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# Transformation of *Azospirillum brasilense* Cd with an ACC Deaminase Gene from *Enterobacter cloacae* UW4 Fused to the *Tet*$^r$ Gene Promoter Improves Its Fitness and Plant Growth Promoting Ability

G. Holguin,[1] B.R. Glick[2]

[1] CIBNOR, Calle Mar Bermejo No. 195, Col. Playa Palo de Sta. Rita, Apartado Postal No. 128, La Paz, BCS, 23090, México
[2] Department of Biology, University of Waterloo, Waterloo ON N2L 3G1, Canada

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## Abstract

It has been reported that PGPB, containing ACC deaminase, can cleave the plant ethylene precursor ACC and thereby lower ethylene concentration in a developing or stressed plant, protecting it against the deleterious effects of stress ethylene and facilitating the formation of longer roots. In a previous work we have demonstrated expression of the ACC deaminase gene (*acdS*) from *Enterobacter cloacae* UW4 under the control of the *lac* promoter in *Azospirillum brasilense* Cd. With the inference that a construct including the ACC deaminase gene under the control of a constitutive promoter weaker than the *lac* promoter might impose less metabolic load on *Azospirillum* and improve its fitness, it was decided to clone *acdS* under the control of a tetracycline resistance gene promoter. The ACC deaminase structural gene was fused to the *Tet*$^r$ gene promoter by overlap extension using PCR, cloned in pRK415, and transferred into *A. brasilense* Cd. The resulting transformants showed lower ACC deaminase activity than those with the *lac* promoter controlled *acdS* gene. However, *acdS* under the control of the *Tet*$^r$ gene promoter imposed lesser metabolic load on *Azospirillum brasilense* Cd. The result was significantly increased IAA synthesis and greater bacterial growth rate, as well as increased ability to survive on the surface of tomato leaves and to promote the growth of tomato seedlings.

## Introduction

Many plant growth-promoting bacteria modify plant growth by lowering concentration of ethylene in plants. The bacteria accomplish this by breaking down the immediate precursor of ethylene, 1-aminocyclopropane 1-carboxylate (ACC), by ACC deaminase [17–19, 22].

Some of the effects that result in lower ethylene levels due to ACC deaminase activity in plant growth-promoting bacteria are reduction in the extent of ethylene inhibition of plant seedling root elongation [15, 18, 22] and amelioration of the inhibitory effects of stress ethylene on plant

*Correspondence to:* G. Holguin; E-mail: glick@sciborg.uwaterloo.ca

growth caused by the presence of heavy metals [12], phytopathogens [37], and flooding [21].

Crop yields are enhanced in several regions of the world by inoculation of the plants with the plant growth-promoting bacterium *Azospirillum* spp. that also reduces the need for chemical fertilizers [1]. Although the presence of ACC deaminase has been detected in a number of soil bacteria and fungi [19], to date it has not been found in plants and is not present in *Azospirillum* [25]. We previously reported expression of the ACC deaminase gene (*acdS*) from *Enterobacter cloacae* UW4 in *A. brasilense* Cd by transferring into the bacterium a construct that involved *acdS* under the control of the *Escherichia coli lac* promoter. This construct conferred upon *A. brasilense* Cd the ability to induce root elongation of canola and tomato seedlings [25]. Here, the preparation of a construct with *acdS* under the control of the tetracycline resistance ($Tet^r$) gene promoter is described. Expression of this construct in *A. brasilense* Cd improves the fitness of the bacterium and its ability to promote plant growth, compared to the construct containing *acdS* under the control of the *lac* promoter. We chose tomato plants as the host plant for these studies because of the high sensitivity of tomato plants to ethylene [22].

## Materials and Methods

### *Bacterial Strains and Growth Conditions*

The bacterial strains used in this study were *Azospirillum brasilense* Cd ATCC 29710, *Escherichia coli* DH5α [23], *A. brasilense* Cd transformed with *acdS* under the control of the *E. coli lac* promoter (Cd/pRKLACC) [25], *A. brasilense* Cd transformed with plasmid pRK415 (Cd/pRK415) [25], and the plant growth promoting bacterium *Enterobacter cloacae* UW4 [15, 34]. The strains were grown as previously described [25] except that the rich medium for the *Azospirillum* strains was Luria broth (Difco Laboratories, Detroit, MI). All strains were activated from frozen stocks (stored at −70°C in Luria broth with 10% glycerol, v/v) by growing them in the appropriate rich medium. The growth medium for the *A. brasilense* Cd and *E. coli* transformed strains was supplemented with tetracycline (20 μg $mL^{-1}$).

