been reported as positive characteristics for symbiotic competitiveness. Nevertheless, beyond this fragmented knowledge, the methodological challenge lies in gaining access into an ever more complete overview of the behavior of rhizobia in the rhizosphere. The main limitations generally have to do with difficulties in obtaining a sufficient quantity of representative samples to undertake classical transcriptomic or proteomic investigations (Jackson and Giddens, 2006; Lozano et al., 2011). Few studies have faced the challenge of focusing on the first hours of rhizosphere colonization by rhizobia. For example, *in-vivo*–gene-expression technologies have been used to characterize the transcription of *Rhizobium leguminosarum* biovar viceae genes induced in the pea rhizosphere (Barr et al., 2008). Subsequently, in a more sophisticated trascriptomic approach, the early adaptation of *R.leguminosarum* biovar *viceae* Rlv 3841 to different plant rhizospheres was investigated, resulting in the

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identification of a number of rhizosphere-induced rhizobial genes, several of which loci were located in the replicon pRL8 (Ramachandran et al., 2011). Based conceptually on a different experimental approach, the screening on an omic scale of mutants created by signature-tagged mutagenesis (STM) can also serve to identify clones that express specific phenotypes (Lehoux et al., 1999; Pobigaylo et al., 2006; van Opijnen and Camilli, 2013). The use of such tools adapted to the analysis of rhizosphere colonization was accordingly able to identify six *R. leguminosarum* biovar viceae mutants with rhizosphere-specific phenotypes and to demonstrate the relevance of arabinose and protocatechuate metabolism to the colonization of pea rhizospheres by those rhizobia (Garcia-Fraile et al., 2015). In the example of *E. meliloti*, the use of STM has thus far concentrated on the screening for genes relevant to symbiosis and competitiveness mediated by the use of signature amplification and microarray technologies (Pobigaylo et al., 2008).

In this report, we present the use of a genome-wide STM approach aimed at the screening of *E. meliloti* mutants affected in the early colonization of two different legume´s rhizospheres. In order to improve the detection of weakly altered phenotypes and also to increase the number of different treatments that are simultaneously practicable, we used STM technologies coupled to both the amplification of signatures by the polymerase-chain-reaction (PCR) and high-throughput second-generation DNA sequencing. Through this approach, we were able to identify several genes that were relevant for the colonization of legume rhizospheres, together with new evidences which are consistent with the existence of ancient plant-bacteria coevolution events at this early stage of the interaction.

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RESULTS

Genome-wide identification of *E. meliloti* mutants altered in the ability to colonize the rhizosphere of *Medicago sativa* (alfalfa)

In order to identify *E. meliloti* genetic determinants associated with the early colonization of the alfalfa rhizosphere, we used a Tn*5*-STM approach that allowed for the simultaneous phenotypic analysis of thousands of independent mutants. A conservation of rhizosphere structure was critical in order to evaluate which mutations altered the ability of rhizobia to colonize roots. Thus, to preserve rhizosphere architecture and nutrient gradients, plants were grown in plastic pots with vermiculite as a carrier support (Materials and Methods). In all the experiments, 15 independent sets of 412 different Tn*5*-signature-tagged rhizobia (Pobigaylo et al., 2006) were used, so that more than 6,000 mutants were analyzed for their colonization capabilities. Two underlying central issues were specifically considered at the time of the experimental design: a) the use of low rhizobial concentrations, in the range of those observed under natural soil conditions (Trabelsi et al., 2009; Somasegaran and Hoben, 2012), in order to avoid artificial interactions that could mask or modify early competitive phenomena; and b) the need nevertheless to preserve an adequate relative representation of each inoculated Tn*5* mutant in the inoculant because the higher the number of different inoculated mutants in the inoculum, or the lower the total rhizobial concentration; the lower will be the representation of each single mutant in the assay. The sampling times were chosen based on the root-colonization kinetics displayed by *E. meliloti* (Fig. S1). A steady increase in root colonization was observed during the first 3 days after inoculation, but thereafter the values plateaued. In view of these kinetics, rhizospheric bacteria were routinely sampled at 3 and 7 days postinoculation (d.p.i.). Fig. 1 summarizes the experimental procedures used and the different treatments performed. To assess which mutant rhizobia were affected in root colonization, the $\log_2$ of the ratio of the number of each mutant in the

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output sample with respect to that same mutant in the input sample (inoculum)—*i. e.*, the M value—was determined (see Materials and Methods). Since any given Tn*5* mutant bore two specific DNA-signature tags (namely, the K and H tags; (Pobigaylo et al., 2006)), the relative amount of each mutant in any given sample was quantified by the high-throughput DNA sequencing of the bar-coded PCR products that separately contained each of the Tn*5* signature tags (Fig. S2; also *cf*. Materials and Methods). The experimental approach used enabled the analysis of 480 independent experimental conditions to be performed in a single sequencing run—*i. e.*, analyses of the colonization of alfalfa roots at two different times (3 and 7 d.p.i.) and the colonization of the nonhost pea roots at 7 d.p.i.; in both instances with 15 different mutant sets and four biological replicas per treatment (*cf*. the experimental scheme in Fig. 1). A mutant was considered to be altered in root colonization when, under any given condition, both of its signatures (the H and the K tags) had changed in representation in the output sample by at least 40% (or a fold change ≤ 0.6) compared to the representation in the input sample—*i. e.*, an M value = $\log_2$(input/output) of ≤ -0.74 for negatively affected mutants (*cf*. Materials and Methods). This cutoff value was chosen because it gave consistent and statistically significant results for both signature tags (with at least $p \leq 0.05$ for one of the signatures, and $p \leq 0.1$ for the other; *cf*. Fig. 2, Panel C). On the basis of this criterion, 128 mutants out of the 6,240 analyzed could be identified as potentially altered in the colonization of alfalfa roots at 3 d.p.i. (*cf*. volcano plot in Fig. 2, panels A and B). An analysis of the insertion sites of these mutants revealed that more than a hundred different genomic regions were affected, in some instances with several insertions within a same gene/operon (constituting a redundancy situation; Table S1). A similar STM experiment, but involving the collection of rhizobia from the rhizosphere at 7 d.p.i., enabled the identification of 148 Tn*5* mutants affected in root colonization, with most (70%) being mutants in the same genes that had been identified at 3 d.p.i. In total, 175 different mutants were found to be impaired in the

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colonization of alfalfa roots considering the two experimental conditions of 3 and 7 d.p.i. The inspection of all Tn*5* insertions listed in Table S1 indicated that 166 mutants contained the Tn*5* insertions within annotated coding regions, and involving 127 different genes. Nine mutants—the last 9 itemized in Table S1—had Tn*5* insertions within intergenic regions and three others within an rDNA (SMc03220).

Validation experiments. Competitiveness of STM-selected mutants against the wild-type rhizobia for root colonization in double-inoculation experiments

In order to assess whether mutants identified by STM were in fact defective in rhizosphere colonization capability, we performed double-inoculation competition experiments using a 1:1 mutant:wild-type ratio at the beginning of the assays (ca. 1×10^5 u.f.c. /ml of each strain) and then counted each competitor rhizobium by plating at 3 d.p.i. (validation assays)(see Materials and Methods). Experiments were performed with 14 mutants covering different M values, and having been recognized by STM as affected at both 3 d.p.i. and 7 d.p.i.. All the mutants tested (with M values ranging from –5.5 to –1.0) were confirmed as being impaired in root colonization compared to the wild-type strain (p <0.05, chi-squared test; Fig. 3). This evaluation, along with the analysis of an extensive number of mutants (*cf.* the following sections), demonstrated that the M values obtained from the STM experiments (M_{STM}) were consistent with their corresponding M values calculated from the validation experiments ($M_{plating}$; Fig. S3). Thus, the STM design presented here proved to be an effective and powerful approach to obtain reliable and quantitative estimations of colonization phenotypes for thousands of mutants in a single sequence run. The transduction of 9 different mutations into the wild-type rhizobia [*flgK* (two independent Tn5 mutants), *motD*, *flgE*, Smc03015, Smc01164, Smc03140, Smc04042, and Smc03113 (the unaffected

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control)] in all instances resulted in colonization phenotypes that were consistent with the behavior of the original mutant (data not shown).

