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Rhizobium leguminosarum Biovar viciae 1-Aminocyclopropane-1-Carboxylate Deaminase Promotes Nodulation of Pea Plants

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Ethylene inhibits nodulation in various legumes. In order to investigate strategies employed by *Rhizobium* to regulate nodulation, the 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene was isolated and characterized from one of the ACC deaminase-producing rhizobia, *Rhizobium leguminosarum* bv. viciae 128C53K. ACC deaminase degrades ACC, the immediate precursor of ethylene in higher plants. Through the action of this enzyme, ACC deaminase-containing bacteria can reduce ethylene biosynthesis in plants. Insertion mutants with mutations in the rhizobial ACC deaminase gene (*acdS*) and its regulatory gene, a leucine-responsive regulatory protein-like gene (*lrpL*), were constructed and tested to determine their abilities to nodulate *Pisum sativum* L. cv. Sparkle (pea). Both mutants, neither of which synthesized ACC deaminase, showed decreased nodulation efficiency compared to that of the parental strain. Our results suggest that ACC deaminase in *R. leguminosarum* bv. viciae 128C53K enhances the nodulation of *P. sativum* L. cv. Sparkle, likely by modulating ethylene levels in the plant roots during the early stages of nodule development. ACC deaminase might be the second described strategy utilized by *Rhizobium* to promote nodulation by adjusting ethylene levels in legumes.

Gram-negative soil bacteria that belong to the family *Rhizobiaceae* are well-known for their ability to infect the root tissues of their compatible host legumes and induce the formation of nitrogen-fixing nodules (34). For more than a decade, the phytohormone ethylene has been known to inhibit nodulation in various legumes (16, 18, 22, 26). Decreased levels of nodulation have been observed after application of exogenous ethylene or 1-aminocyclopropane-1-carboxylic acid (ACC) prior to or at the same time as the addition of rhizobia (18, 22); conversely, nodulation can be promoted when plants are treated with ethylene inhibitors or antagonists (18, 22, 26, 38).

The fate of rhizobial infection in the root hairs of legumes has been proposed to be regulated by the levels of ethylene in the underlying plant cortex (13); a low level of ethylene, allowing proper disposition of the cytoskeleton, is probably required for successful entry of the infection thread in the outermost layer of cortical cells, whereas higher levels of the hormone induce abortion of the infection thread by inducing cross-linking of its matrix glycoproteins. This hypothesis is substantiated by numerous types of evidence. For example, *sickle*, an ethylene-insensitive mutant of barrel medic (*Medicago truncatula*), has very high persistence of infection threads and a hypernodulation phenotype (23); on the other hand, in *brz*, a potential ethylene-oversensitive mutant of *Pisum sativum*, the number of aborted infection threads is much higher than the number in wild-type plants (15).