### *Plasmid isolation and transformation of* Escherichia coli *DH5α*

Plasmid DNA was routinely prepared by the alkaline lysis miniprep method [33]. *E. coli* DH5α cells were transformed with plasmid DNA by treatment of the cells with $CaCl_2$ as described [33].

### *Triparental Mating*

Plasmid DNA was transferred from *E. coli* DH5α to *A. brasilense* Cd by triparental mating using pRK2013 [14] in *E. coli* HB101 as the helper plasmid. The construct pRKTACC (the ACC deaminase gene under the control of the $Tet^r$ promoter cloned in pRK415) was transferred from *E. coli* DH5α to *A. brasilense* Cd as described previously [25]. Transconjugants of *A. brasilense* Cd were selected following growth for 3 days at 30°C on OAB minimal medium [29] supplemented with 3.0 mM ACC in the presence of 20 μg $mL^{-1}$ tetracycline.

### *ACC Deaminase Gene Cloning under the Control of the* $Tet^r$ *Promoter*

The ACC deaminase structural gene *acdS* was fused with the constitutive promoter of the tetracycline resistance gene by overlap extension [26] using the polymerase chain reaction, PCR [31]. The first step in this procedure involved the generation of DNA fragments (the promoter region of the $Tet^r$ gene and the ACC deaminase structural gene, *acdS*) with complementary regions using independent PCR reactions (Fig. 1). In the second step, the two amplified fragments were fused together and amplified. The overlap allowed one strand from each fragment to act as a primer for the other strand, and extension of this overlap resulted in the fused product *pTet-acdS*.

The oligonucleotide primers used according to Fig. 1 were:

(1) 5′-CCC GAA **AGG TAC** CGC CTC ACG -3′

(2) 5′-CG ATT CAG GTT CAT ACT CGC TGC CTT ACT GCG TTA GC -3′

(3) 5′-GC AGT AAG GCA GCG AGT ATG AAC CTG AAT CGT TTT GAA -3′

(4) 5′-GCA ATC **TCG CAT GC**A TGG CGG-3′

The promoter region of the $Tet^r$ gene was amplified with primers 1 and 2, using linearized plasmid pBR322 [8] as template. The first 14 bases of primer 2 are complementary to the first 14 bases of *acdS*. Primer 1 was designed to include the *Kpn*I restriction site AGG TAC marked in bold above. To amplify *acdS*, primers 3 and 4 were used, with pRKACC carrying the ACC deaminase gene of *Enterobacter cloacae* UW4 cloned in the broad-host-range plasmid pRK415 as a template [34]. The first 17 bases of primer 3 are complementary to the first 17 bases of the $Tet^r$ promoter region. Primer 4 was designed to include the *Sph*I restriction site TCG CAT GC marked in bold above. The ribosome binding site in the promoter region of the $Tet^r$ gene was modified from CAG GCA CCG TGT to AAG GCA GCG AGT (enclosed in a rectangle above), the consensus Shine–Dalgarno sequence [38]. To generate the fusion product *pTet-acdS*, the two fragments *pTet* and *acdS* were gel purified and then amplified using primers 1 and 4. For amplification we used the Expand Long Template PCR system (Boehringer Mannheim, Germany) with the following protocol: denaturation at 94°C for 5 min, 30 cycles of 1 min at 94°C, 2 min at 52°C, and 3 min at 68°C, followed by 7 min at 72°C. The oligonucleotides used as primers