Genomic distribution of genetic markers involved in rhizosphere colonization of alfalfa plants. Search for orthologs in other rhizobia and *in-silico* functional analysis

The genomic distribution of the genes which contained Tn*5* insertions that affected root colonization in alfalfa indicated that nearly 80% were located in the *E. meliloti* chromosome (Fig. 4, Panel A), with only 16.2% and 4.4% of the genes being mapped on the pSymB and pSymA plasmids, respectively. The average density of genes for rhizosphere colonization per replicon length is in the relative order: chromosome > pSymB > pSymA (*cf.* the distribution of red dots in Fig. S4). That 94% of all the chromosomal genes listed in Table S1 had been identified as part of the core set of genes shared among the six available *E. meliloti* genomes suggests a common ancestral origin for most of their associated functions. The ancient character of the colonization genes is also reflected in a significant conservation of this orthologs (≥50%) even in distant members of the Rhizobiacea family including mesorhizobia and bradyrhizobia (Fig. S5). In *E. meliloti*, a minor set of genes required for an efficient root colonization are present in the pSymB chromid (22 genes) and in the pSymA megaplasmid (6 genes).

The cluster of orthologous gene (COG) analysis for those genes whose Tn*5* insertions compromised root colonization indicated that about 56% were related to the metabolism of amino acids, carbohydrates, and cellular energy, among other functions (Fig. 4, Panel B). The most negative M values were accordingly observed within these gene disruptions. Of the remaining genes identified, *ca.* 12% were associated with cell motility and chemotaxis and were moderately impaired in root colonization ($-1.5 < M_{STM} < -1$). Transcription, translation, and replication-associated functions were affected in about 7% of the colonization-defective

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mutants. Finally, *ca*. 15% of the compromised genes were distributed in other less represented COGs.

Growth response in different media of mutants impaired in rhizosphere colonization

With the aim at investigating the type of effects that lead to deficiencies in rhizosphere colonization, we analyzed: a) the growth response of 15 selected mutants to different extracellular-nutrient sources that included alfalfa-root exudates, Evans minimal medium, and TY complex medium; along with b) the ability of each mutant to colonize alfalfa roots at 3 d.p.i. (Fig. 5). Mutants could be classified into three different groups according to their behavior under each of the conditions assayed: *Group A* contained the mutants that exhibited a normal growth in complex medium, but that were restricted in their growth in both minimal medium and alfalfa-root exudates. *Group B* comprised the mutants that grew normally in both minimal and complex media, but were affected in their growth and/or survival in alfalfa-root exudates (*i. e.*, SMc00963, SMc00406, SMc02407, SMc03140. SMc02226, and SMb21263). The functional profile of mutants included in this last group suggests that their defects might be related to deficiencies in the use of endogenous rhizospheric compounds for nutrition. *Group C* consisted of mutants characterized by a normal growth under all the conditions tested—thus likely expressing no nutritional deficiencies—but that still were compromised in root colonization. This last group included mutants altered in cell motility (such as *motD*, *SMc*03005, *flgG*, and *cheW1*), along with mutant *fbpB* (SMc00775) encoding an iron permease.

Direct impact of rhizosphere-colonization ability on competitiveness for nodule occupancy

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Previous experiments had indicated that different mutants deficient in root colonization were also impaired in nodulation competitiveness, thus suggesting that events occurring early on in the rhizosphere are relevant in determining which strains will eventually occupy the root nodules (Phillips et al., 1998; Entcheva et al., 2002). The quantitative extent to which increasing degrees of deficiency in root colonization impact root nodulation, however, is still unknown. In order to investigate this question, we tested the nodulation competitiveness of mutants that were altered to different extents in root colonization ($-4 < M < -1$), when they were inoculated with the wild type rhizobia in a 1:1 ratio (Fig. 6, Panel A). Previous analyses had shown that the log of the ratio of genotypes within the root nodules compared to the log of their ratio in the inoculum were in a linear relationship (Beattie et al., 1989; Lagares et al., 1992). Here we show that—except for mutants whose proportions in the root nodules were lower than 15% (*e. g.*, defective in the loci *purL* and SMc00963)—the proportion of a given mutant strain in the rhizosphere at 3 d.p.i. (*i. e.*, its ability to colonize the rhizosphere) and the nodulation competitiveness of that strain are directly proportional to one another ($r^2 = 0.99$, Fig. 6, Panel B). The relevance of the relationship between rhizosphere colonization and nodulation also becomes evident in that 20 of the mutants previously identified by Pobigaylo *et al.* (2008) as being deficient in nodulation competitiveness were also found to be compromised here in their early interaction with roots (*e. g.*, those with Tn*5* insertions in the loci *ilvC*, *ilvD*, *ilvL*, *metA*, *ccmC*, *pyrE*, *thiC*, *trpF*, and *clpA*, among others; Table S1).

Identification of genetic determinants associated with the preferential colonization of host-plant rhizospheres

The specificity in the interaction between legume and rhizobia is expressed very early in symbiosis—and has been shown to be mediated by soluble signals (*e. g.*, flavonoids,

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nodulation factors)—even before physical contact between partners (Janczarek et al., 2014). With an aim at exploring the possible existence of plant specificity during the colonization of alfalfa by *E. meliloti* compared to the interaction with other plants, we investigated whether or not any of the genetic regions affected in the mutants listed in Table S1 were associated with a preferential colonization of host plants compared to the colonization of other nonhost roots. To explore this possibility, STM experiments were also performed with roots of the nonhost pea (*Pisum sativum*) as outlined in the scheme of Fig. 1. Out of the 131 Tn*5*-mutants listed in Table S1, 23 exhibited: a) a significant difference between the abilities to colonize alfalfa and pea roots—*i. e.*, a $\Delta(M_{alfalfa} - M_{pea})$ with a p <0.05 for one of the bar code signatures (either the H or K tag; Fig. S2) and a p <0.1 for the other signature—and b) M_{pea} values not lower than –0.4. Out of these 23 Tn*5*-mutants, 10 were confirmed with a higher degree of impairment in the colonization of alfalfa roots compared to pea roots (Fig. 7) confirming a differential behavior depending on the plant used. Nine of these mutants were altered in genes for motility and chemotaxis (with at least one Tn*5* insertion in the loci *fliF*, *visN*, *fliM*, SMc03023, *fliL*, *flgB*, *flgG*, and *flgE*). None of those nine were motile in agar-plate tests. The only mutant whose mutation was not directly related to motility and chemotaxis, SMc03140—that mutant being motile in agar-plates—also manifested a plant-specific phenotype in being more defective in alfalfa than in pea. In view of these results, and in order to evaluate how extensive was the participation of the specific genes identified in the colonization of different legume roots, similar experiments were performed to evaluate four of the same mutants (*e. g.*, *fliM*, *fliL*, *flgB*, and *flgG*) in their ability to colonize the roots of particular members of the Trifoliae tribe (*Medicago truncatula*, *Trigonella foenum-graecum* L., and *Trifolium repens*) and the Phaseoleae tribe (*Glycine max*; Fig. 8). The results demonstrated that the rhizospheric specificity observed is not in all instances related to the host or nonhost character of the given plant species. Rather, the mutants that were specifically

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compromised in the colonization of *M. sativa* were also deficient in the colonization of roots of other nonhost plant species of the same tribe, such as those of *Trifolium repens*. In contrast, a much lower (and in some cases not detectable) affection was observed for the same mutants in their rhizosphere colonization of *P. sativum* (Fabeae tribe) or *G. max* (Phaseoleae tribe).

