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Full Paper

Engineered ACC deaminase-expressing free-living cells of *Mesorhizobium loti* show increased nodulation efficiency and competitiveness on *Lotus* spp.†

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Ethylene inhibits the establishment of symbiosis between rhizobia and legumes. Several rhizobia species express the enzyme ACC deaminase, which degrades the ethylene precursor 1-cyclopropane-1-carboxilate (ACC), leading to reductions in the amount of ethylene evolved by the plant. *M. loti* has a gene encoding ACC deaminase, but this gene is under the activity of the NifA-RpoN-dependent promoter; thus, it is only expressed inside the nodule. The *M. loti* structural gene ACC deaminase (*acdS*) was integrated into the *M. loti* chromosome under a constitutive promoter activity. The resulting strain induced the formation of a higher number of nodules and was more competitive than the wild-type strain on *Lotus japonicus* and *L. tenuis*. These results suggest that the introduction of the ACC deaminase activity within *M. loti* in a constitutive way could be a novel strategy to increase nodulation competitiveness of the bacteria, which could be useful for the forage inoculants industry.

Key Words——ACC deaminase; competitiveness; ethylene; lotus; *Mesorhizobium loti*

Introduction

Inoculations with rhizobia may be vital to overcome nitrogen deficiencies in soil. However, some strains often fail to nodulate due to competition with native soil populations (Maier and Triplett, 1996; Vlassak and Vanderleyden, 1997). One approach to solve this problem is their genetic enhancement.

The gaseous phytohormone ethylene inhibits the early signal transduction cascade that characterizes and governs the earlier plant physiological changes during the nodulation process of legumes (Nukui et al., 2004; Oldroyd et al., 2001). It affects the infection thread initiation and elongation (Gage, 2004) and limits nodule number and development in *Medicago truncatula* and *M. sativa* (Penmetsa and Cook, 1997; Peters and Crist-Estes, 1989), which form indeterminate nodules, and in *Lotus japonicus*, which forms determinate ones (Nukui et al., 2000). In contrast, no inhibition has been found for *Glycine max* (Schmidt et al., 1999). Some rhizobia have developed mechanisms to lower plant ethylene and improve root nodulation. One of them relies on the constitutive production of rhizobitoxine, an amino-acid found in *Bradhyrhizobium el-*

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† In memoriam Rodolfo Ugalde.

kanii, which inhibits the activity of two enzymes involved in the ethylene biosynthetic pathway (Okazaki et al., 2004; Yuhashi et al., 2000). Mutants unable to synthesize rhizobitoxine are remarkably less competitive than the wild-type strain (Yuhashi et al., 2000). Another mechanism for ethylene diminution involves the production of ACC deaminase, an enzyme that converts 1-aminocyclopropane-1-carboxilate (ACC, the ethylene precursor) into ammonia and α-ketobutyrate (Penrose and Glick, 2003).

This enzyme has been intensively studied in some plant growth-promoting bacteria (PGPR, Glick et al., 1998; Penrose and Glick, 2001). In rhizobia, the gene codifying for ACC deaminase (*acdS*) has been detected in *Rhizobium leguminosarum* (Ma et al., 2003), *B. japonicum* (Kaneko et al., 2002), *M. loti* (Kaneko et al., 2000; Uchiumi et al., 2004), *Rhizobium* sp. (TAL1145) and *Sinorhizobium* sp. (BL3) (Tittabutr et al., 2008). The beneficial effect of this gene expression on bacterial competitiveness was demonstrated in *R. leguminosarum* and *M. loti* mutants (Ma et al., 2003; Uchiumi et al., 2004). In addition, *S. meliloti* strains expressing the *R. leguminosarum acdS* gene showed greater efficiency in nodulating alfalfa and were more competitive than the wild type strain (Ma et al., 2004). The introduction of multiple copies of the *acdS* gene from both *Rhizobium* sp. (TAL1145) and *Sinorhizobium* sp. (BL3) strains into the first one enhanced its symbiotic efficiency on *Leucaena leucocephala* (Tittabutr et al., 2008).