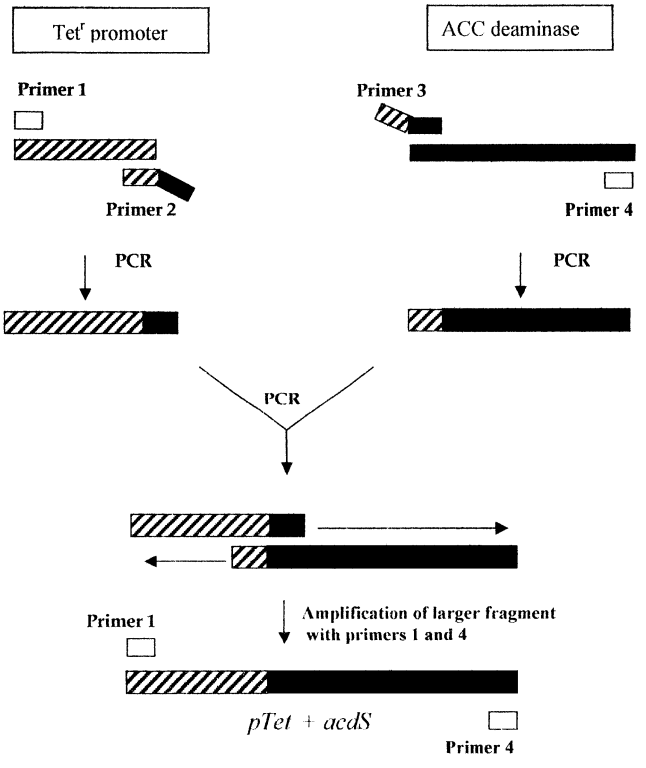


Fig. 1. Fusion of the ACC deaminase structural gene *acdS* with the constitutive promoter of the tetracycline resistance gene, *Tet*$^r$, by overlap extension using PCR. See text for details.

were synthesized by MOBIX, McMaster University, Hamilton, ON, Canada.

### *Cloning of the Fused PCR Product* pTet-acdS *in pRK415*

The PCR product *pTet-acdS* was gel purified and ligated to the vector pGEM-T (Promega, Madison, WI, USA) according to the manufacturer's specifications using a 3:1 molar ratio of the insert DNA to the vector. DNA fragment *pTet-acdS* was excised from plasmid pGEM-T by digestion with *Sph*I (Fig. 2) and ligated into pUC19 digested with *Sph*I. Once it had been demonstrated that *acdS* was expressed in *E. coli* DH5α, the fragment *pTet-acdS* was then excised from pUC19 by double digestion with *Sac*I and *Kpn*I and ligated into pRK415 digested with the same restriction enzymes. After transfer of the plasmid carrying *pTet-acdS* in pRK415 (designated pRKTACC) to *E. coli* DH5α and confirmation of ACC deaminase activity in the transformants, the presence and size of the insert was determined by restriction enzyme digestion and agarose gel electrophoresis. pRKTACC was then transferred from *E. coli* DH5α into *A. brasilense* Cd by triparental mating.

### *ACC Deaminase Assay*

All strains were grown in rich media for 18 h: *Enterobacter cloacae* UW4 was grown in tryptic soy broth (TSB) medium (Difco) and *E. coli* DH5α/pRKTACC, *A. brasilense* Cd/pRKTACC, and Cd/pRKLACC [25] were in Luria Broth (Difco) containing 20 μg mL$^{-1}$ tetracycline. Since *Enterobacter cloacae* UW4 carries the wild-type promoter region which requires ACC induction [21], the cells were harvested by centrifugation, washed with 0.1 M Tris-HCl (pH 7.5), and incubated for another 18 h in minimal medium containing 3 mM ACC as the sole source of nitrogen. In all cases, cell lysates, prepared by vortexing vigorously with 5% (v/v) toluene in minimal medium, were assayed for ACC deaminase activity as described by Honma and Shimomura [28].

The protein concentration in the cell lysates was determined by the procedure described by Bradford [10].

### *IAA Assay*

IAA was detected colorimetrically in the supernatants of the bacterial cultures using Salkowski's reagent [20]. All strains were grown in OAB minimal medium, containing tryptophan, as previously described [25].

### *Acetylene Reduction Assay*

*Azospirillum brasilense* Cd strains were grown in 70-mL serum bottles with nitrogen-free OAB medium (30 ml) for 5 days at 30°C under static conditions. Following this incubation period the cotton stoppers were replaced with rubber stoppers, and 1 mL of air was removed from the bottles with a syringe. One mL of acetylene (0.1 atm) was injected and the bottles were incubated at 30°C for 24 or 48 h.

Ethylene analyses were conducted by gas chromatography using an HP 5890 Series II gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a hydrogen flame ionization detector (FID) and a capillary porous open layer tubular column HP-PLOT/$Al_2O_3$ "S" deactivated, 50 m × 0.32 mm, with film thickness of 8 μm. A detector and injector temperature of 250°C, a column temperature of 100°C, $N_2$ carrier gas and $H_2$ at a flow rate of 30 mL min$^{-1}$, and air flow rate of 300 mL min$^{-1}$ were used. The amount of ethylene was expressed in nanomoles per culture over 24 or 48 h.

