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Disparate role of rhizobial ACC deaminase in root-nodule symbioses

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Abstract The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase converts ACC, a precursor of the plant hormone ethylene, into ammonia and α-ketobutyrate. ACC deaminase is widespread among the rhizobia in which it might play a crucial role in protecting rhizobia against inhibitory effects of ethylene synthesized by the host plant in response to the nodulation process. The beneficial action of this enzyme was demonstrated in several rhizobia such as *Mesorhizobium loti* and *Rhizobium leguminosarum* where knock-out mutants of the ACC deaminase gene showed nodulation defects. The genome of the slow-growing rhizobial species *Bradyrhizobium japonicum* also carries an annotated gene for a putative ACC deaminase (blr0241). Here, we tested the possible importance of this enzyme in *B. japonicum* by constructing an insertion mutant of blr0241 and studying its phenotype. First, the activity of ACC deaminase itself was measured. Unlike the *B. japonicum* wild type, the blr0241 mutant did not show any enzymatic activity. By contrast, the mutant was not impaired in its ability to nodulate soybean, cowpea, siratro, and mungbean. Likewise, symbiotic nitrogen fixation activity remained unaffected. Furthermore, a co-inoculation assay with the *B. japonicum* wild type and the blr0241 mutant for soybean and siratro nodulation revealed that the mutant was not affected in its competitiveness for nodulation and nodule occupation. The results show that the role previously ascribed to ACC deaminase in the rhizobia cannot be generalized, and species-specific differences may exist.

Keywords 1-aminocyclopropane-1-carboxylic acid deaminase · *Bradyrhizobium japonicum* · Ethylene · Rhizobia · Root-nodule symbiosis

1 Introduction

Rhizobia are soil bacteria which can establish a symbiosis with legume host plants, therein acting as nitrogen-fixing endosymbionts. Symbiotic nitrogen fixation takes place in specific plant organs, the root nodules. To adapt to the symbiotic life-style, rhizobia have to cope with extremely low oxygen conditions as well as with host-plant responses to infection. One of the phytohormones that regulate nodulation is ethylene. This small and readily diffusible compound is well known to be involved in many aspects of the plant life cycle, including seed germination, root initiation, root hair development, fruit development and ripening (Abeles et al. 1992). Ethylene is also synthesized in response to abiotic (wounding, hypoxia, chilling) and biotic stresses (pathogen attack) which includes the interaction of plants with rhizobia (Spaink 1997; Abeles et al. 1992). In the latter case ethylene was reported to inhibit the nodulation of legumes (Lee and LaRue 1992; Nukui et al. 2000; Peters and Cristestes 1989). Studies on a *Medicago truncatula* ethylene insensitive mutant showed that during the initiation of symbiosis with *Sinorhizobium meliloti*, ethylene has an inhibitory effect on nodule formation by interfering with root hair deformation, infection thread initiation, calcium spiking, and

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rhizobial proliferation (Penmetsa and Cook 1997; Oldroyd et al. 2001; Tamimi and Timko 2003). In the *Bradyrhizobium japonicum*-legume symbioses, however, the effects of ethylene on nodulation are much less clear. It was previously shown that after inoculation with *B. japonicum*, the *Glycine max* (soybean) roots produce transiently more ethylene (Suganuma et al. 1995), but surprisingly, when an ethylene insensitive soybean mutant was inoculated with *B. japonicum*, no significant increase in nodule number was observed (Nukui et al. 2000; Schmidt et al. 1999). Moreover, the direct application of either Ag^+, an inhibitor of ethylene perception, or 1-aminocyclopropane-1-carboxylic acid (ACC, an intermediate of ethylene biosynthesis) on plant roots had no effect on soybean nodulation. Nevertheless, in the symbiosis between *B. japonicum* and another host plant *Macroptilium atropurpureum* (siratro), ethylene does appear to play an inhibitory role (Nukui et al. 2000).