M. loti MAFF303099 expresses ACC deaminase only as bacteroid (inside the mature nodule), under the activity of a NifA-RpoN dependent promoter (Nukui et al., 2006). *Mesorhizobum* forms a symbiotic association with *Lotus* spp., a plant genus comprising more than 200 species, including *L. japonicus*, a model legume for genetic studies, whose genome has been sequenced (Sato et al., 2008; Szczyglowski and Stougaard, 2008). *L. tenuis* (syn. *L. glaber*) is an important forage legume with high adaptability to several abiotic conditions (Teckle et al., 2006). In addition, genotypes of this species may be propagated by stem cuttings as a way to reduce the intrinsic genetic heterogeneity occurring in available commercial lines (Echeverria et al., 2008). Since inoculated *M. loti* strains have to compete with those naturally occurring in the field, which often present a low nitrogen fixation capacity (Triplett and Sadowsky, 1992), the objective of the present work was the construction of a *Mesorhizobium* strain with the ability to constitutively express ACC deaminase during the free-living stage, additionally to its natural, induced expression as bacteroid, and to study its effect on *Lotus* nodulation. Our hypothesis was that the new ability would improve the infectivity and competitiveness of the engineered strain.

Results and Discussion

Characterization of the M. loti strain MAFF303099 bearing a constitutively expressed acdS gene copy integrated into its chromosome

The *acdS* gene of *M. loti* MAFF303099, transcriptionally fused with the promoter region of the Gentamicin (Gm) cassette (Ugalde et al., 2000) was integrated by a simple recombination event into the chromosome of *M. loti* MAFF303099 (MAFF SR) (Fig. 1A). There were no differences in growth between the recombinant and the wild-type (MAFF Sm) strains (Fig. 2A). In contrast, the ACC deaminase activity of MAFF SR was higher than that of the corresponding control, either in the presence or absence of Gm in the culture medium (Fig. 2B). This result confirms the constitutive activity of the cloned promoter.

Effect of the increased ACC deaminase activity on the symbiosis with Lotus spp.

Infectivity. The number of nodules induced by *M. loti* strains was assessed at 28 days post inoculation (dpi) on *L. japonicus* and at 60 dpi on *L. tenuis*. Both plant species presented a higher number of nodules per root when inoculated with the strain having in-

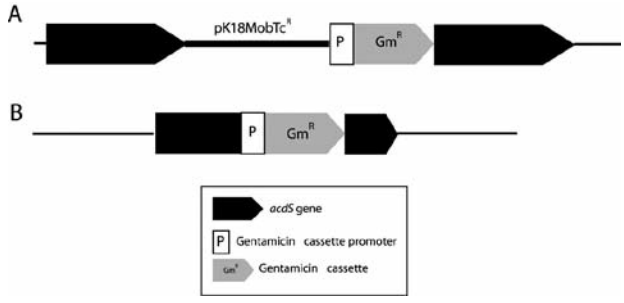

Fig. 1. Physical map of the constructions obtained in this work.

A) MAFF SR. An additional copy of the *acdS* gene under the control of the Gentamicin resistance cassette promoter was inserted into the chromosome of the wild-type MAFF303099. B) MAFF *acdS* mutant strain. The chromosomal copy of the *acdS* gene was interrupted by a Gentamicin resistance cassette in order to generate the dysfunctional mutant.

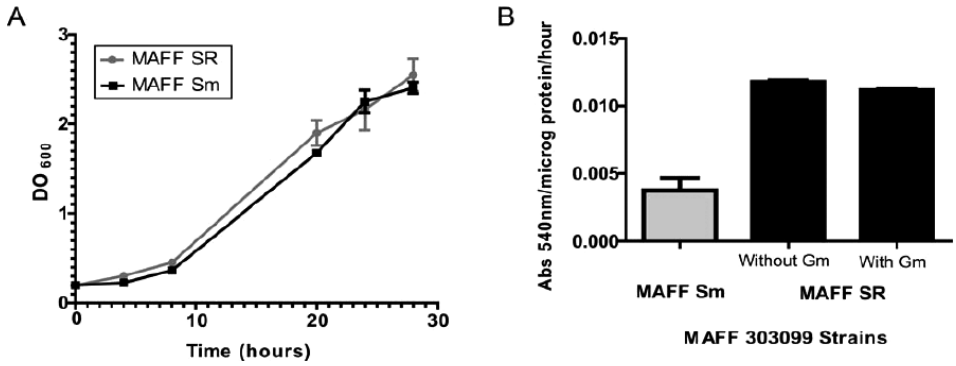

Fig. 2. Growth and ACC deaminase activity for wild-type and SR strains.