On an evolutionary basis, it would have been beneficial for the rhizobia to develop mechanisms by which the levels of plant endogenous ethylene are reduced. One rhizobial species, *Bradyrhizobium elkanii*, appears to have done this by producing rhizobitoxine, an ethylene biosynthesis inhibitor. This compound was found to enhance the nodulation of siratro (*Macroptilium atropurpureum* Urb.) by reducing root ethylene production (38). In this study, we were interested in studying another mechanism known to be used by plant growth-promoting bacteria to decrease ethylene levels in plants (9, 10). These microorganisms, which attach to the surfaces of plant roots or seeds, take up some of the ACC exuded from the plant and degrade it through the action of ACC deaminase, an enzyme which converts ACC to ammonia and α-ketobutyrate. In order to maintain the equilibrium between internal and external ACC levels, more ACC is exuded by the plant and drawn away from the ethylene biosynthesis pathway (9, 24); this mechanism effectively reduces the amount of ethylene evolved by the plant. Thus, plants inoculated with ACC deaminase-producing bacteria have longer roots in gnotobiotic conditions (10) and are better able to resist the inhibitory effects of ethylene stress on plant growth imposed by heavy metals (3), pathogens (36), and flooding (12). In a survey of 13 wild-type *Rhizobium* spp., we found 5 species which had ACC deaminase activity (21). One of these five rhizobia was *Rhizobium leguminosarum* bv. viciae 128C53K. Whereas *Enterobacter cloacae* UW4 (an organism which produces high levels of ACC deaminase) had an ACC deaminase activity of 21.23 ± 0.17 μmol of α-ketobutyrate · h^{-1} · mg of protein^{-1}, 128C53K had an activity of 1.06 ± 0.17 μmol of α-ketobutyrate · h^{-1} · mg of protein^{-1}. We postulated that these strains, which have ACC deaminase activity, are able to lower ethylene levels in legumes and overcome some of the inhibitory effects of ethylene on nodulation. Here, we describe cloning of the ACC deaminase gene and its regulatory region from *R. leguminosarum* bv. viciae 128C53K, as well as the involvement of ACC deaminase in the enhancement of nodulation in pea plants.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference(s)
Rhizobium leguminosarum bv. viciae strains		
128C53K	AcdS$^+$, nodulates *P. sativum* L. cv. Sparkle	Lipha Tech
128Sm	AcdS$^+$, nodulates *P. sativum* L. cv. Sparkle, Sm^r	This study
128Sm*acdS*Ω::Km	AcdS$^-$ *acdS*Ω::Km Gm^s Sm^r Km^r	This study
128Sm*acdS*Ω::Km(pSPacdS)	AcdS$^+$ Tc^r Sm^r Km^r	This study
128Sm*lrpL*Ω::Km	AcdS$^-$ *lrpL*Ω::Km Gm^s Sm^r Km^r	This study
128Sm*lrpL*Ω::Km(pSPlrpL)	AcdS$^+$ Tc^r Sm^r Km^r	This study
128Sm(pWM2)	ACC deaminase high-expression mutant, Tc^r Sm^r Km^r	This study
Enterobacter cloacae UW4	AcdS$^+$, plant growth-promoting bacterium	10
Escherichia coli strains		
DH5α	SupE44 Δ*lacU169*(ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	28
S17-1	Mobilizing strain with chromosomally integrated RP4 derivative	32
Plasmids		
pUC19	Ap^r	28
pWM1	pUC19 containing the *acdS* and *lrpL* genes of *R. leguminosarum* bv. viciae 128C53K on a 4-kb DNA fragment	This study
pJQ200KS	*mob* Gm^r *ori sacB*	27
pHP45Ω-Km	Ap^r Km^r	8
pJQacdSΩ::Km	*R. leguminosarum* bv. viciae 128Sm *acdS* gene disrupted by a kanamycin resistance gene inserted into pJQ200KS, Km^r Gm^r	This study
pJQlrpLΩ::Km	*R. leguminosarum* bv. viciae 128Sm *lrpL* gene disrupted by a kanamycin resistance gene inserted into pJQ200KS, Km^r Gm^r	This study
pSP329	RP4 replicon, broad-host-range vector, Tc^r	5, 29
pWM2	pSP329 containing the *acdS* and *lrpL* genes of *R. leguminosarum* bv. viciae 128Sm, Tc^r	This study
pSPacdS	pSP329 containing the *acdS* gene of *R. leguminosarum* bv. viciae 128Sm, Tc^r	This study
pSPlrpL	pSP329 containing the *lrpL* gene of *R. leguminosarum* bv. viciae 128Sm, Tc^r	This study

MATERIALS AND METHODS

Growth conditions. (i) Bacteria. The bacterial strains used in this study are shown in Table 1.

R. leguminosarum bv. viciae 128C53K and mutants derived from this strain were grown at 25°C in TY medium (2) or M9 minimal medium (2) supplemented with 0.3 μg of biotin ml^{-1}. Appropriate antibiotics were added to the media when it was necessary. *E.cloacae* UW4 was grown at 30°C in TSB medium (Difco Laboratories, Detroit, Mich.) or DF minimal medium (2). *Escherichia coli* DH5α and S17-1 and transformants carrying different plasmids were grown at 37°C in Luria broth (Difco Laboratories) with appropriate antibiotics.

(ii) Plants. *P. sativum* L. cv. Sparkle was grown in a controlled environmental growth room under cool white fluorescent lights (approximately 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a cycle consisting of 16 h of light at 22°C and 8 h of darkness at 18°C (14).

Detection of ACC deaminase in *Rhizobium* spp. Rhizobial cells were grown in 5 ml of TY medium with appropriate antibiotics at 25°C for 3 days until they reached the stationary phase. To induce ACC deaminase activity, the cells were resuspended in 2 ml of M9 minimal medium supplemented with 5 mM ACC and then incubated for 40 h at 25°C with shaking (100 rpm) (21). ACC deaminase activity was determined by measuring the production of α-ketobutyrate (17).

Western blots were also used to detect the ACC deaminase protein. An antibody was raised from rabbits and directed against the *E. cloacae* UW4 ACC deaminase. L-Leucine (1 or 2 mM) was added together with 5 mM ACC to M9 minimal medium when the regulatory effect of L-leucine on expression of the ACC deaminase gene of *R. leguminosarum* bv. viciae 128C53K was investigated.