Ethylene is synthesized in plants via three key reactions. First, the amino acid methionine is converted to *S*-adenosyl-L-methionine (*S*-AdoMet) by the *S*-AdoMet synthetase. Second, the *S*-AdoMet is converted to ACC by the ACC synthase. Finally, ACC is converted to ethylene, carbon dioxide and hydrogen cyanide by the action of the ACC oxidase (Abeles et al. 1992; Lin et al. 2009).

Several soil-borne microorganisms use external mechanisms to influence ethylene levels in plants, either by synthesizing rhizobitoxine, a competitive inhibitor of the ACC synthase (Yasuta et al. 1999), or by expression of an ACC deaminase (AcdS) which degrades ACC into ammonia and α-ketobutyrate (Ma et al. 2002). Regardless of whether ACC is constitutively present or newly synthesized in response to nodulation, the model of Glick et al. (1998) proposes that ACC is exuded from plant roots and taken up by the bacteria in which it would be metabolized as an energy and/or nitrogen source. This degradation would help lowering the amounts of ethylene provided by the plant. Hence, ACC deaminase would protect plants against the inhibitory effects of ethylene synthesized in response to stresses incurred by infecting bacteria.

The ACC deaminase is an oligomeric enzyme that carries pyridoxal 5-phosphate as an essential co-factor. The enzyme does not have a high affinity for its substrate ACC, with K_m values varying between 1.5 and 17.5 mM (Honma and Shimomura 1978). This suggests that the enzyme can also degrade other substrates (Walsh et al. 1981). A broad range of organisms such as yeast and other fungi as well as few plants and several plant growth-promoting bacteria possess an ACC deaminase gene (Minami et al. 1998; Shah et al. 1998; Sterky et al. 1998; Jia et al. 2000; Riemenschneider et al. 2005).

The present paper serves two purposes. On the one hand, it explores for the first time the possible function of the *B. japonicum* ACC deaminase in the interaction with host legumes. On the other hand, as our data are in contrast with those obtained with other rhizobia, it summarizes the current knowledge with respect to the role of ACC deaminase in nodulation, which unveiled a certain degree of non-uniformity.

2 Materials and methods

2.1 Bacterial strains, media and growth conditions

Luria-Bertani (LB) medium was used for growth of *Escherichia coli* (Miller 1972). Cells of the *B. japonicum* wild-type strain 110*spc*4 and the blr0241 mutant strains were grown under aerobic condition with rigorous shaking (180 rpm) in PSY medium (Regensburger and Hennecke 1983) containing 0.1 % L-arabinose and the antibiotic spectinomycin (100 μg ml^{-1}). When appropriate, kanamycin (100 μg ml^{-1}) was used for selections. Yeast-extract mannitol medium (Daniel 1972) supplemented with 10 mM KNO_3 was used for anaerobic growth. Such cultures were grown with moderate shaking (80 rpm) in 500-ml rubber-stoppered serum bottles containing 200 ml medium and a gas atmosphere consisting of 100 % N_2.