A) Comparison of growth culture kinetic between MAFF Sm and MAFF SR strains. Each value is the average of two determinations. B) ACC deaminase activity in MAFF Sm and MAFF SR strains, cultured in the presence or in the absence of Gentamicin. The results are the average of two determinations.

creased ACC deaminase activity (Fig. 3A and B). No differences were observed in the plant growth. This result extends to *L*. *tenuis* the direct relationship between the increased ability to degrade the ethylene precursor and the improved strain infectivity, previously demonstrated in *L*. *japonicus* (Uchiumi et al., 2004). To provide additional evidence about the occurrence of this relationship in *L*. *tenuis*, a further experiment was performed, where the nodulation level induced by a *M*. *loti* strain with its *acdS* gene disrupted (MAFF *acdS*) was compared with that induced by MAFF Sm. To avoid the genetic heterogeneity of the plant material, we used *L*. *tenuis* plants obtained from stem cuttings of two different genotypes (Echeverria et al., 2008). After 28 dpi, plants inoculated with the strain bearing the disrupted *acdS* gene presented a significantly lower number of nodules than those inoculated with the wild-type strain (Fig. 3C). This result was independent of the *L*. *tenuis* plant genotype and supports our previous finding of a direct relationship between ACC deaminase activity and root infectivity, also in this plant species. However, nodulation levels in genotype 19 were higher than those in genotype 30, what puts forward the possibility that infectivity may greatly vary within populations of *L*. *tenuis* and shows the convenience of using a more genetically homogeneous plant material for this kind of experiment.

Competitiveness for nodule occupancy. Earlier experiments demonstrated that the modified strain possesses a higher infectivity than the wild-type. Since *M*. *loti* MAFF303099 has a natural capacity to express ACC deaminase only into the active nodule, the obtained results indicate that an additional beneficial effect comes from expressing this activity also in earlier steps of the nodulation process. The capacity of a modified rhizobium to express the ACC deaminase in the free-living state allows it to lower the ACC levels in the rhizosphere. According to the model postulated by Glick et al. (1998) for the PGPR, a lowering of the ACC levels in the rhizosphere will conduce to a lowering of plant ethylene that could result in a positive effect for nodulation. However, this would favor nodulation by any compatible rhizobium in the rhizosphere, not only the engineered one. Therefore, we asked whether the constitutive expression of ACC deaminase during the free-living stage may confer a competitive advantage to the engineered bacteria over the wild-type strain. To test this hypothesis, seedlings of both *L*. *japonicus* and *L*. *tenuis* were co-inoculated with a mixture of MAFF Sm and MAFF SR bacterial suspensions. Table 1 shows the percentage of *M*. *loti* MAFF SR in the inocula used on *L*. *japonicus* and in that used on *L*. *tenuis*, and the percentage of nodules occupied by *M*. *loti* MAFF SR at 28 dpi, in the respective plants. Results indicate that the constitutive expression of ACC deaminase confers a competitive advantage to the engineered bacteria over the wild-type strain suggesting a more local effect. This also was suggested for the effect of the rhizobitoxine of *Bradyrhizobium elkanii* (Okazaki et al., 2004).

Strain competitiveness to colonize the root surface. There exists the possibility that the higher competitiveness might be due to the new capacity of the modified strain to use constitutively the ACC as a nutrient source, as suggested by Glick et al. (1998). To test whether the observed higher nodulation competitive-