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DISCUSSION

In this work we searched—on a genomic scale—for the genetic information required by the model rhizobium *E. meliloti* to colonize the rhizospheres of host and nonhost plants. The previous experimental approaches to characterize genetic markers involved in rhizosphere colonization by rhizobia have been systematically focused on the analysis of individual mutants (Ormeño-Orrillo et al., 2008) or recombinant strains (Robleto et al., 1997). Using a phenomic approach, we could identify the collection of genetic markers involved in the rhizobial colonization of rhizospheres. By means of a similar strategy, several mutants of *R. leguminosarum* bv *viciae* with decreased rhizosphere fitness had been recently reported (Garcia-Fraile et al., 2015). The experimental protocol that was used in the present work combined the power of high-throughput nucleotide sequencing to quantify (*i. e.*, count) the presence of specific mutants in the rhizosphere, with the possibility of simultaneously investigating the colonization phenotype of *ca*. four hundred different mutants per inoculum at a total rhizobial concentration of *ca*. 10^5 total c.f.u./mL. Such experimental condition was used in order to avoid the masking of relevant colonization phenotypes by the use of unnaturally high rhizobial titers. Through the analysis of 15 independent mutant sets we could screen the phenotype of more than 6,000 Tn*5* insertions distributed all over the genome at comparable densities (Fig. S4) and covering more than 40% of the annotated open reading frames (ORFs). A major advantage in the use of a Tn*5*-STM technology was the associated possibility to easily recover each mutant of interest from the stock collection for the validation assays. On the basis of the analyses at both 3 and 7 d.p.i. more than hundred genes proved to be relevant for the colonization of the alfalfa rhizosphere. The defective colonization phenotype was validated without exception in randomly selected mutants, with $M_{plating}$ values that correlated very well with the M_{STM} values (*cf.* Fig. 6, Panel B). The robustness of the technology and the conditions used were also supported by the

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identification of several defective mutants with independent Tn*5* insertions within the same gene (redundancy). The interruption of most of the 136 genes identified interfered with root colonization at both of the times sampled (*i. e.*, at 3 and 7 d.p.i.), with only certain mutants being more adversely affected at one of the two postinoculation times. This observation suggests that most of the genes identified were likely associated with the initial stages in the rhizobia's approach to the root, thus compromising the colonization phenotypes at both 3 and 7 d.p.i.. The results of the screening revealed that *E. meliloti* devotes more than 2% of its genome to encoding functions that are relevant to the colonization of roots, thus providing quantitative evidence in support of the importance of that phenotype to the bacterial interaction with plants. That no mutations had strong positive effects on rhizosphere colonization by the rhizobia was also remarkable. The volcano plots (M values vs. *p*-values) in Fig. 2 evidenced the absence of *E. meliloti* Tn*5* mutants with strongly improved fitness in the rhizosphere (no mutants with $M_{STM} > 1.5$), suggesting that during their evolution rhizobia have minimized the presence of genes deleterious to the early interaction with roots. Experiments to evaluate nodulation competitiveness of mutants with different negative M values revealed how a rapid access to the root environment determined the ability of rhizobia to compete for nodule occupancy (Fig. 6). While certain *E. meliloti* infection-defective mutants are known to be affected in nodulation competitiveness (Lagares et al., 1992), we have now characterized here a broad set of genetic determinants that modulate competitiveness for nodulation from the very early interaction of rhizobia with roots, even before physical contact between both symbionts occurs.

In an attempt to recognize general phenotypic groups affected in root colonization in our set of mutants, we identified: (a) auxotrophs (certain mutants from *Group A*, Fig. 5), (b) mutants having no detectable differences in their growth in minimal medium or in root exudates compared to the wild-type strain (*e. g.*, the mutants from the *Group C* of Fig. 5, such

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as those affected in motility and/or chemotaxis), and (c) other mutants that were impaired in their growth in root exudates as the only C- and N-source (*e. g.*, the mutants from *Group B* of Fig. 5, such as those compromised in the loci SMc00406, SMc03140, and SMc02226). The biochemical analysis of mutants from *Group B* is expected to elucidate as-yet-unknown rhizobial activities associated with the response to and use of rhizospheric metabolites of plant origin. The genes from this group might also be associated with an enhancement of the rhizobial tolerance to abiotic stresses generated by compounds in the root exudates, such as the locus SMc00406 encoding a ferredoxin protein and the downstream ORF SMc00407 encoding a putative glutathione S-transferase. The *in-silico* global prediction of functions associated with the complete set of genes listed in Table S1 indicated that more than 50% of the insertions that disturbed root colonization were related to metabolic functions; with half of those being involved in the metabolism of amino acids (Fig. 4, Panel B), thus pointing to the more relevant pathways for rhizobial life in the rhizosphere. This finding is in agreement with previous transciptomic results obtained from the interaction between *R. leguminosarum* and pea roots (Ramachandran et al., 2011). We do not know yet which ones and how many of the *E. meliloti* genes required to colonize the root are inducible by interaction with plant compounds. Ramachandran *et al.* (2011) had already reported 138 up-regulated genes at 7 d.p.i. when *R. leguminosarum* bv. *viciae* Rlv3841 was inoculated onto pea roots. We observed several differences upon comparison of the rhizosphere-induced genes in *R. leguminosarum* bv. *viciae* with the *E. meliloti* genes that affected the colonization of alfalfa roots in this study. Although the genes for motility and chemotaxis were relevant in our phenotypic analysis (12% of the mutants in Table S1), those loci were down-regulated in *R. leguminosarum* bv. *viciae* in the root environment (as could be expected for rhizobia that had already reached the rhizosphere). Conversely, some genetic markers overexpressed in the pea rhizosphere were not present in our STM experiments. Within this group, of particular

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interest was the absence of *nod* mutants in our screening, thus making unlikely any significant role of those genes in the early rhizobial approach to the roots. Though *nodD3* and *nodJ* mutants had been found to have attenuated symbiotic phenotypes (Honma and Ausubel, 1987; Pobigaylo et al., 2008), deficiencies in those loci do not appear to be associated with a diminished rhizospheric colonization. Observations like these indicated that conjoint transcriptomic and phenomic approaches are both complementary and necessary.

That some of the defective genes in the Tn*5* mutants listed in Table S1 had been previously associated with the colonization of alfalfa roots supported the robustness and validity of the experimental approach used here. Examples of those genes include *putA* (metabolism of proline, and stachydrine; (Phillips et al., 1998)) and *bioN* (SMc00963; (Entcheva et al., 2002)). Furthermore, in agreement with our results, the role of motility and chemotaxis for rhizosphere colonization was also previously demonstrated in other systems such as *Pseudomonas fluorescens*-alfalfa, -tomato, and -potato (De Weger et al., 1987; De Weert et al., 2002; Capdevila et al., 2004). That other genes found to be relevant to the colonization of pea roots by *R. leguminosarum* bv. *viciae*—such as *araE* (arabinose catabolism) and *pcaM* (protocatechuate transport; (Garcia-Fraile et al., 2015))—did not prove to be as relevant for the colonization of alfalfa roots by *E. meliloti* is indeed of interest. In view of these data, future studies should be performed to investigate if the compositional profiles of root exudates from different legumes correlate with the catabolic capabilities of the associated symbiont rhizobia.

The role of the amino-acid metabolism in relation to symbiosis has been extensively studied, particularly with respect to the late stages of the symbiotic interaction (*i. e.*, infection, nodulation (Dunn, 2014)). Beyond their nutritional role, certain rhizobial genes related to amino-acid metabolism—such as *leuA* (Sanjuán-Pinilla et al., 2002), *metZ* (Taté et al., 1999), and *cysG* (Tate et al., 1997)—have proved to be relevant in preinfection processes.