2.2 DNA methods and construction of blr0241 mutant strains

Genomic DNA was isolated from *B. japonicum* wild-type strain 110*spc*4 as previously described (Hahn and Hennecke 1984). Recombinant DNA work was carried out according to standard procedures (Sambrook and Russell 2001). *B. japonicum* blr0241 mutant strains 6316 and 6317 were constructed by marker insertion mutagenesis. The 5′ and 3′ parts of the blr0241 gene were amplified by PCR using the following primer pairs (added restriction sites underlined): blr0241_up_f/*Pst*I (AACTGCAGGGCGTCGTCG AACAGGCATC) and blr0241_up_r/*Kpn*I (GGGGTACCTCCTCGCGCTTGGCATAGAT) for the 5′ region; blr0241_down_f/*Kpn*I (GGGGTACCCTG CAACTCCGGCCTCGCCTA) and blr0241_down_r/*Xba*I (GCTCTAGATGGCTTCGAGACGCGCGGTGAG) for the 3′ region. The 793-bp blr0241-5′ region and the 690-bp blr0241-3′ region were cloned in the pGEM-T Easy vector (Promega Corp., Madison, WI, U.S.A.). After verification by sequencing, both products were eventually cloned tail-to-head into pSUP202pol6K (Zufferey et al. 1996). In two separate constructions, a 1,260 bp *Kpn*I kanamycin resistance cassette (*aph*II) from pBSL86 (Alexeyev 1995) was inserted in either orientation into the unique *Kpn*I site between the blr0241-5′ and -3′ regions. The resulting plasmids pRJ6316 and pRJ6317 were mobilized into the *B. japonicum* wild-type strain 110*spc*4 (Hahn and Hennecke 1984) yielding mutants 6316 and 6317 with *aph*II in the opposite and same directions as blr0241, respectively. The correct genomic integrations were confirmed by PCR. The

*aph*II insertion point is between genome coordinates 235,837 and 235,838 (http://genome.kazusa.or.jp/rhizobase/).

2.3 Plant material, inoculation, and growth conditions

Seeds of soybean (*G. max* (L.) Merr. cv. Williams), cowpea (*Vigna unguiculata* (L.) Walp. cv. Red Caloona), mungbean (*Vigna radiata*), and siratro (*M. atropurpureum* [DC.] Urb.) were surface-sterilized as recently described (Koch et al. 2010). Germination, inoculation, and growth of the plants were done as previously described (Hahn and Hennecke 1984; Göttfert et al. 1990). Nitrogenase activity in root nodules was determined at day 21 (soybean, cowpea, mungbean) or day 31 (siratro) post inoculation (dpi). For soybean, cowpea and siratro 10 plants inoculated with *B. japonicum* wild type 110*spc*4 and 10 plants inoculated with the blr0241 mutant strain were tested. For mungbean 6 plants inoculated with *B. japonicum* wild type and 6 plants inoculated with the blr0241 mutant strain were tested. Nitrogenase activity was measured with the acetylene reduction assay (Hahn and Hennecke 1984; Göttfert et al. 1990). For the *B. japonicum* wild type 110*spc*4 versus mutant 6316 competition assay, two respective strain ratios (1:1 and 1:10) were tested for inoculation of soybean or siratro seedlings. Each ratio with 100 *B. japonicum* cells per seedling was applied to three soybean and four siratro plants. All of the nodules per plant were harvested at 21 dpi for soybean and 31dpi for siratro. Total nodules from one plant were surface-sterilized for 5 min in 100 % ethanol, washed 6 times with water, and then crushed. Bacteria were diluted and plated on selective agar plates. The competitiveness was assessed by comparing the proportional presence of each strain in the inoculum at time 0 to the proportional nodule occupancy of each strain after recovery from the nodules at 21 or 31 dpi.

2.4 ACC deaminase activity assay

The enzyme sources were crude extracts from either culture-grown cells or legume nodules. The *B. japonicum* wild-type strain 110*spc*4 and mutant strains 6316 and 6317 were grown in anoxic conditions for optimal induction of the blr0241 gene. Early stationary-phase cells were collected by centrifugation at 1,845 *g* for 10 min at 4 °C. Nodules of cowpea, soybean, siratro and mungbean infected either with the *B. japonicum* wild type 110*spc*4 or with strain 6316 were disrupted as described previously (Pessi et al. 2007). The cell sediment corresponding to a 50-ml anoxic culture was used for enzymatic tests as previously described (Penrose and Glick 2003). The substrates tested were from the following sources: ACC (Merck, Darmstadt, Germany); β-chloro-D-alanine hydrochloride and D-Serine (Sigma-Aldrich Co., St. Louis, MO, U.S.A.).