### *Survival of* Azospirillum *in soil*

Ten grams of Pro-Mix "BX," a peat-based general-purpose growth medium (Premier Horticulture, Ltd, Rivière du Loup, Quebec, Canada) was spread evenly in plastic Petri dishes (100 × 15 mm) saturated with sterile distilled water, and inoculated with 12 mL of a bacterial suspension prepared as described below, with the cell concentration adjusted with phosphate buffer (pH 6.8, 0.05 M, supplemented with 1.92 mM NaCl, 20 mM fructose, and 18.7 mM $NH_4Cl$) to $10^7$ cfu mL$^{-1}$. The petri dishes were incubated at 26°C for a period of 1.5 months. The soil was kept moist throughout the experiment by adding sterile distilled water when required. The survival of bacteria in soil was determined by bacterial counts using the plate-count method [9].

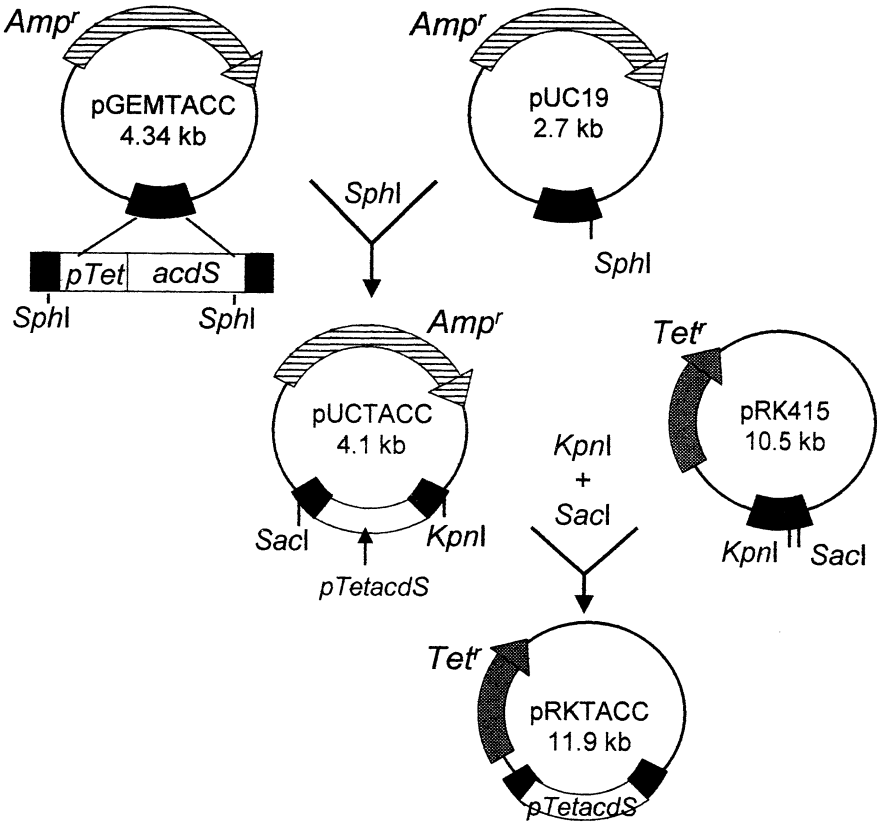


Fig. 2. Cloning of *pTet-acdS* in pRK415. pGEMTACC represents *pTet-acdS* cloned in pGEM-T, while pUCTACC and pRKTACC stand for *pTet-acdS* cloned in pUC19 and in pRK415, respectively.

### *Survival of* Azospirillum *on tomato leaves*

Nutrient-poor basaltic soil (3% organic matter; 0.05% total nitrogen) from Baja California Sur, Mexico [6], was mixed 1:1 (v/v) with vermiculite (Vil Vermiculite, Inc., Montreal, Quebec, Canada) and autoclaved three times for 30 min each to eliminate spores of any microorganisms. Soil (180 g) was saturated with sterile distilled water, poured into 300-mL Erlenmeyer flasks, and used as a growth medium for aseptic tomato seeds. Seeds were disinfected by immersion in 1% sodium hypochlorite for 5 min, and then thoroughly rinsed with sterile distilled water. Seeds were plated on Luria agar to test surface sterilization. Flasks containing the seeds were covered with a double layer of Parafilm and placed in a growth chamber at 26°C with an average daytime light illumination of 130 μmol $m^{-2}$ $s^{-1}$ for 14 h and 10 h of darkness. The Parafilm was replaced every 3 days to allow air exchange. Once the tomato seedlings reached the second leaf stage, 100 μL of a bacterial suspension was distributed onto the leaves and the plants were incubated for 120 h. To prepare the bacterial suspensions, *A. brasilense* Cd, *A. brasilense* Cd/pRKLACC, *A. brasilense* Cd/pRKTACC, and *A. brasilense* Sp245/pRKLACC cells were grown overnight in LB medium, washed twice in the phosphate buffer described previously, and resuspended in the same buffer solution at a concentration of $10^7$ cfu $mL^{-1}$.