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Our results clearly demonstrated the relevance of amino-acid metabolism to rhizobial fitness during rhizosphere colonization. Similarly, amino-acid biosynthesis has also shown to be required for an efficient root colonization of tomato roots by *Pseudomonas flourescens* (Simons et al., 1997). Moreover, recent metabolomic analyses indicated that branched-chain amino acids were observed at relatively high levels in the rhizosphere of *Medicago truncatula* (Watson et al., 2015), with mutations in certain of the corresponding biosynthetic genes accordingly producing some of the greatest impairments in rhizosphere colonization observed—*i. e.*, the *ilvL2, leuA, ilvD2, ilvC,* and *leuB* mutants with M_{STM} values ranging between –4.10 and –5.86. Our results suggest that either the amount of these amino acids in the rhizosphere is insufficient to satisfy the early symbiotic requirements, or that this biosynthetic route plays an additional nonnutritional role in rhizosphere colonization. With respect to valine metabolism in particular, whether the impaired rhizosphere colonization by *iolA* mutants results exclusively from the function of the *iolA*-gene product in the catabolism of inositol (Kohler et al., 2010), in the catabolism of valine (Kohler et al., 2011), or in another yet unknown process is still not clear. While *iolR*, *iolA*, *iolC*, *iolD*, and *iolE* mutants have previously proved to be unable to grow with inositol as the sole carbon source and highly deficient in nodulation competitiveness (Kohler et al., 2011); we did not observe any defect in rhizosphere colonization in independent mutants bearing Tn*5* insertions within the *iolB, iolD,* and *iolE*. This observation contrasts with the decrease in rhizosphere colonization observed in the *iolR* mutant (Table S1) and to a less extent in the *iolA* mutant and at the same time points to the participation of *iolR* as well as *iolA* in other cellular processes unrelated to inositol metabolism that could be necessary for rhizosphere colonization.

Concerning cdiGMP, a second messenger associated to diverse cellular processes, the STM library included 21 mutants with Tn5 insertions within 12 different GGDF- and/or EAL domain-encoding genes. In our STM experiments none these mutants showed to be impaired

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in rhizosphere colonization. We did not investigate, however, the effects of double or multiple mutants in those same genes. While no effects in symbiosis were previously observed in single inoculation assays(Schäper et al., 2016), a decreased competitiveness for nodulation was reported for some single mutants affected in cdiGMP metabolism (Wang et al., 2010). Such results and our data taken together suggest that any role in symbiosis of individual genes related to cdiGMP metabolism should be related to associative stages that take place later than rhizosphere colonization (i.e. root adhesion, infection).

The chromosomal location of most genomic localization of the Tn*5* insertions listed in Table S1 implies that the genetic information for an efficient colonization of plant roots is an ancestral character in *E. meliloti*, and most likely also in other rhizobia and rhizobacteria that share several homologues to the genes identified here. The ancestral character of the chromosome, together with the patchy distribution of the colonization genes within the chromosome—*i. e.*, therefore not having been simultaneously acquired by horizontal gene transfer—strongly suggests that the bacterial ability to colonize rhizospheres could have been acquired even earlier than the rhizobial association with legumes (*i. e.*, before 60 million years ago; (Hirsch et al., 2001; Sprent, 2007). Previous results along with those from this work have indicated that specific rhizospheric preferences exist between certain legumes and rhizobia. Thus, the mutation of two *R. leguminosarum* genes specifically up-regulated in the pea rhizosphere reduced colonization of the pea but not the alfalfa rhizosphere (Ramachandran et al., 2011). Conversely, mutations in *E. meliloti* motility genes reduced the colonization of alfalfa, *Trigonella foenum-graecum* L., and clover roots, but did much less or not at all with respect to pea and soybean roots (this work). According to the currently accepted dating for the evolution of legumes, the specific *E. meliloti* phenotype associated with the root colonization of members of the Trifolieae tribe (*Medicago*, *Trigonella*, *Trifolium*) should have likely been acquired between 17 to 45 million years ago after the

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divergence of the Phaseoleae and the Fabeae tribes (http://www.timetree.org/, (Hedges et al., 2015)) but before the time at which the rhizobia became specialized in the nodulation of alfalfa and clover (Fig. 9, gray shadowed area). Thus, modern day recognition of clover rhizospheres by *E. meliloti* is likely reminiscent of an ancient bacterial adaptation to the Trifolieae ancestors.

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EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Fifteen sets of *E. meliloti* signature-tagged mutants from a previously reported mini-Tn5-transposon library were used (from mutant mix 1 to mutant mix 15 (Pobigaylo et al., 2006)). *E. meliloti* cells were grown at 28 °C in tryptone-yeast (TY) complex medium (Beringer, 1974) or in Evans defined minimal medium (Evans et al., 1970), containing glucose (10 g/L) and ammonium chloride (0.7 g/L) as the respective carbon and nitrogen sources, as indicated. For solid media, 15 g of agar per liter of medium was added. When required, the media were supplemented with neomycin (120 µg/mL) and/or streptomycin (400 µg/mL). Transductions were done with the phage ΦM12 as previously described (Finan et al., 1984).

Collection of alfalfa-root exudates and exudate-growth assays

For exudate-growth assays, alfalfa-root exudates were collected in Fåhraeus medium (Fahraeus, 1957), as described in Supplementary methods. 40 µL of an inoculum containing *ca*. 1 x 10^5 colony-forming units (c.f.u.)/mL of a 1:1 ratio of wild-type *E. meliloti* 2011 that was green-fluorescent-protein–positive (GFP+)(Pistorio et al., 2002) and a given mutant was mixed with 800 µL of exudates in a 48-well plate and incubated under the same growth conditions as for bacterial cultivation. Four days after inoculation, dilutions were plated in TY medium supplemented with streptomycin and counted under a fluorescence stereomicroscope (Leica MZFL III). The proportion of wild-type and mutant rhizobia were calculated as the ratio of GFP+/total bacteria or as (1 – GFP+)/total bacteria, respectively.

Screening of STM mutants in colonization experiments

Seed sterilization protocol and plant cultivation conditions are described in

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Supplementary methods. In order to prepare the initial inoculum, each of the 15 different STM mutant mixes were freshly grown in TY to an optical density at 600 nm [OD_{600}] roughly between 0.2–0.3. Each mutant-mix culture was diluted in a flask containing 3 L of Fåhraeus medium to a concentration of approximately 1 x 10^5 c.f.u./mL, and the resulting dilution used to flood vermiculite-containing plastic pots (4 replicates pots for each condition to be studied). The flooded pots were then drained. A part of each original culture was stored and used as a reference (*i. e.*, constituting the input sample or inoculum). After inoculation, 10–12 alfalfa seedlings were planted in each pot. In the pea-containing pots, 4 seedlings were used. Plants were grown for 3 or 7 days depending on the condition studied. For the 3-days-postinoculation (3 d.p.i.) experimental group, plants were stirred gently to remove the bulk of vermiculite carrier adhering to the root and were then harvested in 10 mL tubes containing 5 mL of Fåhraeus medium. The tubes were then shaken for 20 s to extract the bacteria from the rhizosphere. For the 7 d.p.i. group, plants were removed from the pots and the aerial parts cut off. The roots were stirred gently and harvested in 50 mL tubes containing 10 mL of Fåhraeus medium and were then also shaken for 20 s to extract bacteria from the rhizosphere (those being the 7 d.p.i. output samples). The samples extracted from the plants were all treated as follows: 1.8 mL were mixed with 200 μL 10X suspension buffer (0.5 M Tris-HCl, 0.1 M ethylenediaminetetraacetic acid, 0.5 M NaCl; pH 8) and centrifuged for 10 min at 14,000 x g. Of the supernatant, 1.8 mL was discarded and the remaining 200 μL of 1X suspension buffer containing the bacteria extracted from roots was stored at –80 °C until the time of DNA extraction. DNA extraction method is described in Supplementary methods.

Amplification of mutant signatures by qPCR through the use of bar-coded oligonucleotides

As previously reported, different mixes of 412 mini Tn*5* mutants were constructed for

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STM studies (Pobigaylo et al., 2006). The identity of any individual mutant within a given mix could be achieved by the identification of either of the two (H and K) DNA signatures present within the mini Tn5. In order to identify and quantify specific mutants in any mutant mix (*i. e.*, in initial inocula, or in extracts of plant rhizospheres at the end of the experiments), the H and K signatures were independently amplified by real-time PCR (qPCR) through the use of specific pairs of 5′–bar-coded primers P1, P2, P3, and P4 as summarized in Fig. S2 (Pobigaylo et al., 2006). For the primer bar-coding, different randomly generated 4-base-pair sequences were added to primers P1, P2, P3, and P4, followed by the addition of a terminal adenine. All 62 different primers used in this work are listed in Table S2. The bar codes present in primers P2 and P4 indicated the mutant-mix number, while the bar codes present in primers P1 and P3 indicated the specific experimental condition used and the replica number. qPCR amplification conditions are described in Supplementary methods.