3 Results and discussion

3.1 A survey on the occurrence and function of the ACC deaminase gene (*acdS*) in the Rhizobiales

The *acdS* gene is widely spread among rhizobia (Online Resource 1). Presence of *acdS* in *Rhizobium* spp. was first reported by Ma et al. (2003a). In addition, an *acdS*-like gene can be detected with a database homology search (http://www.ncbi.nlm.nih.gov) in the genomes of *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, some *Sinorhizobium* strains as well as in the β-rhizobial strains *Burkholderia phymatum* and *Burkholderia tuberum* (Onofre-Lemus et al. 2009). Most of the genomes tested have only one copy of *acdS*. Few of the rhizobial *acdS* genes have been characterized by mutational analysis (Online Resource 1). A *Mesorhizobium loti acdS* knock-out mutant showed a decreased nodulation ability and was less competitive then the wild-type strain (Kaneko et al. 2000; Uchiumi et al. 2004) while a mutant constitutively expressing the *acdS* gene induced more nodules and was more competitive than the wild type (Conforte et al. 2010). Similarly, *R. leguminosarum* bv. viciae, mutants of either *acdS* or the associated regulatory gene (*lprL*) led to a decreased nodule number (Ma et al. 2003b). However, these mutations did not affect nitrogen fixation, indicating that the ACC deaminase does not play a role in late steps of nodule development. A double knock-out mutant of *acdS* and *lrpL* of *A. tumefaciens* D3 significantly lost the ability to improve root elongation of canola seedlings as compared with the wild type (Hao et al. 2011). Furthermore, alfalfa nodulation by *Sinorhizobium meliloti* strain Rm1021, which lacks *acdS*, was improved by the introduction of *acdS* from *R. leguminosarum* bv. viciae through plasmid transfer (Ma et al. 2004). Experiments along the same line were performed by Tittabutr et al. (2008) who introduced *acdS* from *Sinorhizobium* sp. strain BL3 in multiple copies into *Rhizobium* sp. strain TAL1145, a strain that exhibits only a low ACC deaminase activity. The resulting bacterial hybrid showed an increased ACC deaminase activity and led to an enhanced nodule number, nodule dry weight, and root dry weight in symbiosis with *Leucaena*. An *acdS* mutant of *Rhizobium* sp. strain TAL1145, however, did not cause a defective symbiotic phenotype on *Leucaena*. Another type of *acdS*-associated phenotype was observed after co-inoculation of mungbean seedlings with *acdS*-free *Rhizobium phaseoli* and the *acdS*-containing *Pseudomonas syringae*, which resulted in an increased host resistance to salt stress (Ahmad et al. 2011). Collectively, these reports argued for a beneficial role of ACC deaminase in the nodulation process. Yet, deviating findings—especially in the *Bradyrhizobium*-soybean symbiosis—, and the absence of *acdS* in some rhizobia made us wonder as to whether the reported positive effects can be generalized for every rhizobia-legume system.

3.2 Analysis of the *B. japonicum acdS* genomic region

A putative ACC deaminase gene (blr0241; Fig. 1) has been annotated in the *B. japonicum* genome (Kaneko et al. 2002). This gene encodes a 337-amino acid protein that has 87 % and 74 % positional amino acid sequence identity with the ACC deaminases of *M. loti* MAFF303099 and *R. leguminosarum*, respectively. A domain analysis of the Blr0241 protein shows the presence of a highly conserved region around a lysine at position 50 which is the essential amino acid that forms a covalent bond with the enzyme co-factor, pyridoxal-5′-phosphate (Yao et al. 2000; Fujino et al. 2004). Furthermore, it was previously shown that the tyrosine residues Y268 and Y294 are conserved in the known ACC deaminases (Karthikeyan et al. 2004). An alignment of the Blr0241 amino-acid sequence with the ACC deaminase sequences of *Pseudomonas* sp. and *Hansenula saturnus* (of which enzyme crystal structures are known) showed that these two residues are also conserved in *B. japonicum* ACC deaminase.