Isolation of the ACC deaminase gene from *R. leguminosarum* bv. viciae 128C53K. An 800-bp DNA fragment of the *E. cloacae* UW4 ACC deaminase structural gene was amplified from the genomic DNA of UW4 by PCR by using 5′-TA(CT)GC(CG)AA(AG)CG(ACGT)GA(AG)GA(CT)TGCAA-3′ and 5′-C CAT(CT)TC(AGT)ATCAT(ACGT)CC(GA)TGCAT-3′ as the primers (31). The PCR product was labeled with digoxigenin (Roche Molecular Biochemicals, Laval, Quebec, Canada) and used as a hybridization probe for the genomic DNA of 128C53K. Both hybridization and washing were carried out at 60°C.

Based on the Southern hybridization results (data not shown), *R. leguminosarum* DNA fragments (approximately 4 kb) from genomic DNA digested with *Bam*HI were ligated to pUC19 to construct a partial DNA library in *E. coli* DH5α. This library was screened by colony hybridization, and the DNA sequence of the 4-kb insert of a positive clone, plasmid pWM1, was determined.

Isolation of a streptomycin-resistant derivative of *R. leguminosarum* bv. viciae 128C53K. Spontaneous streptomycin-resistant mutants of 128C53K were isolated on TY agar supplemented with 200 μg of streptomycin ml^{-1}. One stable strain which had the same level of ACC deaminase activity and nodulated cv. Sparkle to the same extent as 128C53K was chosen and designated 128Sm. Strain 128Sm was used for all subsequent experiments.

Construction of *R. leguminosarum* bv. viciae 128Sm mutants. (i) Construction of gene-disrupting mutants. The *acdS* and *lrpL* genes were disrupted by a kanamycin resistance gene and ligated into plasmid pJQ200KS (27). The resulting plasmids, pJQacdSΩ::Km and pJQlrpLΩ::Km, were then transferred by conjugation into 128Sm. Transconjugants that were kanamycin resistant and sucrose resistant were tested for gentamicin sensitivity to confirm that a double-crossover had occurred. The knockout mutants were designated 128Sm*acdS*Ω ::Km and 128Sm*lrpL*Ω::Km.

(ii) Complementation of the ACC deaminase and leucine-responsive regulatory protein (LRP)-like protein-negative mutants. A DNA fragment carrying the *acdS* gene and its 5′ upstream regulatory region (length, 314 nucleotides) from *R. leguminosarum* bv. viciae 128C53K was amplified from pWM1 by PCR by using the following primers: 5′-GCCGGTAATGTAGCCCTCCTC-3′ and 5′-G TCGAGAAGACAGCCTCATCC-3′. The PCR product was ligated into plasmid pSP329 (5, 30), and the resulting plasmid, pSPacdS, was introduced into 128Sm*acdS*Ω::Km by conjugation.

A 1.1-kb DNA fragment containing the *lrpL* gene was excised from pWM1 with *Kpn*I and ligated into pSP329. The resulting plasmid, pSPlrpL, was then introduced into 128Sm*lrpL*Ω::Km by conjugation to complement the mutant.

(iii) Construction of an ACC deaminase high-expression mutant. The 4-kb DNA fragment containing the *acdS* and *lrpL* genes from 128C53K was inserted into plasmid pSP329 to construct pWM2, which was then introduced into 128Sm by conjugation.

All molecular manipulations were performed as described by Sambrook and

Russell (28). Deduced amino acid sequences were compared with the sequences in the National Center for Biotechnology Information GenBank database by using the program Advanced BLAST. Putative promoters were deduced by using the program Promoter Predictions at the Lawrence Berkeley National Laboratory server.

Measurement of ethylene production in intact cv. Sparkle seedlings. The ethylene synthesized by cv. Sparkle, inoculated 3 days after planting with either the wild-type *Rhizobium* or the ACC deaminase knockout mutant, was determined. Two and three days after inoculation plants were incubated in sealed 25-ml glass containers at room temperature for 4 h. Gas samples were analyzed with a gas chromatograph (model GC-17A; Shimadzu, Tokyo, Japan) equipped with a hydrogen flame ionization detector and an AT-1 column (0.53 mm by 15 m; Alltech Associates Inc.). Eighteen plants were analyzed in three different replicates.

Nodulation assay. Nodulation assays were performed with cv. Sparkle by using the procedures described by Guinel and Sloetjes (14). The plants were harvested on day 28 of growth, the number of nodules was determined for each plant, and shoot dry weights were determined by baking the shoots at 105°C for approximately 4 days until no further reduction in weight was noted. The nitrogenase activities of the nodules were determined by using an acetylene reduction assay (33) prior to measurement of the number of nodules and the shoot dry weight. The ethylene concentrations were determined by comparing the areas of the ethylene peaks generated from the reduction of acetylene to the area of the peak of a 100-ppm ethylene standard (Alltech Associates, Inc.) by using the gas chromatograph as described above except that it was equipped with an HP-PLOT/Al203 column (0.53 mm by 50 m; Hewlett-Packard Co., Palo Alto, Calif.).