Mixes of PCR products, high-throughput DNA sequencing, and sequence processing

In order to obtain a similar number of reads for each experimental condition, equivalent amounts of each one of the PCR-amplification products were mixed. To this end, the maximum intensity achieved in the qPCR reaction—*i. e.*, the fluorescence intensity obtained for each tube in the plateau—was taken into consideration. In order to mix comparable amounts of all PCR products, volumes between 2.5 and 4.5 µL of each sample were mixed. Samples from treatments corresponding to mutant set 11 in the 3 d.p.i. alfalfa group were not sequenced since the qPCR evidenced that the initial amount of DNA was insufficient to insure that the signatures would be representative. DNA sequencing specifications are described in Supplementary methods. Sequence analysis was done using Galaxy platform (Blankenberg et al., 2010) as described in Supplementary methods.

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Data analysis

The consolidated data for each experimental condition and each mutant mix consisted in a 412 x 8 count matrix (for either the H or the K data) including: 412 rows (*i. e.*, for the different mutants analyzed), 4 columns for the input replicates, and 4 columns for the output replicates. A spreadsheet template was used for the statistical calculations. For each experimental condition and mutant mix the proportion of a specific mutant in the mix was calculated as the number of counts for that mutant (*i. e.*, its sequence reads) over the total number of counts obtained for the corresponding experimental condition. The proportions of input and output replicates were separately averaged. The M_{STM} value for a given "*i*" mutant under a specific condition was calculated as follows:

$M_{STM} = \log_2$ {[proportion of mutant "i" in the output sample (rhizosphere)]/[proportion of mutant "i" in the input sample (inoculum)]} / {[proportion of wild-type strain in the output sample (rhizosphere)]/[proportion of wild-type strain in the input sample (inoculum)]}

For each mutant under all the conditions studied, M_{STM} values were obtained for both the H and the K signature. The statistical significance for differences between M_{STM} values was estimated by the Student t test.

Validation assays. Direct estimation of the competitiveness for root colonization by individual coinoculation experiments

For the validation experiments the same plant inoculation and cultivation system used in the STM experiments was employed. In this instance, the vermiculite-containing pots were inoculated with a 1:1 mixture of wild-type *E. meliloti* 2011-GFP+ and a given mutant (*ca*. 2 x 10^5 c.f.u. for both). The initial inoculum and the bacteria recovered from the rhizosphere after 3 or 7 d.p.i. were diluted and plated in TY medium supplemented with streptomycin. The

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GFP+ and the total bacteria were counted in a fluorescence stereomicroscope (Leica MZFL III) and the respective proportions of wild-type or mutant bacteria calculated as [(GFP+)/total bacteria] or as [(1 – GFP+)/total bacteria]. These proportions were used to calculate M values that were based on the results of the colony counts ($M_{plating}$).

Evaluation of nodulation competitiveness

In the experiments to evaluate the nodulation competitiveness of mutants compromised to a different extent in their rhizosphere colonization, plants were inoculated with a 1:1 mixture of wild-type strain to mutant rhizobia (neomycion-resistant) and then were grown for 4–5 weeks until mature nodules developed. The nodules were harvested, surface-sterilized with H_2O_2 (20 %) for 10 min, and washed with abundant sterile water. The sterilized nodules were then crushed in 50 μL of Fåhraeus medium and plated in TY supplemented with neomycin, where only the mutants were able to grow. For each competition experiment, 18 to 35 nodules collected from 30 to 40 plants were analyzed and the mean value for the percent of the nodules occupied by the mutant calculated.

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AUTHOR CONTRIBUTION: MES, MJL, JLL, WD, FJA, and JN did the STM experiments, amplifications, and validation experiments. MJL and GP processed the HiSeq sequencing results. JS sequenced the Tn*5*-insertion sites. MES, MJL, GTT, MP, and FDP did data analysis and interpretation. MES, MJL, AB and AL conceived the experiments and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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FIGURE LEGENDS

Fig. 1. General experimental design for the identification of *E. meliloti* Tn*5* mutants affected in rhizosphere colonization through an STM approach coupled with high-throughput DNA sequencing. The scheme summarizes the experimental design used to evaluate the root-colonization phenotype of more than 6,000 Tn*5* mutants distributed in 15 sets of *ca*. 412 mutants each (Pobigaylo et al., 2006). Experiments were performed in plastic pots containing sterile vermiculite inoculated by flooding with *ca*. 10^5 rhizobia c.f.u./mL (input sample). The proportion of each mutant in the input and output samples—the latter recovered from roots at 3 d.p.i. or 7 d.p.i.—was estimated by bar-coded PCR of specific Tn*5* signatures followed by high-throughput DNA sequencing (Illumina-HiSeq) of the amplified products (*cf*. details in Materials and Methods). For each set of mutants, under any of the indicated experimental conditions, four biological replicas were included. A decreased abundance of a specific mutant in the output sample compared to the abundance of the same mutant in the inoculum was indicative of a Tn*5* insertion that possibly affected root colonization. The experiments were performed with the roots of alfalfa and pea as host and nonhost plants, respectively.

Fig. 2. Selection of miniTn*5* mutants affected in rhizosphere colonization on the basis of their M_{STM} and *p* values. The panels in the figure represent the volcano plots of the M_{STM} values for the K (Panel A) and the H (Panel B) signatures evaluated at 3 d.p.i. versus their corresponding statistical significances (*p* values). In Panel A, the p value of the K tag is plotted on the y-axis versus the M value of the K tag on the x-axis; in Panel B, the p value of the H tag on the y-axis is plotted versus the M value of the H tag on the x-axis; and in Panel C, the p value of the H tag on the y-axis is plotted versus the p value K tag on the x-axis. The data for each panel represent all those mutants whose number of sequence reads was higher

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than 70. The gray shadowed boxes in panels A and B contain the group of negatively affected mutants that were associated with an $M_{STM} < -0.7$ and a $p < 0.1$ for either the K signature (Panel A, 223 mutants) or the H signature (Panel B, 209 mutants). Panel C plots the *p* values of those mutants from A and B that have in both signatures (K and H) M_{STM} values < -0.7, and the light-blue shadowed region includes only those mutants with at least $p \leq 0.05$ in one of the signatures, and $p \leq 0.1$ in the other (Those mutants correspond to the ones listed in Table S1.).

Fig. 3. Evaluation of specific mutants in the ability to colonize alfalfa roots in competition experiments against the wild-type rhizobia. One-day-old alfalfa seedlings were planted in plastic pots with vermiculite that had been previously inoculated with a mix of wild-type *E. meliloti* 2011 (GFP+):mutant rhizobia at a 1:1 ratio. The proportions of mutants in the inoculum and in the rhizosphere were calculated as the corresponding ratios of nonfluorescent/total rhizobia. Mutant 1.01.E07 was used as an unaffected control for root colonization ($M_{STM} = 0.11$). The results for each mutant correspond to the average of three biological replicates. All the mutants proved to be significantly affected in root colonization ($p < 0.05$, Chi-squared test). Indicated on the x-axis for each mutant is the gene disrupted by the Tn*5* insertion, the M_{STM} corresponding to an average of the M values obtained from colonization experiments in alfalfa at 3 and 7 d.p.i. (*cf*. the values in Table S1), and the $M_{plating}$ for the present colonization trial with these mutants. The bars represent standard errors. NS, insertion site unknown.