The gene located upstream of blr0241—i.e., bll0240 (Fig. 1)—is transcribed in the opposite direction and is predicted to code for a regulatory protein (AcdR) of the Lrp/AsnC family. Prigent-Combaret et al. (2008) noticed that *acdR* homologs are frequently associated with *acdS* in bacteria. These two genes co-occur also in some species of *Pseudomonas, Burkholderia*, and *Ralstonia*. The *R. leguminosarum* homolog of bll0240 was found to be required for expression of *acdS* (Ma et al. 2003b). AcdR of *P. putida* UW4 can bind to the *acdS* regulatory region, and a potential second regulatory protein, called AcdB was identified in this bacterium (Cheng et al. 2008). By analogy, although an AcdB homolog is absent in *B. japonicum*, the Bll0240 protein may act as a transcriptional regulator of the *acdS* gene in *B. japonicum*. Previous global transcript expression studies, using a custom-made Affymetrix GeneChip (Hauser et al. 2007), showed that bll0240 and blr0241 do not share the same expression profile. In contrast to bll0240, the *acdS*-like blr0241gene is highly expressed and up-regulated (20-fold change) in soybean symbiosis as compared with the expression in culture-grown, aerobic cells (Online Resource 2; Pessi et al. 2007). We also performed a tiling analysis of blr0241 expression. Interestingly, expression strength depended on the oxygen-level. The *acdS* gene was up-regulated during free-living micro-oxic (4.8-fold change) and anoxic growth (12-fold change) as well as in symbiosis (Online Resource 2, and Fig. 1). Induction under oxygen limitation clearly depended on the transcription factors RpoN (σ^{54}) and NifA (data not shown, but cf. Hauser et al. 2007 and Pessi et al. 2007). However, in contrast to *M.*