Half of the leaves were used for examination by scanning electron microscopy, and the other half for counting the number of cells attached to the leaves by the dilution plate-count method.

### *Bacterial Counts on Leaves and Roots of Tomato Plants*

For counting the number of cells attached to leaves or roots, the sample was rinsed twice with 0.03 M $MgSO_4$, vortexed, and sonicated for 3 min using light sonication (Branson Sonifier Cell Disrupter, model 200, Branson Sonic Power Company, Danbury Conn., USA). The sample was then serially diluted and counted by the plate-count method on solid Luria broth medium. In the case of the *A. brasilense* Cd transformants, the medium was supplemented with 20 μg $mL^{-1}$ tetracycline

### *Effect of Inoculation on Pot-Grown Tomato Plants with* Azospirillum brasilense *Cd Transformants*

Tomato seeds (*Lycopersicum esculentum* Mill. cv. Heinz 1439 VF) were surface disinfected as described above and incubated for 2–3 h at 30°C with 10 mL of either 0.03 M $MgSO_4$ or a bacterial suspension in 0.03 M $MgSO_4$. The bacterial suspension was prepared by growing the bacteria overnight in Luria broth (supplemented with 20 μg $ml^{-1}$ tetracycline in case of the *Azospirillum* transformants), washing the cells twice with 0.03 M $MgSO_4$, resuspending them in the same solution, and adjusting the concentration of cells to $10^8$ cfu $mL^{-1}$.

Tomato seeds were germinated and grown in 60 × 30 × 5.5 cm boxes containing vermiculite and placed in a growth chamber under the conditions specified above. The plants were watered once a week with half-strength Hoagland's solution [24]. For dry

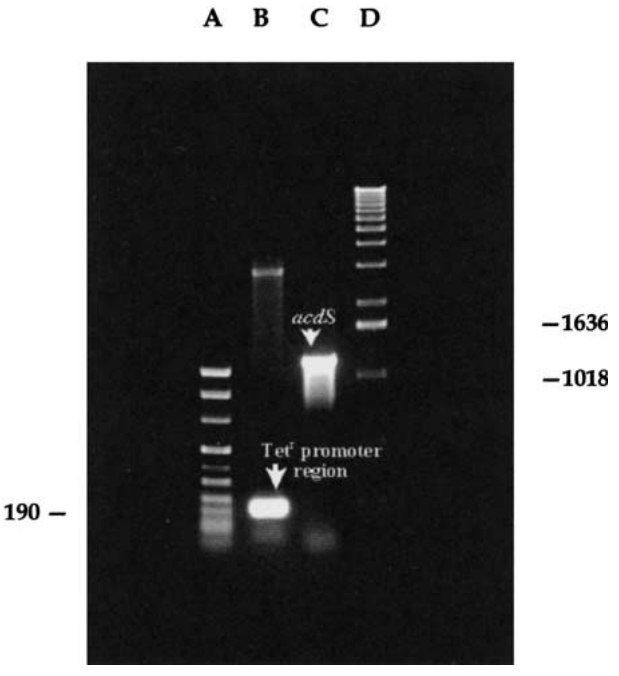


Fig. 3. Amplification of the ACC deaminase structural gene *acdS* and the tetracycline resistance promoter region *pTet* by PCR. Lanes A and D show the DNA molecular weight marker; lane B shows a band (194 bp) marked with an arrow that corresponds to *pTet*; lane C shows a band marked with an arrow (1171 bp) that corresponds to *acdS*.