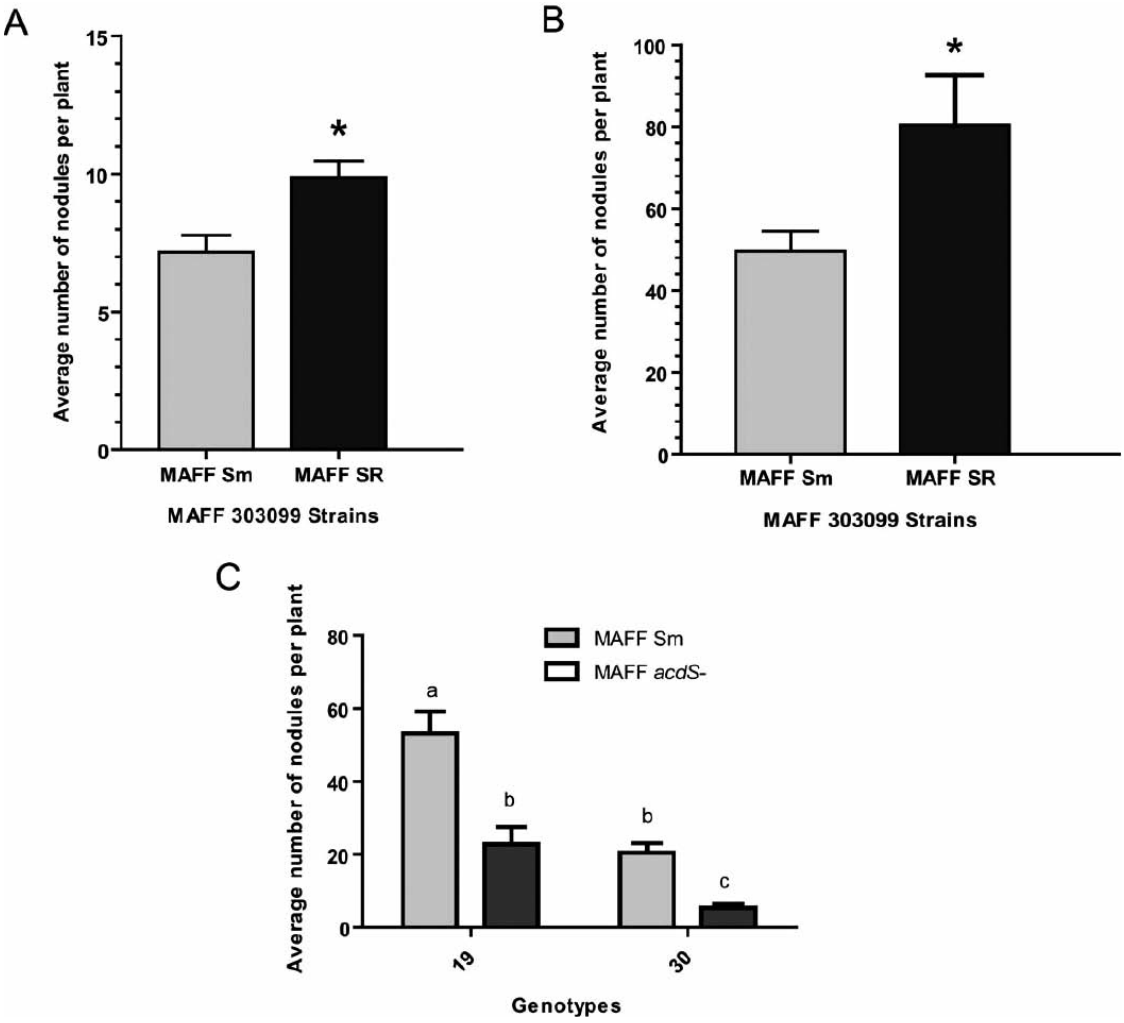

Fig. 3. Infectivity of wild-type and SR strains.

A) Number of nodules induced on a plant population of *L. japonicus* by MAFF Sm and by MAFF SR at 28 dpi. B) Number of nodules induced at 60 dpi on a plant population of *L. tenuis* (cv. Pampa INTA) by the MAFF Sm and the MAFF SR. Results are the average of three experiments. Asteriks mean significantly different at $p \leq 0.05$. C) Number of nodules induced on two different *L. tenuis* genotypes by the MAFF Sm and the MAFF *acdS* mutant strains. Results are the average of three experiments. Bars with the same letter are not significantly different ($p \leq 0.05$).

Table 1. Competitiveness for nodule occupancy.

Inoculated plant	% of *M. loti* MAFF SR in the co-inoculation mixture	% of nodules occupied by *M. loti* MAFF SR at 28 dpi
L. tenuis	48	70±7
L. japonicus	22	67±11

Values for % of nodules occupied by MAFF SR represent the mean of three experiments.

ness is due to a higher colonization due to this capacity, a bacterial suspension containing a mixture of the wild type and recombinant strain (MAFF SR) in a 0.25 : 0.75 proportion was inoculated on *L. japonicus* roots and changes in that proportion were followed during 22 days. Figure 4 shows that the relative amounts of recombinant and wild type strains in the bacterial population on the root suffered respectively a slight decrease and increase during the time course experiment. This result, although it suggests that the MAFF SR strain has less capacity for long-term survival, rules out the possibility that the observed positive effect of the increased ACC deaminase activity on

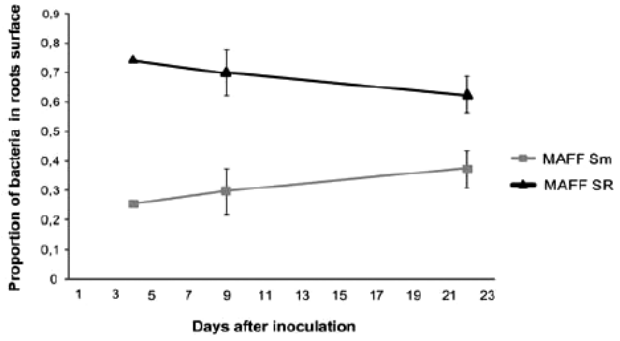

Fig. 4. Relative proportion of MAFF Sm and MAFF SR strains on *L*. *japonicus* roots.