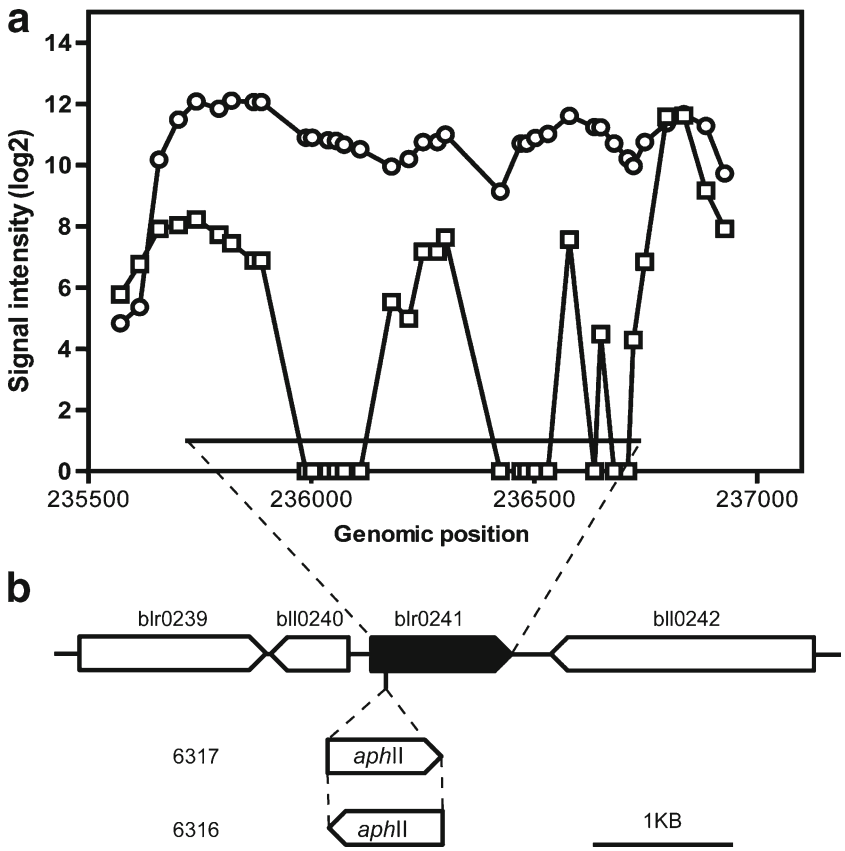

Fig. 1 Transcription analysis and genomic environment of blr0241. **a** Hybridization signal intensities derived from individual oligonucleotide probe pairs of the blr0241 region, using *B. japonicum* mRNA from aerobic culture (□) and from soybean root-nodules (○). For better visualization, individual data points were connected by solid lines. The open reading frame of blr0241 is indicated by a line above the genome coordinates. **b** Map of the blr0241 genomic region and the insertion mutations. The black arrow indicates the blr0241 reading frame. The orientation of the inserted resistance cassette (*aph*II) is shown together with the corresponding *B. japonicum* strain numbers

loti acdS (Nukui et al. 2006) the promoter region of blr0241 shows no predicted NifA or RpoN consensus binding sites suggesting that this gene might not be a direct target of these regulatory proteins. A proteomics study of *B. japonicum* revealed that ACC deaminase is abundant in root-nodule bacteroids of soybean, siratro, and cowpea (853, 477, 527 spectral counts, respectively) (Online Resource 2; Delmotte et al. 2010; Koch et al. 2010). Intuitively, the strong upregulation of blr0241 in *B. japonicum* bacteroids would be consistent with an important function of the ACC deaminase in soybean root-nodule symbiosis. To test this inference, blr0241 was mutated and the effect of the mutation was phenotypically analyzed.

3.3 Construction and phenotypical analysis of a *B. japonicum* ACC deaminase mutant

Knock-out mutants of blr0241 were constructed (strains 6316 and 6317) in which the *acdS*-like gene was disrupted at nucleotide position 116 after the translational start codon by inserting a kanamycin resistance gene (*aph*II) (Fig. 1). The correct genomic structures of the mutants were confirmed by PCR (data not shown). The growth rates of the mutants in oxic and micro-oxic conditions were not impaired (data not shown). However, a moderate growth delay was observed in anoxic, denitrifying conditions (Fig. 2), suggesting that the blr0241 gene product might play a supportive role while *B. japonicum* is growing anoxically. Furthermore, the colony forming units (CFU) of the mutant growing in complex PSY medium in aerobic condition decreased 2.3-fold as compared with the wild-type, i.e., at an optical density (OD_{600}) of 1 the wild-type CFU was 10^9 bacteria per ml, whereas that of strain 6316 was 4.4×10^8.

In order to unveil a possible role of blr0241 during symbiosis, seedlings of soybean, siratro, cowpea and mungbean plants were inoculated with the *B. japonicum* wild type and strain 6316. After 21 days (soybean, cowpea, and mungbean) and 31 days (siratro), whole-nodule symbiotic nitrogen fixation activity was measured with the standard acetylene reduction test. Plants inoculated with the mutant did not exhibit a defect in symbiotic nitrogen fixation as compared with plants inoculated with the wild type. Moreover morphology, number and dry weight of nodules induced by the mutant were wild-type like. To test whether the blr0241 mutant has perhaps a more subtle nodulation deficiency that escaped detection, a competition assay on soybean and siratro was performed between the wild type and 6316 strain. Figure 3 clearly shows that the mutant was as competitive as the wild type in colonizing soybean and siratro root nodules. Hence, the *B. japonicum acdS*-like gene does not appear to play a crucial role in the initiation and formation of a root nodule symbiosis. These results were unexpected in view of the fact that deletion of *acdS* in other rhizobia led to noticeable defects in terms of nodule number, shoot dry weight, or competition efficiency (Ma et al. 2003b; Uchiumi et al. 2004). This raised concerns about whether or not the *B. japonicum acdS*-like gene indeed encodes a functional ACC deaminase.