Statistics. The data were analyzed by analysis of variance, and the treatment means were compared by Tukey's honestly significant difference (HSD) multiple-comparison test. All hypotheses were tested at the 95% confidence interval ($\alpha = 0.05$).

Nucleotide sequence accession numbers. The nucleotide sequences of the ACC deaminase structural gene (*acdS*) and the LRP-like protein gene (*lrpL*) have been deposited in the GenBank database under accession numbers AF421376 and AY172673, respectively.

RESULTS AND DISCUSSION

Sequence analysis of the *acdS* gene and its regulatory region. The analysis of the DNA sequence of the 4-kb insert from pWM1 revealed two open reading frames (ORFs) that were 1,002 nucleotides long (ORF1) and 474 nucleotides long (ORF2). The deduced amino acid sequence of ORF1 exhibited 64% identity and 79% similarity with the ACC deaminase from *E. cloacae* UW4; therefore, the gene was determined to be the ACC deaminase structural gene (*acdS*) (GenBank accession no. AF421376). High levels of similarity were also found when the sequence of the protein product of *acdS* from *R. leguminosarum* bv. viciae 128C53K was compared with the sequences of the ACC deaminases from various microorganisms, such as the ACC deaminase from *Pseudomonas* sp. strain ACP (67% identity and 81% similarity) and the ACC deaminase from *Hansenula saturnus* (58% identity and 75% similarity),.

The potential protein encoded by ORF2 exhibited similarity with the LRPs from *E.coli* (44% identity and 62% similarity), *Bradyrhizobium japonicum* (67% identity and 76% similarity), and other bacteria. This gene was therefore designated the LRP-like protein gene (*lrpL*) (GenBank accession no. AY172673). The *lrpL* gene is transcribed in the direction opposite that of the *acdS* gene, and there is a 150-bp DNA fragment between the translation start codons of the two genes.

LRP is a global regulatory protein in *E. coli* that affects the expression of many proteins and operons (4, 6). Many of the genes or operons regulated by LRP are also subject to control by L-leucine (4, 6). In *E.cloacae* UW4, the *acdS* gene is under the control of *acdR*, encoding an LRP-like protein, which is located upstream of *acdS*, and L-leucine inhibits expression of the *acdS* gene (11, 20). In order to investigate whether L-leucine has a regulatory effect on ACC deaminase expression in *R. leguminosarum* bv. viciae 128C53K, 1 or 2 mM L-leucine was added to M9 minimal medium together with 5 mM ACC during induction of the enzyme before the ACC deaminase activities of the differently induced cells were determined. In the presence of 1 and 2 mM L-leucine, expression of ACC deaminase, as estimated by measuring the enzyme activity, was reduced about 25 and 40%, respectively, compared to the level of expression in cells induced in the absence of L-leucine. It is likely that as in *E. cloacae* UW4 (11, 20), L-leucine regulates transcription of the *acdS* gene through the upstream LRP-like protein in *R. leguminosarum* bv. viciae 128C53K. We postulate that the LRP-like protein–ACC complex depends on transcription of the *R. leguminosarum* bv. viciae*acdS* gene; L-leucine competitively binds to the LRP-like protein to make it unavailable for binding ACC, thus inhibiting ACC deaminase expression. The experimental finding that 2 mM L-leucine resulted in a higher degree of reduction of ACC deaminase activity in *R. leguminosarum* bv. viciae 128C53K than 1 mM L-leucine resulted in is consistent with this hypothesis.

TABLE 2. ACC deaminase activities of *R. leguminosarum* by viciae 128Sm and mutants induced by 5 mM ACC[a]

Strain	ACC deaminase activity (μmol of α-ketobutyrate $\cdot$ h^{-1} $\cdot$ mg of protein^{-1})
UW4	20.48 ± 1.93
128Sm	1.56 ± 0.23
128Sm(pWM2)	3.97 ± 0.29
128Sm*acdS*Ω::Km	ND
128Sm*lrpL*Ω::Km	ND
128Sm*acdS*Ω::Km(pSPacdS)	3.24 ± 0.24
128Sm*lrpL*Ω::Km(pSPlrpL)	1.81 ± 0.19

[a] The activity found in *E. cloacae* UW4 induced by 5 mM ACC was used as a positive control. The values are means ± standard errors of the means for three independent assays. ND, no ACC deaminase activity was detected in the ACC deaminase-negative and LRP-like protein-negative mutants.