A mixture of wild-type and recombinant strain, in a 0.25 : 0.75 proportion, was inoculated on *L*. *japonicus* roots and the proportion of each one at the root surface was determined at different post inoculation times.

nodulation was due to an improved ability to colonize the root surface by the recombinant strain. This is in line with Belimov et al. (2009), who observed that genetic modification had no effect on the root colonization ability of the PGPR ACC deaminase-expressing *Pseudomonas brassicacearum* in tomato.

In conclusion, we have constructed a new strain of *M*. *loti* with the ability to express ACC deaminase in free-living conditions in addition to its natural bacteroid state-expressing capacity. The obtained modified strain was able to induce more nodules (both on the model *L*. *japonicus* and on the economically important forage *L*. *tenuis)* and also presented higher competitiveness compared with the wild type strain. Consequently, the introduction of the ability to express ACC deaminase in the free-living state, in addition to the natural bacterial expression into active nodules, could constitute a good strategy to increase the competitiveness of inoculants applied to fields. With this objective in mind, our next step is to obtain a modified strain with constitutive ACC deaminase expression but without the information for Gentamicin resistance. However, rigorous experimentation must be done before this strain may become a realistic and efficient inoculant option for plant growth promotion in the field, including the assessment of the nitrogen fixing capacity and survival length of mutants, compared with those of wild-type strains, as well as the description of potential adverse effects of the integrated *acdS* gene and mechanisms intervening in the improvement of *M*. *loti* nodulation competitiveness on *Lotus* spp.).

Experimental Procedures

Bacterial strains and growth media. *Escherichia coli* strains were grown at 37°C in Luria-Bertani media. *M*. *loti* strains were grown at 28°C in AB minimal medium (Douglas et al., 1985) supplemented with sucrose (0.5%, wt/vol). When necessary, antibiotics were added to the following final concentration (μg/ml): Gentamicin (Gm) at 15, Ampicilin (Amp) at 100, Streptomycin (Sm) at 250, and tetracycline (Tc) at 10 for *E*. *coli* or 1 for *M*. *loti*.

Construction of the MAFF SR strain. A DNA fragment was amplified from *M*. *loti* MAFF303099 by polymerase chain reaction (PCR) with the *pfu* enzyme and with the primers 5′-GGATCCTTGCGTGGCTATTTC-3′ and 5′-TTAACAACTGCCCGCTGA-3′. The PCR amplified a DNA fragment of 1,153 bp, spanning from 27 bp upstream of the ATG to 76 bp downstream of the stop codon of the *M*. *loti acdS* gene. The PCR product was ligated into pBluescript II KS (+/−) (Stratagene) digested with *Eco*RV (pBlue-acdS). The construction was sequenced to eliminate the possibility of any mutation generated during amplification. Then, the construction was digested with *Eco*RI and *Hind*III and the resulting fragment was cloned into pBBR1MCS-4 (Kovatch et al., 1995), which was digested with the same enzymes, in the opposite direction with respect to the transcription of the endogenous promoter of the vector multiple cloning site. The resulting plasmid was called pBBR*acdS*. A Gm cassette was digested from plasmid pSPG1 (Ugalde et al., 2000) with *BamH*I. This cassette is devoid of a transcriptional terminator sequence (Ugalde et al., 2000) and has constitutive promoter activity in rhizobium. pBBR*acdS* was digested with *BamH*I upstream of the *acdS* ATG and ligated to the Gm cassette. Constructs in which the transcription of *acdS* was in the orientation of the Gm promoter were selected. The construction in the pBBR1MCS-4 vector was digested with *Xba*I and *Hind*III and the Gm::*acdS* construct was ligated into pK18mobTc (Sánchez et al., 2009), a suicide vector in rhizobium obtaining the pK18mobGm::*acdS* plasmid. pK18mobTcGm::*acdS* was used to transform *E*. *coli* S17 λpir and then introduced into *M*. *loti* MAFF303099 by biparental mating. Gm-resistant clones were isolated and a simple recombination event was selected by searching Tc resistant strains. On this basis a recombinant strain called MAFF SR was selected. Figure 1A shows an schematic representation of the new construction inte-

grated into the *M*. *loti* chromosome.