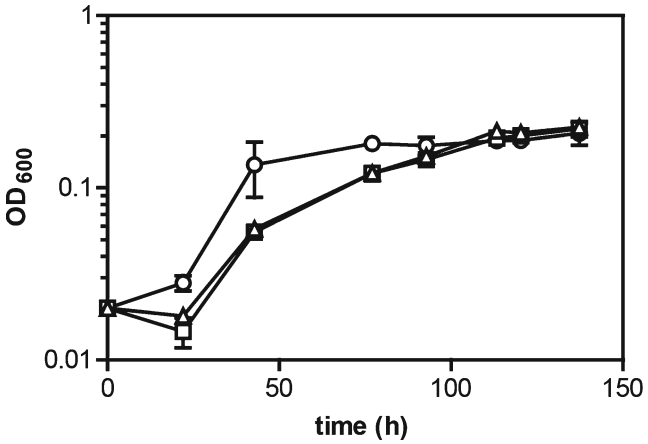

Fig. 2 Growth of the *B. japonicum* wild type (○) and mutant strains 6316 (□) and 6317 (Δ) in anoxic condition with nitrate as the terminal electron acceptor

3.4 *B. japonicum* ACC deaminase activity assay

To prove that the *acdS* gene encodes a functional enzyme in *B. japonicum*, the ACC deaminase activity was assayed. Measurements were done with the *B. japonicum* wild type and the insertion mutant 6316, both grown under anoxic free-living condition and in symbiosis with different host plants. In anoxic, free-living culture, the wild type had an activity of

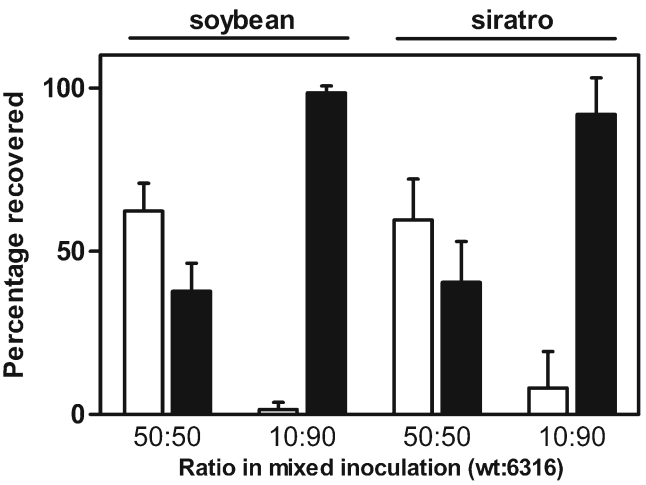

Fig. 3 Competition assay for soybean and siratro inoculation with the *B. japonicum* wild type (wt) and mutant 6316. The mixed inoculum contained wild type and strain 6316 in two different ratios as indicated. The percentage of in-nodule occupancy was determined by re-isolation of viable bacteria (from all nodules from each plant) and identification of their antibiotic resistance profile. The columns reflect the recovery of the wild type (*white bars*) and the mutant (*black bars*) as % of total isolates

1.49 μmoles α-ketobutyrate/mg/h whereas the insertion mutant showed no activity beyond background levels (Table 1). The ACC deaminase activity in root nodules varied depending on the host plant (Table 1). While the wild type-infected nodules consistently displayed a higher activity than mutant-infected nodules, we noticed a substantial background activity in the latter. One explanation could be that this activity stems from a host plant ACC deaminase, although we did not find an *acdS*-like gene in the sequenced soybean genome by homology search (Schmutz et al. 2010). Alternatively, this unexpected activity might be explained by the presence of plant compounds other than α-ketobutyrate, having aldehyde or keto groups that would be detected by the 2,4-dinitrophenylhydrazine reagent in the assay.