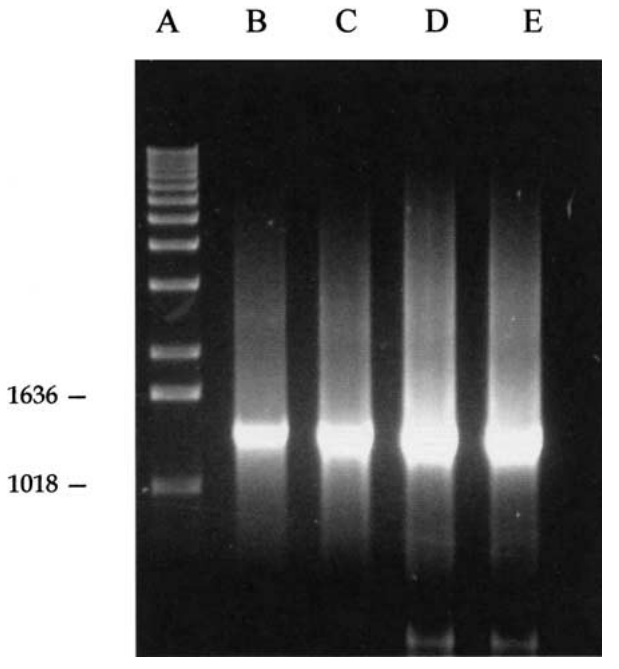


Fig. 4. Amplification of *pTet-acdS* by PCR. Lanes B, C, and D show a band (1334 bp) located between 1636 and 1018 bp that corresponds to the fused product *pTet-acdS*.

weight measurements, each plant was dried separately in an oven at 70°C for 24 h.

### *Scanning Electron Microscopy*

After 24, 48, and 96 h of incubation with bacteria, some tomato leaves were excised, rinsed, and then vortexed in 0.05 M phosphate buffer pH 6.8 and fixed in 2.5% glutaraldehyde (Sigma, St. Louis, MO, USA) in the same buffer. The leaves were dehydrated by passage through increasing concentrations of ethanol in water. The final wash was in 100% acetone. The samples were dried in a critical point dryer (Denton, DCP-1, Cherry Hill, NJ, USA) in a $CO_2$ atmosphere. The dried samples were affixed to stubs with conductive, self-sticking adhesive tabs and coated with 30 nm gold film (Polarum, Watford, UK) before being examined by SEM (Hitachi S-570, Japan) at 60 kV.

### *Experimental Design*

Experiments were repeated at least twice. Each treatment consisted of a minimum of three replicates. Experiments to determine the effects of inoculation with *Azospirillum* on tomato plants were carried out with 10 seedlings per treatment. Statistical significance was determined by one-way analysis of variance (ANOVA) at $P \leq 0.05$ followed by Tukey's range test. Graphs were plotted using Sigma Plot, Version 3.0 (Jandel Corporation). The curves in Fig. 7 were plotted using a smooth curve fit by Sigma Plot.

## Results

### *Construction of acdS under the Control of* pTet *and Cloning of the* pTet-acdS *Fusion Fragment in pRK415*

The ACC deaminase gene, *acdS*, was fused successfully with the promoter of the tetracycline resistance gene *pTet* by overlap extension using PCR (Fig. 1). The first part of the procedure involved two PCR steps: one to amplify the structural gene *acdS* and the other to amplify *pTet* (Fig. 3). The second step consisted of a third PCR step to generate the fusion fragment *pTet-acdS*.

When the PCR products *acdS* and *pTet* were analyzed by agarose gel electrophoresis, the bands (Fig. 3) corresponded to the predicted sizes of the DNA fragments (1171 and 194 bp, respectively). Similarly, gel electrophoresis of the PCR product *pTet-acdS* revealed a band the predicted size for the fused product (1334) (Fig. 4). The size of the fused fragment *pTet-acdS* did not equal the sum of starting fragments, i.e. 1171 and 194 = 1365 bp), since the primers used to generate *pTet-acdS* overlap in 31 bp, resulting in a smaller fused fragment of 1334 bp.

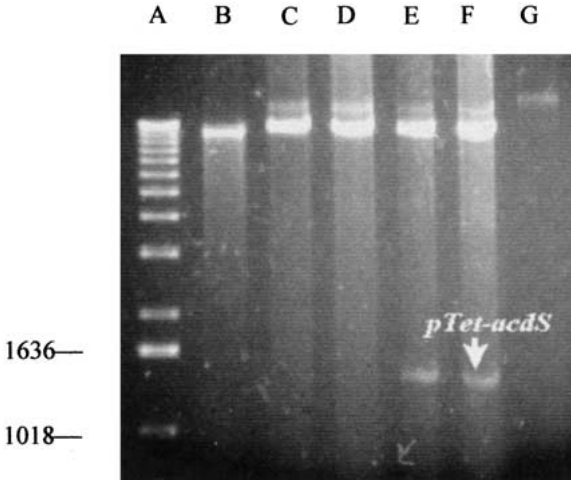


Fig. 5. DNA electrophoresis of pRKTACC isolated from *Azospirillum brasilense* Cd/pRKTACC transformants. Lane A, molecular weight marker; lane B, pRK415 cut with *Sac*I; lanesC and D, *A. brasilense* Cd/pRKTACC digested with *Sac*I; lanes E and F, *A. brasilense* Cd/pRKTACC cut with *Kpn*I and *Sac*I, allowed excision of the insert pTet-*acdS* from pRKTACC; lane G, *A. brasilense* Cd cut with *Kpn*I and *Sac*I.