Construction of the MAFF acdS mutant. A Gm cassette, devoid of a transcriptional terminator sequence (Ugalde et al., 2000), was digested with *BamH*I, extreme filled and ligated into a unique *Stu*I site of the *acdS* insert into the pBlue-*acdS* plasmid. The gene fragment containing the Gm cassette was cut out of pBlue-acdS::Gm with *Pst*I and *Hind*III and ligated to the pK18mobTc (Sánchez et al., 2009), digested with the same enzymes. The resulting plasmid, pK18mobTc-*acdS*::Gm, was used to transform the *E*. *coli* S17 λpir strain and then introduced into *M*. *loti* MAFF303099 by biparental mating. Gm-resistant clones were isolated and double recombination events were selected by testing sensitivity to Tc. On this basis, a mutant named MAFF *acdS* was selected. The occurrence of a double crossover event was corroborated by PCR analysis. Figure 1B shows a schematic representation of the Gm cassette integration into the *M*. *loti* genome.

Analysis of ACC deaminase activity. ACC deaminase activity was measured, at an exponential phase of bacterial growth (0.8−1.0 OD), according to Penrose and Glick (2003).

Nodulation assays. Germinated seeds: *L*. *japonicus* Gifu and *L*. *tenuis* (cv. Pampa INTA) seeds were surface sterilized and pre-germinated following Lepek et al. (1990). Three-day-old seedlings were planted (5 plants per jar) in autoclaved modified Leonard jars filled with vermiculite and B & D 1/4 (Broughton and Dilworth, 1971) solution supplemented with 0.1 mM KNO_3, and inoculated with *M*. *loti* strains at an OD of 0.6 (200 μl/plant). The number of nodules per plant was determined at 28 days post inoculation (dpi) in the case of *L*. *japonicus* and at 60 dpi for *L*. *tenuis*. Results are the average of three experiments.

Stem cuttings: Two genotypes of *L*. *tenuis* (19 and 30) differing in their salt tolerance capacity (Echeverria et al., 2008) were vegetatively propagated according to Mujica and Rumi (1998). Nineteen-day-old rooted cuttings were transferred to modified Leonard jars filled with sterile vermiculite and Evans solution (Evans and Hoagland, 1927), and inoculated with *M*. *loti* strains at an OD of 0.6 (1 ml/plant). The number of nodules per plant was determined at 28 dpi. Results are the average of three experiments.

Competition assay for nodule occupancy. The two strains, wild type (MAFF Sm) and MAFF SR, were mixed together and used to inoculate *Lotus* spp. plants, as described previously (D'Antuono et al., 2005). The exact proportion of each strain in the mixture was determined by dilution and plating in media with the corresponding antibiotics. At 28 dpi, 10 nodules from each of three experiments were harvested, sterilized, and crushed separately in AB minimal medium. To determine the proportion of nodules occupied by each strain, serial dilutions were plated on AB (0.5% sucrose) minimal medium with Gm or Sm respectively. Nodulation results are the average of the three experiments.

Evaluation of strain competitiveness to colonize the root surface. The colonization competitiveness between the wild-type and MAFF SR strains was assessed according to Wolfgang et al. (1996) with some modifications. Seeds of *L*. *japonicus* were sterilized and germinated as above. Two-day-old seedlings were transferred to a Leonard jar filled with vermiculite and B&D 1/4 solution and roots were inoculated with a bacterial suspension containing a mixture (0.25 : 0.75) of the wild type and recombinant strain on day 0. The exact proportion of each strain in the mixture was determined by dilution and plating in media with the corresponding antibiotics. Different days after the inoculation, plants were harvested and shaken in order to remove the vermiculite. The root was excised from the stem and placed in a 1.5 ml tube containing 1 ml of distilled water plus 0.01% Tween 20 and vortexed for 10 min. In order to calculate the relative proportion of each strain that colonized the root surface, an appropriate dilution of the suspension was placed in AB sucrose 0.5% agar and incubated. Grown colonies were then sub-cultured in the same medium supplemented with either Gm or Sm. Each treatment consisted of at least three replicate Leonard jars, each containing three plants.

Statistical analysis. Differences were analyzed using Duncan's test.

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References

Belimov, A. A., Dodd, I. C., Hortzeas, N., Theobald, J. C., Safronova, V. I., and Davies, W. J. (2009) Rhizosphere bacteria containing 1-aminocyclopropane-1-carboxylate deaminase increase yield of plants grown in drying soil via both local and systemic hormone signaling. *New Phytol*., **181**, 413–423.