Intriguingly, it was shown previously with the ACC deaminase from *Pseudomonas* sp. that the enzyme can use substrates other than ACC, such as D-serine and D-β-chloroalanine (Walsh et al. 1981). Therefore, we also tested the *B. japonicum* enzyme present in crude cellular extracts for activity with these two substrates. A low but significant activity was detected with D-serine as the substrate which corresponded to about 10 % of the activity measured with ACC as the substrate. D-β-chloroalanine was not used as a substrate. The low activity measured with D-serine corroborates the notion that the Blr0241 protein might be a slightly promiscuous enzyme, just like the *Pseudomonas* sp. ACC deaminase (Walsh et al. 1981). Thus, the possibility cannot be excluded that the blr0241-encoded enzyme uses not only ACC but also other compounds that might be present in the host plant or in the bacterium.

3.5 Concluding remarks

The use of ACC by plant-associated, Gram-negative bacteria would require (i) that plants secrete ACC despite the necessity to convert it to ethylene *in planta*, and (ii) that bacteria take up ACC through the outer and the cytoplasmic membranes so that it can be used intracellularly as a substrate for degradation. None of these transport processes has ever been demonstrated in any plant-microbe system. Therefore, it remains a mystery how ACC reaches ACC deaminase on its way from the site of synthesis to the site of degradation. Nevertheless, there are reports that show for rhizobia a beneficial effect of possessing ACC deaminase. In the present paper, however, the data obtained with *B. japonicum*-legume symbioses do not support such a claim. The most clear-cut result was that a *B. japonicum acdS* (blr0241) knock-out mutant displayed an unaltered, wild-type behavior in the processes of nodulation and symbiotic nitrogen fixation with four different host plants. The lack of any effect, which was additionally confirmed in a more sensitive nodulation competition assay, might have been expected at least for the *B. japonicum*-soybean symbiosis, because previous investigations had already shown that soybean nodulation was independent of ethylene signaling (Schmidt et al. 1999). Yet, the nodulation process on siratro, another host of *B. japonicum*, did show an involvement of the ethylene signaling pathway (Nukui et al. 2000), which we cannot support due to the lack of an effect caused by the blr0241 mutation on siratro nodulation and the absence of a competition defect in comparison to the wild type. By and large, the beneficial effect of ACC deaminase seen in several rhizobia-legume symbioses cannot be extrapolated uncritically to all nodulating plants.

The question arises as to why the expression of the *B. japonicum* blr0241 gene is strongly up-regulated under low oxygen conditions such as it prevails in root nodules (Hauser et al. 2007; Pessi et al. 2007). One possibility is that rhizobial ACC deaminase plays an important role in micro-oxic or anoxic metabolism rather than attenuating plant-internal ethylene synthesis. Moreover, one might even go as far as to suggest that a hitherto unidentified metabolite other than ACC is the true in-vivo substrate for rhizobial ACC deaminase. What speaks in favor of this statement is the remarkable substrate promiscuity of the enzyme and the very poor affinity constant for ACC (Honma and Shimomura 1978; Walsh et al. 1981). In this context, it is interesting to note that the *Arabidopsis thaliana* ACC deaminase had originally been discovered as a D-cysteine desulfhydrase (Riemenschneider et al. 2005). In our own study, we confirmed that the Blr0241 protein uses D-serine as a substrate, although less efficiently than ACC. This might be interpreted to mean that the true substrate for ACC deaminase has yet to be discovered, and the corresponding substrate conversion might be unrelated to the nodulation process. In line with this inference, we made the disillusioning experience that strong expression of a gene in bacteroids is no guarantee for its importance in symbiosis.

Table 1 Determination of ACC deaminase activity (μmol 2-ketobutyrate formed h^{-1} mg $protein^{-1}$) in *B. japonicum* cells grown free-living and in symbiosis

Growth condition	Wild type	Strain 6316
Free-living		
In anoxic culture	1.49±0.01	0.053±0.0
Symbiotic		
In soybean nodules	0.47±0.0	0.17±0.0
In mungbean nodules	1.52±0.0	0.45±0.11
In siratro nodules	0.78±0.04	0.48±0.08
In cowpea nodules	1.02±0.05	0.59±0.016

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