The fusion fragment *pTet-acdS* was first cloned in pGEM-T, then subcloned into pUC19 and then into pRK415 (Fig. 2). The resulting plasmid pRKTACC was used to transform *E. coli* DH5α and was then transferred to *A. brasilense* Cd (Fig. 5) by conjugation.

### *Growth, IAA Synthesis, ACC Deaminase Activity, and Nitrogen Fixation of Nontransformed and* Transformed Azospirillum brasilense *Cd*

Nontransformed *A. brasilense* Cd reached the log phase earlier than *A. brasilense* transformed with pRKTACC, pRKLACC, or pRK415 (Fig. 6A). However, after 16 h, *A. brasilense* Cd/pRKTACC and Cd/pRK415 attained similar cell density as the nontransformed strain.

Cultures of *A. brasilense* Cd/pRKLACC in OAB minimal medium supplemented with tryptophan synthesized reduced amounts of IAA compared to that of cultures of nontransformed *A. brasilense* Cd, *A. brasilense* Cd/pRKTACC, and Cd/pRK415 (Fig. 6B).

The ACC deaminase activity in *A. brasilense* Cd/pRKTACC was significantly ($P \leq 0.05$) lower than in Cd/pRKLACC (Fig. 6C). No significant difference was found between the ACC deaminase activity in Cd/pRKLACC and *Enterobacter cloacae* UW4.

Acetylene reduction by the nontransformed *A. brasilense* Cd strain was significantly higher than that of either of the transformed strains Cd/pRKLACC and Cd/pRKTACC, measured after periods of 24 and 48 h. No significant difference was found between the ethylene synthesis of *A. brasilense* Cd/pRKLACC and *A. brasilense* Cd/pRKTACC (Fig. 6D).

### *Survival of Nontransformed and transformed* A. brasilense *Cd*

Analysis of survival in soil showed that the cell density of *A. brasilense* Cd/pRKLACC increased during the first 10 days, after which it abruptly declined (Fig. 7A). The population of *A. brasilense* Cd/pRKTACC remained stable during the first 20 days but subsequently declined and stabilized after 30 days. The cell density of *A. brasilense* Cd/pRK415 declined during the first 20 days and then remained relatively stable. In contrast, the population of nontransformed *A. brasilense* Cd did not suffer abrupt changes and decreased slowly during the entire incubation period.

Inoculation of *A. brasilense* Cd in leaves of axenically grown tomato plants showed that both the nontransformed *A. brasilense* Cd and the transformed strains had the ability to attach to the leaves of tomato plants and to survive on the surface of the plant for a period of at least 14 days (Figs. 7B, 8a–f). The cell density values of nontransformed *A. brasilense* Cd and Cd/pRKTACC were similar throughout the duration of the experiment. The cell density of Cd/pRKLACC decreased dramatically compared to the cell density of either nontransformed *A. brasilense* Cd or Cd/pRKTACC.

### *Scanning Electron Microscopy*

Observations by scanning electron microscopy (SEM) (Fig. 8a–f) complemented results from bacterial counts on agar plates (Fig. 7B) and showed that all strains were capable of establishing on the leaf surface after 1 day of inoculation. The colonization pattern of both transformed and nontransformed *A. brasilense* was in the form of cell aggregates distributed across the leaf surface. All cells produced fibrils that connected the cells to each other and to the surface of the leaves (Fig. 8a–f). No bacteria were found colonizing leaf surface of the noninoculated plants (Fig. 8g, h).

Four days after inoculation, most cells of Cd/pRKLACC were found embedded in a sheath (Fig. 8d). This material of unknown nature concealed the cells, making it difficult