Construction and characterization of the ACC deaminase and LRP-like protein-negative mutants. To investigate both the effect of the LRP-like protein in *R. leguminosarum* bv. viciae 128Sm on regulation of *acdS* gene expression and the involvement of the ACC deaminase in nodulation of pea plants, knockout mutants of the *acdS* and *lrpL* genes were constructed by using the gene disruption method. The structures of the mutants were confirmed by Southern hybridization and PCR (data not shown), and the presence of the ACC deaminase protein in the knockout mutants was examined by Western blotting and enzyme assays. Compared to the parent strain, 128Sm, which had an activity of 1.56 ± 0.23 μmol of α-ketobutyrate $\cdot$ h^{-1} $\cdot$ mg of protein^{-1}, no ACC deaminase was detected in either of the mutants when they were induced with 5 mM ACC (Table 2). This suggests that as previously observed with *E. cloacae* UW4 (11, 20), the *lrpL* gene located in the region immediately upstream of the *acdS* gene is required for expression of the ACC deaminase in 128Sm.

The growth rates of *R. leguminosarum* bv. viciae 128Sm*acdS*Ω ::Km and 128Sm*lrpL*Ω::Km in TY medium were determined. The doubling time of both of the knockout mutants was approximately

TABLE 3. Nodulation of *P. sativum* L. cv. Sparkle inoculated with *R. leguminosarum* bv. viciae 128Sm and mutants[a]

R. leguminosarum bv. viciae strain	Normalized no. of nodules compared to the no. produced by the wild-type bacterium	Shoot dry wt ($mg \cdot plant^{-1}$)	Total nitrogenase activity ($\mu mol \cdot h^{-1} \cdot plant^{-1}$)	Nitrogenase sp act ($\mu mol \cdot h^{-1} \cdot g$ [dry wt] of $shoots^{-1}$)
128Sm	1.00 ± 0.05 A	395.92 ± 22.13 A	0.69 ± 0.13 A	1.74
128Sm(pWM2)	0.97 ± 0.08 A	393.54 ± 30.36 A	0.73 ± 0.12 A	1.85
128Sm*acdS*Ω::Km	0.74 ± 0.06 B	304.42 ± 18.22 B	0.56 ± 0.03 B	1.84
128Sm*lrpL*Ω::Km	0.65 ± 0.05 BC	278.56 ± 16.51 B	0.51 ± 0.09 B	1.83
128Sm*acdS*Ω::Km(pSPacdS)	1.05 ± 0.06 A	403.87 ± 28.79 A	0.71 ± 0.09 A	1.76
128Sm*lrpL*Ω::Km(pSPlrpL)	0.98 ± 0.04 A	385.73 ± 31.24 A	0.69 ± 0.06 A	1.79

[a] Three independent assays were performed, and in each assay there were 12 plants per treatment. The values are means ± standard errors of the means for a pool of 36 plants. The average number of nodules formed by wild-type *R. leguminosarum* bv. viciae 128Sm per plant was defined as 1.0 (typically approximately 240 nodules per plant). Individual numbers of nodules from all the plants inoculated with either the wild-type strain or the mutants were normalized accordingly. Within columns values that are followed by different letters are statistically significantly different; values that are followed by the same letter are not statistically significantly different.

8.9 h, which is very similar to that of parent strain 128Sm. The growth curves of the parent strain and the knockout mutants indicated that the growth of the mutants in rich medium was not affected by inactivation of the genes (data not shown).

Complementation of the two knockout mutants. *R. leguminosarum* bv. viciae 128Sm*acdS*Ω::Km and 128Sm*lrpL*Ω::Km were transformed with pSPacdS and pSPlrpL, respectively. ACC deaminase assays showed that both transconjugants regained the ability to produce ACC deaminase (Table 2); 128Sm*lrpL*Ω::Km(pSPlrpL) had an ACC deaminase activity similar to that of 128Sm, whereas 128Sm*acdS*Ω::Km(pSPacdS) had an ACC deaminase activity that was twice that of 128Sm. Presumably, the multicopy plasmid carrying the *acdS* gene conferred a higher level of expression of ACC deaminase, which resulted in greater enzyme activity.

ACC deaminase activity of high-expression mutant of *R. leguminosarum* bv. viciae 128Sm. *R. leguminosarum* bv. *viciae* 128Sm containing plasmid pWM2 was tested by the enzyme assay for the production of ACC deaminase. Compared to parent strain 128Sm, 128Sm(pWM2) had an ACC deaminase activity that was approximately 2.5 times the wild-type level (Table 2), which was comparable to the ACC deaminase activity of 128Sm*acdS*Ω::Km(pSPacdS).