Fig. 4. Genomic location and functional prediction of the *E. meliloti* Tn*5*-mutated genes associated with rhizosphere colonization. Panel A: Genomic location of Tn*5* insertions in the mutants listed in Table S1. The results are expressed as the percent of Tn*5* insertions

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located in each *E. meliloti* replicon (pSymA, pSymB, or chromosome). Panel B: Functional prediction of the Tn*5*-disrupted genes listed in Table S1 according to their associated cluster of orthologous genes (COGs). The pie chart to the left illustrates, for genes that affect rhizsphere colonization, the percent contribution of each of the functional categories itemized on the right. Numbers in brackets indicate the percent contribution of each functional category when considering all Tn5-mutated genes in the STM library.

Fig. 5. Growth phenotypes in root exudates and in culture media of selected *E. meliloti* mutants affected in rhizosphere colonization. Sixteen selected mutants from those listed in Table S1 were characterized for growth in root exudates and in different culture media. As in Fig. 3, also included in the assay as a positive control was the Tn*5*-mutant strain 1.01.E07 (disrupted in open-reading frame SMc03113) that was unaffected in rhizosphere-colonization ability (M_{STM} = 0.11). Mutant and wild-type rhizobia were inoculated in comparable amounts into each nutrient source (1:1 ratio, at concentrations that ranging between 10^4 and 10^5 c.f.u./mL each). The gray bars represent the percentage of mutant rhizobia in the inoculum, the green bars the percentage of mutants in the alfalfa-root exudates at 4 d.p.i., and the blue bars the percentage of mutants in the alfalfa rhizosphere at 3 d.p.i. The ability of each mutant to grow in Evans minimal medium or in TY medium is indicated below the figure. Whereas 3 of the mutants failed to grow in Evans minimal medium, all the mutants grew in TY medium. As indicated in the text, the colonization-defective mutants were classified into three different groups (A–C, bracketed above the figure) according to the behavior observed under each of the conditions studied. The final values are the average of three biological replicates. The bars indicate standard error. All the mutants proved to be significantly affected in root colonization (blue bars; $p < 0.05$, Chi-squared test). NS, insertion site unknown.

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Fig. 6. Nodulation competitiveness of *E. meliloti* mutants with different degrees of disability in rhizosphere colonization. Mutants with increasing degrees of disability in rhizosphere colonization were inoculated with the wild-type rhizobia in a 1:1 ratio onto alfalfa plants. As in Figs. 3 and 5, also included in this experiment as a positive control was the Tn*5*-mutant strain 1.01.E07 (disrupted in open-reading frame SMc03113) that was unaffected in rhizosphere-colonization ability (M_{STM} = 0.11). Panel A: In the figure, the percent of each of the mutant strains occupying the nodules on the left y-axis (blue bars) and the M value for that same mutant on the right y-axis (red bars) are plotted for each of the mutant strains indicated on the x-axis. Panel B: Relationship between the capability of a mutant to occupy the rhizosphere (2^M) and its competitiveness for nodulation. In the figure, the percentage of nodules containing mutant strains is plotted on the y-axis as a function of the proportion of mutants present in the rhizosphere on the x-axis. The least-squares–regression equation is shown below in the figure, and the straight line representing the corresponding locus of points is drawn in black.

Fig. 7. Evaluation of the ability of specific mutants to colonize alfalfa and pea roots in competition experiments against the wild-type rhizobia. Alfalfa and pea seedlings were planted in plastic pots with vermiculite that had been previously inoculated with a mix of wild-type *E. meliloti* 2011 (GFP+):mutant rhizobia in a 1:1 ratio. The proportion of each mutant in the inoculum and in the rhizosphere at 7 d.p.i. were calculated as the corresponding ratios of nonfluorescent/total rhizobia. In the figure, the % of a given signature-tagged–mutagenesis mutant in the wild type–mutant mixture in the inoculum (light brown bars), in the alfalfa rhizosphere at 7 d.p.i. (blue bars), or in the pea rhizosphere (gray bars) is plotted on the y-axis for each of the mutants listed on the x-axis. The bars represent standard errors. The results for each mutant correspond to the average of three biological replicas. All

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mutants proved to be significantly defective in the colonization of alfalfa roots ($p < 0.05$, Chi-squared test). In addition, all mutants showed to be significantly more affected in alfalfa than in pea ($p < 0.05$, Chi-squared test)(plant-specificity effect).

Fig. 8. Evaluation of the ability of specific mutants to colonize different legume roots in competition experiments against the wild-type rhizobia. Seedlings of the indicated plants were planted in plastic pots with vermiculite that had been previously inoculated with a mix of wild-type *E. meliloti* 2011 (GFP+):mutant rhizobia in a 1:1 ratio. The proportions of mutants in the inoculum and in the rhizosphere at 7 d.p.i. were calculated as the corresponding ratios of nonfluorescent/total rhizobia. In the figure, the percent of a given bar-coded mutant in the inoculum and in the rhizospheres of the indicated plants is plotted on the y-axis for each of the mutants listed on the x-axis. Key to bar colors: light brown, inoculum; blue, *M. sativa* (alfalfa); light gray, *Medicago truncatula*; yellow, *Trifolium repens* (clover); blue-green, *Trigonella foenum-graecum* L.; medium gray, *Pisium sativum* (pea); dark gray, *Glycine max* (soybean). The bars represent standard errors. The results correspond to the average of three biological replicas for all the experiments except for the *fliM* mutant in *Trifolium repens* and the SMc03140 mutant in *Trifolium repens* and in *Trigonella foenum-graecum* L. that correspond to the average of two biological replicas. All the mutants proved to be significantly defective in the colonization of alfalfa roots ($p < 0.05$, Chi-squared test).

Fig. 9. Time-scaled schematic representation illustrating the diversification of the Fabeae, Phaseoleae, and Trifolieae tribes; indicating the estimated period (gray-shadowed box) for the appearance of the specific rhizospheric effects described in this work for *E. meliloti* and different members of the Trifolieae tribe. The estimations of node positions were performed according to TimeTree (www.timetree.org) based on data

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previously published (Hedges and Kumar, 2009; Hedges et al., 2015). In brief, two species or higher taxa are queried in the time tree of life (TTOL) to find their most recent common ancestor (MRCA). When one or both of the species is not present in the TTOL, the taxonomy from the National Center for Biotechnology Information is scanned to find the closest relatives of the species or higher taxa that was requested. Those taxa are used as proxies to find the MRCA for the given query. Finally, the divergence time for the MRCA is retrieved from the TimeTree database. In the figure, the time scale is expressed in millions of years ago (MYA). The red dots indicate the estimated divergence positions for the taxa of interest with their confidence intervals in brackets. The gray-shadowed box denotes the time period where the specific rhizospheric effect between *E. meliloti* and members of the Trifolieae tribe (see text) could have appeared.