Broughton, W. J. and Dilworth, M. J. (1971) Control of leghemoglobin synthesis in snake beans. *Biochem. J*., **125**, 1075–1080.

Douglas, C. J., Staneloni, R. J., Rubin., R. A., and Nester, E. W. (1985) Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. *J. Bacteriol*., **161**, 850–860.

D'Antuono, A. L., Casabuono, A., Couto, A., Ugalde, R. A., and Lepek, V. C. (2005) Nodule development induced by *Mesorhizobium loti* mutant strains affected in polysaccharide synthesis. *Mol. Plant-Microbe Interact*., **18**, 446–457.

Echeverria, M., Scambato, A. A., Sannazzaro, A., Maiale, S., Ruiz, O., and Menéndez, A. B. (2008) Phenotypic plasticity with respect to salt stress response by *Lotus glaber*: The role of its AM fungal and rhizobial symbionts. *Mycorrhiza*, **18**, 317–329.

Evans, H. M. and Hoagland, D. R. (1927) The synthesis of vitamin E by plants grown in cultures solution. *Am. J*. *Physiol*., **80**, 702–704.

Gage, D. J. (2004) Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev*., **68**, 280–300.

Glick, B. R., Penrose, D. M., and Li, J. (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *J. Theor. Biol*., **190**, 63–68.

Kaneko, T., Nakamura, Y., Sato, S., Asamizu, E., Kato, T., Sasamoto, S., Watanabe, A., Idesawa, K., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Mochizuki, Y., Nakayama, S., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M., and Tabata, S. (2000). Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Res*., **7**, 331–338.

Kaneko, T., Nakamura, Y., Sato, S., Minamisaura, K., Uchiumi, T., Sasamoto, S., Watanabe, A., Idesawa, K., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Mochizuki, Y., Nakayama, S., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M., and Tabata, S. (2002) Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. *DNA Res*., **9**, 189–197.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II, and Peterson, K. M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCs, carrying different antibiotic-resistance cassettes. *Gene*, **166**, 175–176.

Lepek, V., Navarro de Navarro, Y., and Ugalde, R. A. (1990) Synthesis of β(1-2)glucan in *Rhizobium loti*. Expression of *Agrobacterium tumefaciens chvB* virulence region. *Arch. Microbiol*., **155**, 35–41.

Ma, W., Charles, T. C., and Glick, B. R. (2004) Expression of an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene in *Sinorhizobium meliloti* increases its ability to nodulate alfalfa. *Appl. Environ. Microbiol*., **70**, 5891–5897.

Ma, W., Guinel, F. C., and Glick, B. R. (2003) *Rhizobium leguminosarum* biovar viciae 1-aminocyclopropane-1-carboxylate deaminase promotes nodulation of pea plants. *Appl. Environ. Microbiol*., **69**, 4396–4402.

Maier, R. J. and Triplett, E. W. (1996) Toward more productive, efficient, and competitive nitrogen-fixing symbiotic bacteria. *Crit. Rev. Plant Sci*., **15**, 191–234.

Mujica, M. M. and Rumi, C. P. (1998) A technique of vegetative propagation by stem cuttings was fitted to *Lotus tenuis*. *Lotus Newsl*., **29**, 411.

Nukui, N., Ezura, H., and Minamisawa, K. (2004) Transgenic *Lotus japonicus* with an ethylene receptor gene *Cm-ERS*/*H70A* enhances formation of infection threads and nodule primordia. *Plant Cell Physiol*., **45**, 427–435.

Nukui, N., Ezura, H., Yuhashi, K., Yasuta, T., and Minamisawa, K. (2000) Effects of ethylene precursor and inhibitors for ethylene biosynthesis and perception on nodulation in *Lotus japonicus* and *Macroptilium atropurpureum*. *Plant Cell Physiol*., **41**, 893–897.

Nukui, N., Minamisawa, K., Ayabe, S., and Aoki, T. (2006) Expression of the 1-aminocyclopropane-1-carboxylic acid deaminase gene requires symbiotic nitrogen-fixing regulator gene *nifA2* in *Mesorhizobium loti* MAFF303099. *Appl. Environ. Microbiol*., **72**, 4964–4969.

Okazaki, S., Nukui, N., Sugawara, M., and Minamisawa, K. (2004) Rhizobial strategies to enhance symbiotic interactions: Rhizobitoxine and 1-aminociclopropano-1-carboxilate deaminase. *Microbes Environ*., **19**, 99–111.