Measurement of ethylene produced by pea plants. In order to investigate the ethylene production by pea plants, at 2 and 3 days after inoculation with either the wild type or the ACC deaminase knockout mutant of *R. leguminosarum* bv. *viciae* 128Sm intact cv. Sparkle seedlings were examined and compared to uninoculated seedlings. Despite all attempts to increase the sensitivity of the gas chromatograph, the lowest level of ethylene that could be reliably determined with the instrument was approximately 0.2 ppm, whereas the plants that were inoculated with rhizobia and the plants that were not inoculated with rhizobia produced ethylene at levels that were lower than 0.1 ppm and thus barely detectable by the instrument used. The detection limit of the method used for ethylene determination was above the levels produced by the pea plants; therefore, no conclusion concerning ethylene evolution could be made for the inoculated pea seedlings.

Lee and LaRue (19) found that uninoculated cv. Sparkle plants produced levels of ethylene similar to the levels produced by plants just after inoculation. Based on these observations, we postulated that *R. leguminosarum* bv. viciae 128Sm has a very localized effect on reducing the ethylene production in pea plants through the action of ACC deaminase; probably the bacterial cells act only around the infection threads (13). Therefore, the reduction in ethylene production by the *Rhizobium* strain was undetectable when the ethylene levels of the intact seedlings were measured.

Involvement of the rhizobial ACC deaminase in nodulation. To delineate the involvement of ACC deaminase from *R. leguminosarum* bv. viciae 128Sm in the nodulation process, the two knockout mutants which did not produce ACC deaminase and one mutant which overexpressed the protein were examined in nodulation assays with *P. sativum* L. cv. Sparkle. The nodulation of *P. sativum* has previously been reported to be inhibited by exogenous ethylene; the number of nodules was reduced by one-half when as little as 0.07 ppm of ethylene was applied continuously to the pea rhizosphere, and this inhibition was overcome by application of 1 μM Ag^+, an antagonist of ethylene action (18).

Table 3 shows the results of the nodulation assay performed with pea plants inoculated with wild-type *R. leguminosarum* bv. viciae 128Sm, as well as with the mutants constructed. As described below, ACC deaminase in strain 128Sm promotes the nodulation of pea plants, probably by lowering the ethylene levels in the plants. The number of nodules formed on the plants inoculated with the ACC deaminase-negative mutant was approximately 25% less than the number found on plants inoculated with the parent strain. The plants inoculated with the LRP-like protein-negative mutant formed even fewer nodules (about 35% less than the wild type). A significant decrease ($P < 0.05$) was also found in shoot dry weights (Table 3) when the plants were inoculated with the two knockout mutants. The shoot dry weights of plants inoculated with the ACC deaminase-negative mutant were approximately 23% less than the shoot dry weights of plants inoculated with the wild-type strain, while the shoot dry weights of the plants inoculated with the LRP-like protein-negative mutant were approximately 30% less (Table 3). The genetically complemented derivatives of the two knockout mutants performed like the wild-type strain, indicating that the nodulation efficiency of the mutants on pea plants was restored along with their ability to produce ACC deaminase. These data confirmed that ACC deaminase is involved in the control of nodulation in pea plants.

The total nitrogenase activity of the nodules (Table 3) formed by the two knockout mutants showed a degree of reduction similar to that of the wild-type strain. However, the

fact that the specific nitrogenase activities were not altered in these mutants suggests that regulation by ACC deaminase is involved only in the early stages of nodule development, not in nodule function (i.e., nitrogen fixation inside the bacteroids). Guinel and Geil (13) proposed that the main site of ethylene action was at the interface between the epidermis and the outermost cortical cell layer when the infection threads progress towards the nodule primordia. It is possible that *R. leguminosarum* bv. viciae 128Sm can finely reduce the ethylene level in pea roots through the action of its ACC deaminase, which would allow progress of the infection threads inside the cortex.

The ACC deaminase high-expression mutant of *R. leguminosarum* bv. viciae 128Sm performed like the wild-type strain for all of the parameters tested in the nodulation assay, suggesting that under laboratory conditions, above a certain threshold, a higher level of ACC deaminase activity does not enhance nodulation further. It is possible that plants use a regulatory mechanism other than ethylene, such as a cytokinin-mediated regulatory process (37), to control the number of infections by *Rhizobium* to reach an optimal number of nodules, so that the host plants can obtain enough fixed nitrogen and at the same time withstand the energy cost of nitrogen fixation.