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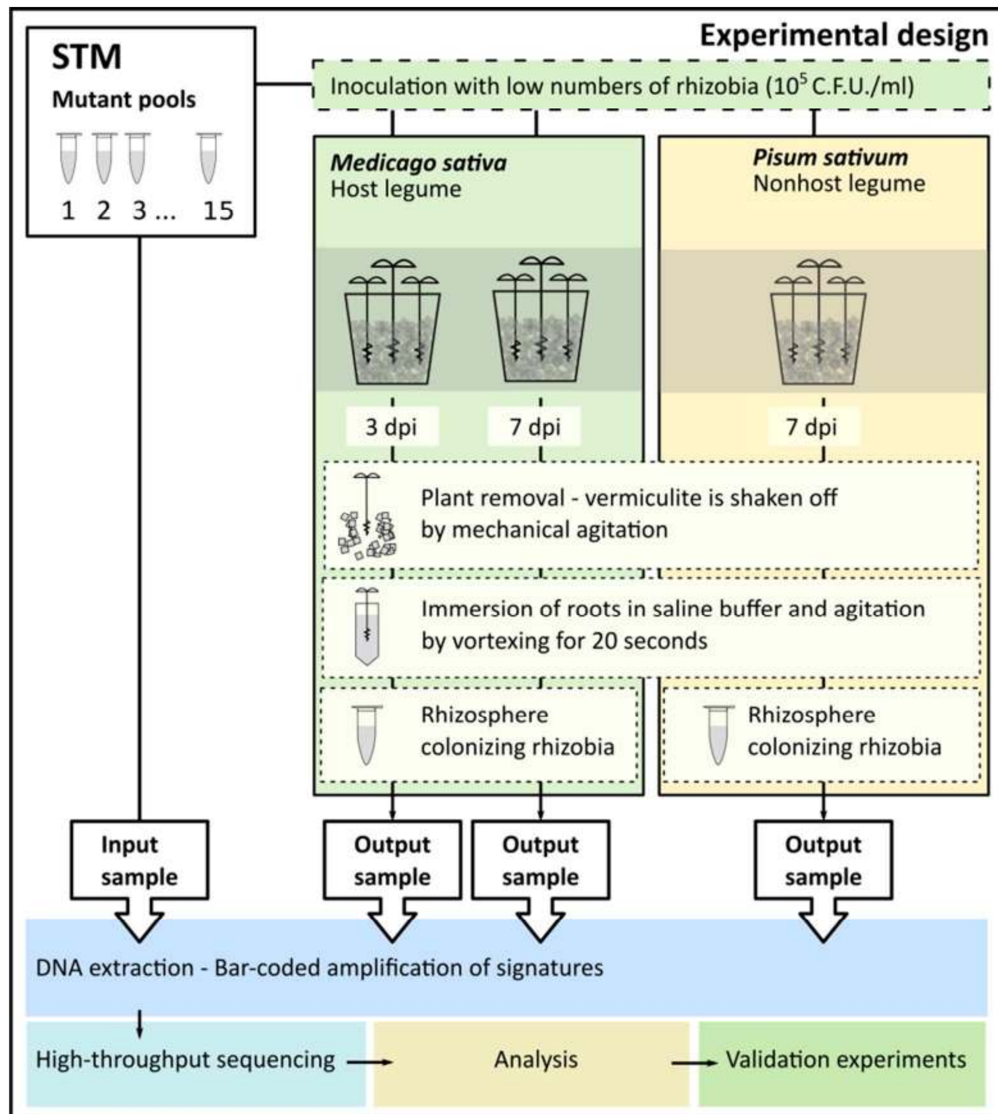

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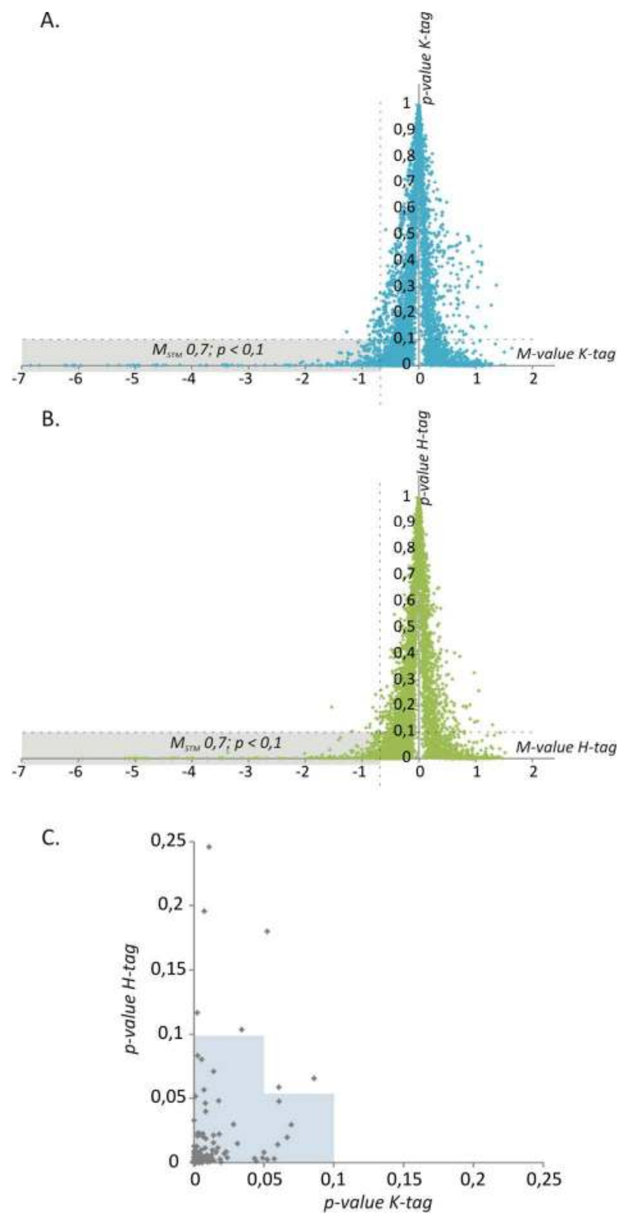

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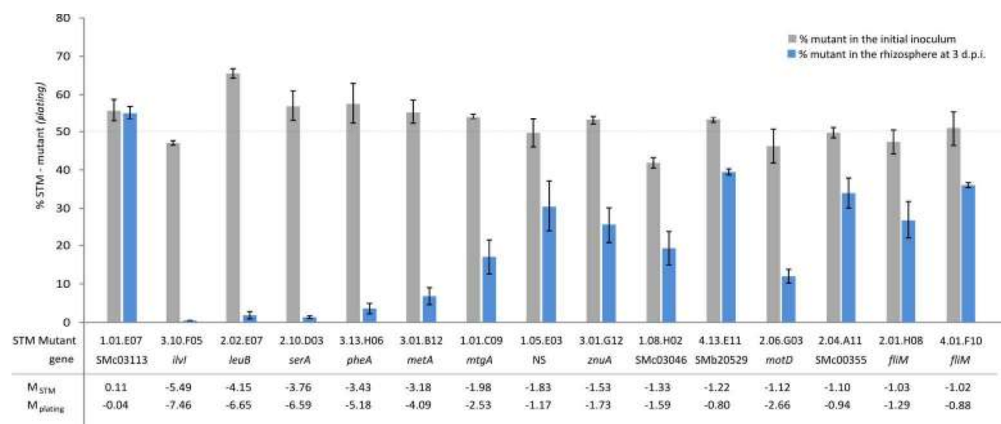

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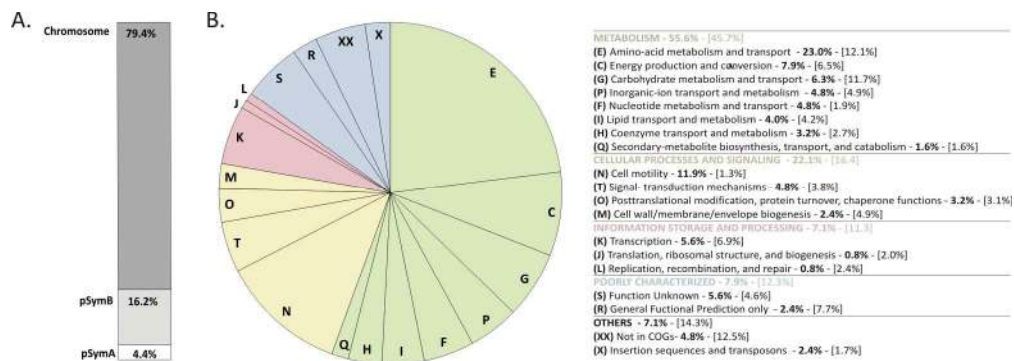

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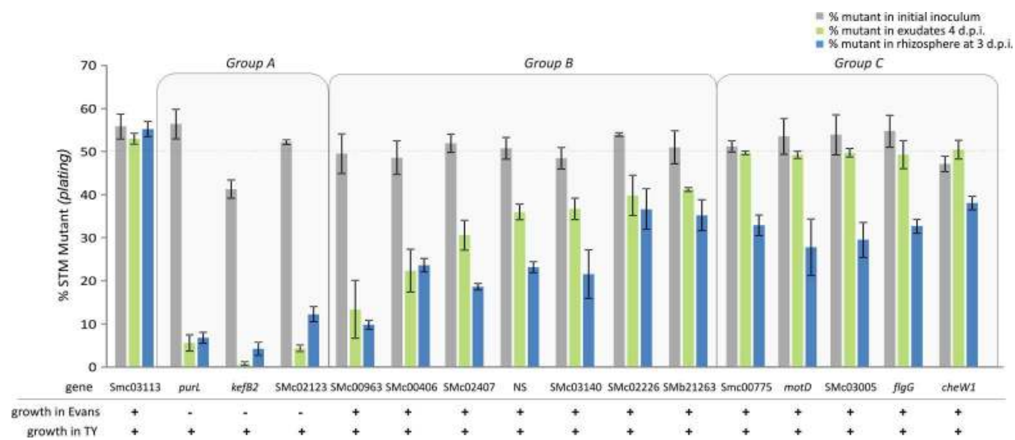