Oldroyd, G. E., Engstrom, E. M., and Long, S. R. (2001) Ethylene inhibits the Nod factor signal transduction pathway of *Medicago truncatula*. *Plant Cell*, **13**, 1835–1849.

Penmetsa, R. V. and Cook, D. R. (1997) A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science*, **275**, 527–530.

Penrose, D. M. and Glick, B. R. (2001) Levels of ACC and related compounds in exudate and extracts of canola seeds treated with ACC deaminase-containing plant growth-promoting bacteria. *Can. J. Microbiol*., **47**, 368–372.

Penrose, D. M. and Glick, B. R. (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol. Plant*., **118**,10–15.

Peters, N. K. and Crist-Estes, D. K. (1989) Nodule formation is stimulated by the ethylene inhibitor aminoethoxyvinylglycine. *Plant Physiol*., **91**, 690–693.

Sánchez, C., Iannino, F., Deakin, W. J., Ugalde, R. A., and Le-

pek, V. C. (2009) Characterization of the *Mesorhizobium loti* MAFF303099 type-three protein secretion system. *Mol. Plant Microbe-Interact*., **22**, 519–528.

Sato, S., Nakamura, Y., Kaneko, T., Kato, T., Nakao, M., Sasamoto, S., Watanabe, A., Ono, A., Kawashima, K., Fujishiro, T., Katoh, M., Kohara, M., Kishida, Y., Minami, C., Nakayama, S., Nakazaki, N., Shimizu, Y., Shinpo, S., Takahashi, C., Wada, T., Yamada, M., Ohmido, N., Hayashi, M., Fukui, K., Baba, T., Nakamichi, T, Mori, H., and Tabata, S. (2008) Genome structure of the legume *Lotus japonicus. DNA Res*., **15**, 227–239.

Schmidt, J. S., Harper, J. E., Hoffman, T. K., and Bent, A. F. (1999) Regulation of soybean nodulation independent of ethylene signaling. *Plant Physiol*., **119**, 951–959.

Szczyglowski, K. and Stougaard, J. (2008) *Lotus* genome: Pod of gold for legume research. *Trends Plant Sci*., **13**, 515–517.

Teckle, N. L., Real, D., and Colmer, T. D. (2006) Growth and ion relations in response to combined salinity and waterlogging in the perennial forage legumes *Lotus corniculatus* and *Lotus glaber*. *Plant Soil*, **289**, 369–383.

Tittabutr, P., Awaya, J. D., Li, Q. X., and Borthakur, D. (2008) The cloned 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene from *Sinorhizobium* sp. strain BL3 in *Rhizobium* sp. strain TAL1145 promotes nodulation and growth of *Leucaena leucocephala*. *Syst. Appl. Microbiol*., **31**, 141–150.

Triplett, E. W. and Sadowsky, M. J. (1992) Genetics of competition for nodulation of legumes. *Annu. Rev. Microbiol*., **46**, 399–428.

Uchiumi, T., Ohwada, T., Itakura, M., Mitsui, H., Nukui, N., Dawadi, P., Kaneko, T., Tabata, S., Yokoyama, T., Tejima, K., Saeki, K., Omori, H., Hayashi, M., Maekana, T., Sriprang, R., Muruoka, Y., Tajima, S., Simomura, K., Nomura, M., Suzuki, A., Shimoda, Y., Sioya, K., Abe, M., and Minamisawa, K. (2004) Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome. *J. Bacteriol*., **186**, 2439–2448.

Ugalde, J. E., Czibener, C., Feldman, M. F., and Ugalde, R. A. (2000) Identification and characterization of the *Brucella abortus* phosphoglucomutase gene: Role of lipopolysaccharide in virulence and intracellular multiplication. *Infect. Immun*., **68**, 5716–5723.

Vlassak, K. M. and Vanderleyden, J. **(**1997) Factors influencing nodule occupancy by inoculant rhizobia. *Crit. Rev. Plant Sci*., **16**, 163–229.

Wolfgang, R. S., Joseph, C. M., and Phillips, D. A. (1996) Biotin and other water-soluble vitamins are key growth factors for alfalfa root colonization by *Rhizobium meliloti* 1021. *Mol. Plant-Microbe Interact*., **9**, 330–338.

Yuhashi, K., Ichikawa, N., Ezura, H., Asao, S., Minakawa, Y., Nukui, N., Yasuta, T., and Minamisawa, K. (2000) Rhizobitoxine production by *Bradyrhizobium elkanii* enhances nodulation and competitiveness on *Macroptilium atropurpureum*. *Appl. Environ. Microbiol*., **66**, 2658–2663.