Ethylene production can be induced by many environmental stresses, such as pathogens, flooding, heavy metals, and mechanical wounding (1). Although a strain that expresses high levels of ACC deaminase does not promote nodulation under normal laboratory conditions, it is possible that a higher level of ACC deaminase activity would help the infecting rhizobial cells decrease the larger amount of ACC produced by plants under such stress conditions and thus obtain better nodulation efficiency.

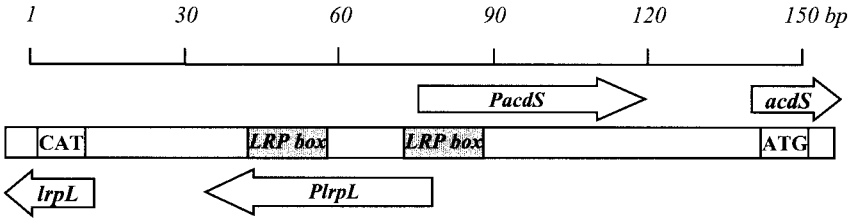
In addition to the *acdS* gene isolated from *R. leguminosarum* bv. viciae 128C53K in the work described here, there are four other potential ACC deaminase genes that were revealed by genome sequencing of four rhizobial strains, *Agrobacterium tumefaciens* C58, *B.japonicum* USDA110, and *Mesorhizobium loti* MAFF303099 and ICMP3150. The deduced amino acid sequences of these genes are highly conserved (average level of similarity, about 80%). Sequence comparisons also revealed the presence of a gene encoding a truncated ACC deaminase on symbiotic plasmid pNGR234a of *Sinorhizobium* sp. strain NGR234. The deduced 42-amino-acid sequence encoded by this partial gene exhibited 79% identity and 91% similarity with the sequence of the ACC deaminase from *R. leguminosarum* bv. viciae 128C53K. It is interesting that the gene encoding the ACC deaminase in *M. loti* ICMP3153 and the DNA fragment encoding the truncated ACC deaminase in *Sinorhizobium* sp. strain NGR234 are located on the symbiotic island (35) and the symbiotic plasmid, respectively, in these bacteria. Our Southern hybridization results also showed that the *R. leguminosarum* bv. viciae 128C53K *acdS* gene is located on one of the endogenous large plasmids (>100 kb) (data not shown). This suggests the possibility that there was horizontal transfer of the putative ACC deaminase genes from other microorganisms, particularly other soil bacteria.

It was recently reported that an ACC deaminase gene was found in *M. loti* ICMP3153 on a symbiotic island called R7A and could be under control of NifA and RpoN (35). It is therefore possible that this ACC deaminase gene is only switched on in bacteroids. When the promoter region of the ACC deaminase homologous gene in *M. loti* MAFF303099 was analyzed, a potential RpoN recognition site (5′-TGGAAC-GAGTT-TTGC-3′) and a NifA binding site (5′-TGT-N_{13}-AC A-3′) were detected. These sequence data indicate that the putative ACC deaminase gene in *M. loti* MAFF303099 could also be under the control of NifA and expressed only in the nodules. This suggestion is consistent with the experimental finding that no ACC deaminase production was observed in *M. loti* MAFF303099 cells induced by ACC (21).

Mounting experimental evidence from different groups shows that the responses of different legumes to ethylene are quite diverse with respect to nodulation (13, 22). For example, nodulation of soybean seems to be insensitive to ethylene (22, 30); in contrast, ethylene is essential for nodulation in *Sesbania* (7). Previous results showing that only a fraction of the rhizobial strains tested had an active ACC deaminase are consistent with the different roles of ethylene in regulating nodule development in different legumes (21). The amino acid sequences deduced from the DNA sequences encoding ACC deaminases are highly homologous in these rhizobial strains, whereas the transcriptional regulators of the genes are quite diverse. It is possible that after these rhizobia obtained the ACC deaminase gene by horizontal transfer from other soil bacteria, their regulation systems evolved later depending on the different responses of the host legumes to ethylene. Therefore, ACC deaminases in different rhizobium species could be designed to perform different functions in various aspects of nodulation depending on the effect of ethylene on the specific host legumes.