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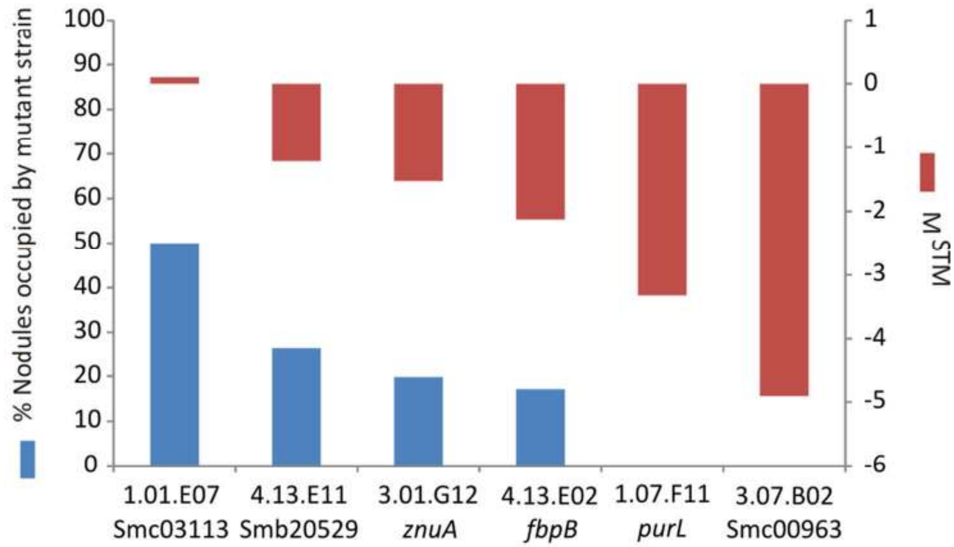

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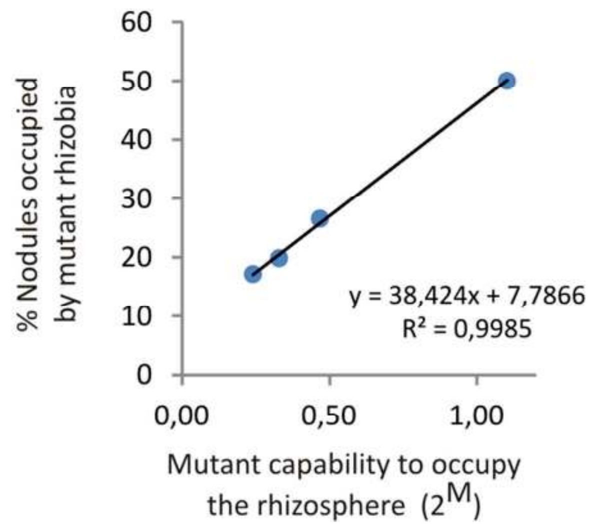

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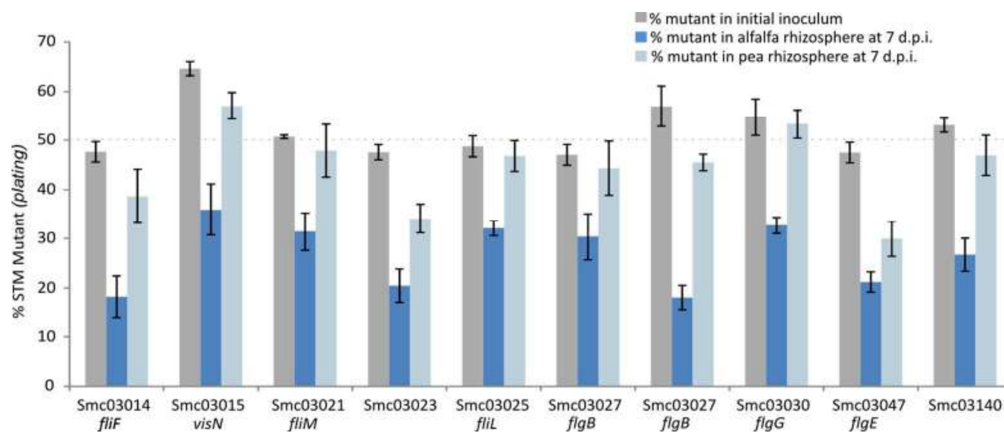

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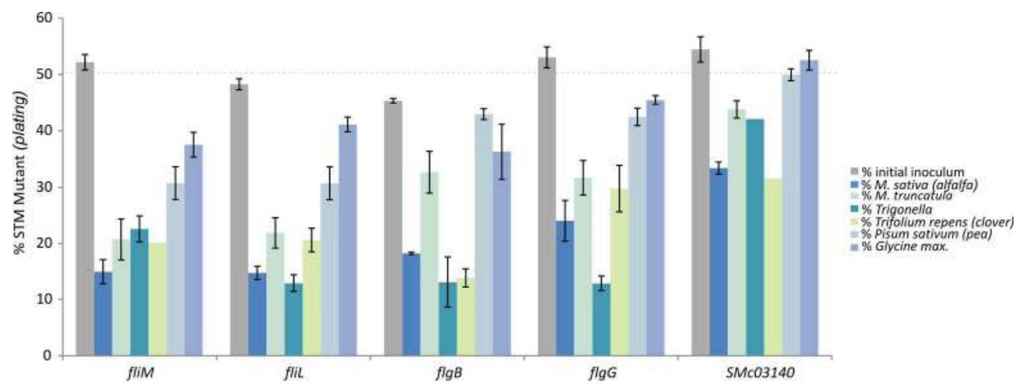

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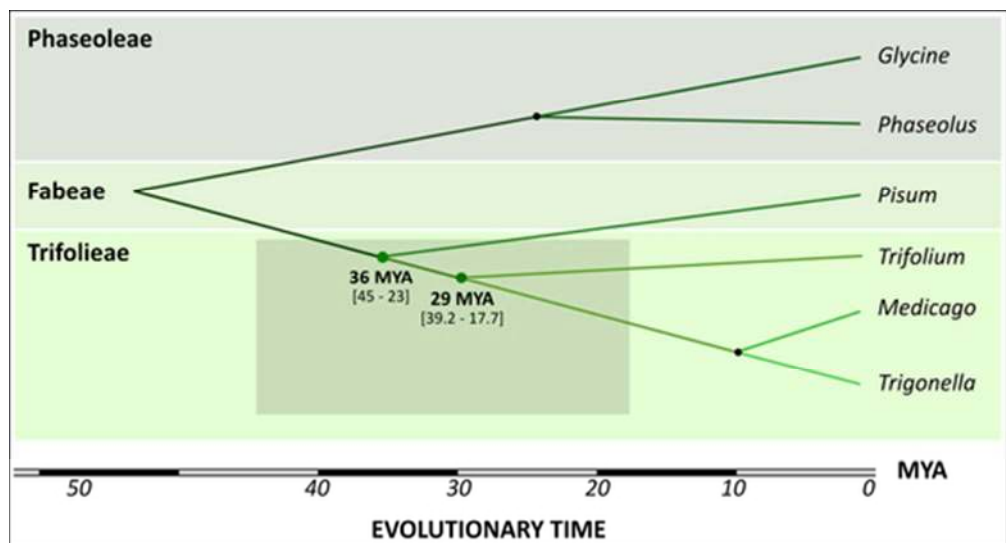

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