Potential involvement of LRP-like protein in nodulation. In *E. coli*, LRP is a major regulatory protein involved in the expression of numerous operons, such as *ilvIH*, *serA*, *livKHMGF*, and *lysU* (4). LRP acts as either an activator or a repressor of gene transcription by binding to a highly conserved 15-nucleotide DNA sequence called an LRP box. For some of the operons regulated by LRP, multiple LRP boxes bind cooperatively to the regulatory protein and lead to the formation of a large nucleoprotein complex (6). When the DNA sequence upstream of both the *acdS* and *lrpL* genes of *R. leguminosarum* bv. viciae 128C53K was analyzed, two 15-nucleotide sequences that might contain LRP boxes, based on the consensus sequence of the LRP-binding sites in *E. coli* (6), were found (Fig. 1). In addition, potential promoter regions of both genes were predicted.

To investigate whether the LRP-like protein activates the transcription of the *acdS* gene by binding to the two potential LRP boxes, three PCR products containing different lengths of the upstream region of the *acdS* gene were used to complement the ACC deaminase knockout mutant of *R. leguminosarum* bv. viciae 128Sm. The longest PCR product contained both of the potential LRP boxes, as well as a 180-nucleotide sequence upstream of them. The second PCR product contained both of the potential LRP boxes but no further upstream regions. The shortest PCR product contained only one potential LRP box. Complementation experiments showed that only the longest PCR product restored ACC deaminase production in the mutant, whereas introduction of the other two DNA fragments did not result in any ACC deaminase production. The results suggest that the 314-nucleotide up-



Potential LRP-binding sites:

Start	End	Sequence
40	54	5′ CGAAAAATTACGCCG 3′
71	85	5′ AAGCAAAATTAGAGA 3′

FIG. 1. Nucleotide sequence between the *R. leguminosarum* bv. viciae 128Sm *acdS* and *lrpL* genes. The predicted promoters of both genes (*PacdS* and *PlrpL*) and two potential LRP-binding sites are indicated. The arrows indicate the directions of transcription. The two potential LRP-binding sites were determined based on a consensus sequence (YAGHAWATTWTDCTR, where Y is T or C, H is not G, W is A or T, D is not C, and R is A or G), which was derived from an analysis of 63 DNA sequences that bind to LRP in *E. coli* (6). In the sequences of both of the potential binding sites, 11 of 15 nucleotides were identical to nucleotides in the consensus sequence.

stream region of the *acdS* gene, including the two potential LRP-binding sites, is required for its transcription in *R. leguminosarum* bv. viciae 128Sm. It is possible that binding of the LRP-like protein to the potential LRP boxes requires sequences upstream of the binding sites.

It was reported by Perret et al. (25) that a gene highly homologous to the *lrp* gene of *Escherichia coli* was isolated from the symbiotic plasmid of *Sinorhizobium* sp. strain NGR234. Transcription of this gene is induced by the flavonoids produced by the host legumes; the same flavonoids stimulate the production of the Nod factor and initiate the nodulation process. A mutant of this *lrp*-like gene, constructed in *Sinorhizobium* sp. strain NGR234, was used to inoculate different host legumes. The mutant caused a 4.5-day delay in the nodulation of *Vigna unguiculata*, and the number of nodules on *Calopogonium caeruleum* was decreased by about 25% compared with number obtained with the wild-type bacterium (25). However, on *Leucaena leucocephala* and *Pachyrhizus tuberosus*, the numbers of nodules were greater than the number obtained with the wild-type bacterium. Although it is not clear how this *lrp*-like gene modifies the efficiency of nodulation, it is possible that some nodulation genes are transcriptionally regulated by LRP (25). In the present study, the fact that pea plants inoculated with the LRP-like protein-negative mutant did not perform as well as plants inoculated with the ACC deaminase-negative mutant (although the results were not significantly different for all of the parameters tested) suggests that the LRP-like protein might play some other role(s) in the nodulation process in addition to regulating transcription of the ACC deaminase gene.

The gene described here is the first ACC deaminase gene from *Rhizobium* that has been isolated and studied to determine its function. We postulate that *R. leguminosarum* bv. viciae 128C53K can finely adjust the ethylene production in pea roots through the action of its ACC deaminase, to obtain an optimal level which allows progression of the infection threads inside the cortex and the formation of functional nodules. It has been suggested that the rhizobia that do not contain ACC deaminase are unable to nodulate their cognate legumes to the same extent that they might be able to if they possessed this enzyme. Moreover, it should be possible to genetically engineer strains that normally lack ACC deaminase with the gene encoding this enzyme with the expectation that the transformed strain will nodulate its cognate legume to a greater extent than the nontransformed strain. In this regard, preliminary results show that *Sinorhizobium meliloti* Rm1021, which does not have ACC deaminase activity, can nodulate its host legume, alfalfa, more efficiently when it is transformed with the *acdS* and *lrpL* genes from *R. leguminosarum* bv. viciae 128C53K (W. Ma, T. C. Charles, and B. R. Glick, unpublished data).

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