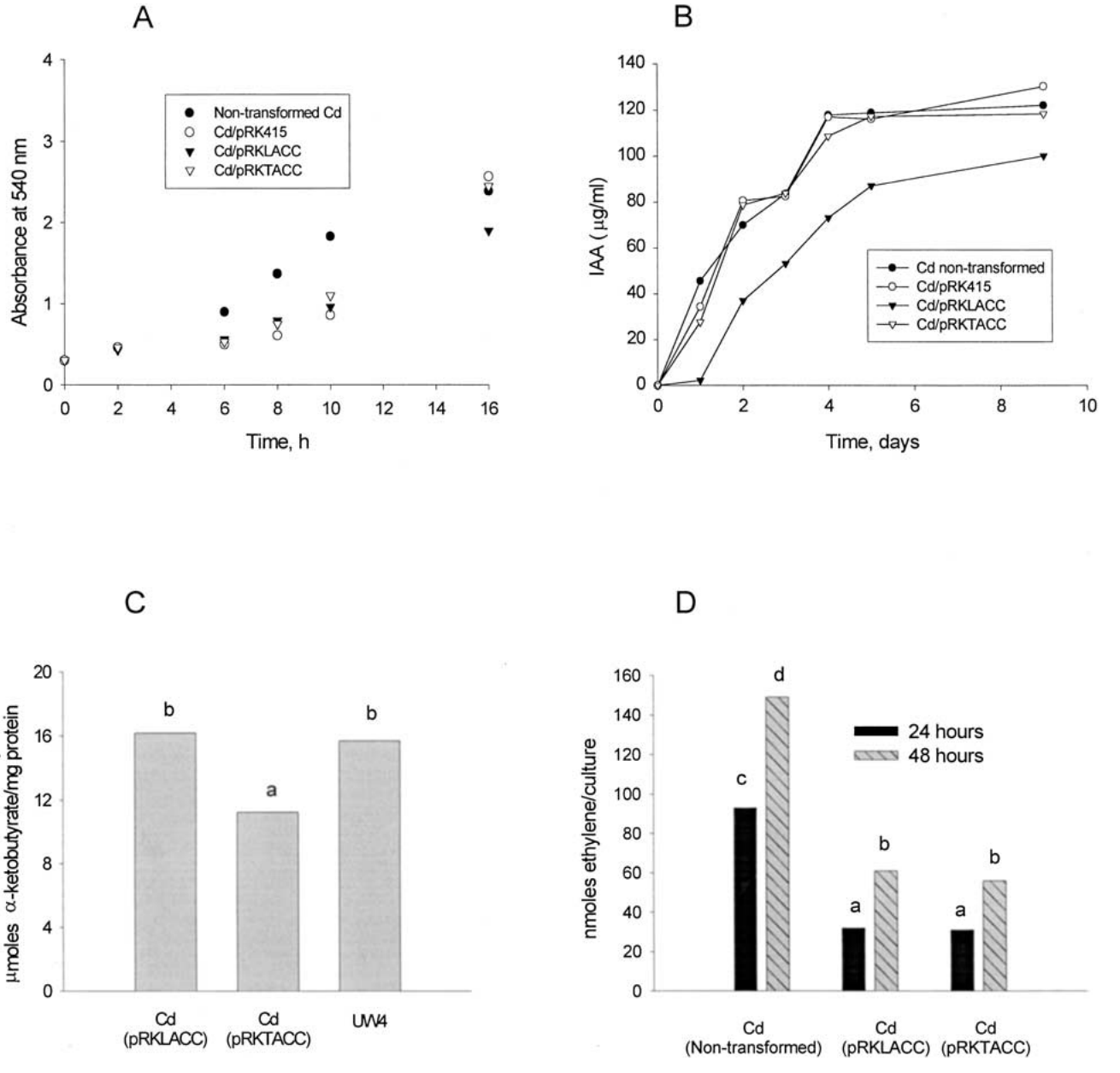


Fig. 6. (A) Growth, (B) IAA synthesis, (C) ACC deaminase activity, and (D) acetylene reduction of the *A. brasilense* Cd transformants and the nontransformed *A. brasilense* Cd strain. Growth and IAA synthesis were determined in cells growing in OAB minimal medium. Treatments marked with different letters show statistically significant differences.

to detect and visualize the bacteria when taking the photographs.

### *Plant Growth Promotion of* A. brasilense *Cd, Cd/pRKLACC, and Cd/pRKTACC*

The dry weight of 15-day-old tomato seedling roots was significantly higher in inoculated plants as compared to that of nontreated plants (Fig. 9A). Interestingly, seedlings treated with *A. brasilense* Cd/pRKTACC exhibited a significant increase in the dry weight of roots as compared to that of plants treated either with Cd/pRKLACC or with the nontransformed Cd strain. The dry weight of shoots of seedlings treated with nontransformed *A. brasilense* Cd was significantly higher than those of nontreated plants. While the dry weight of shoots treated with Cd/pRKTACC was greater than the dry weight of shoots from any other treatment, the difference was not statistically significant (Fig. 9B).

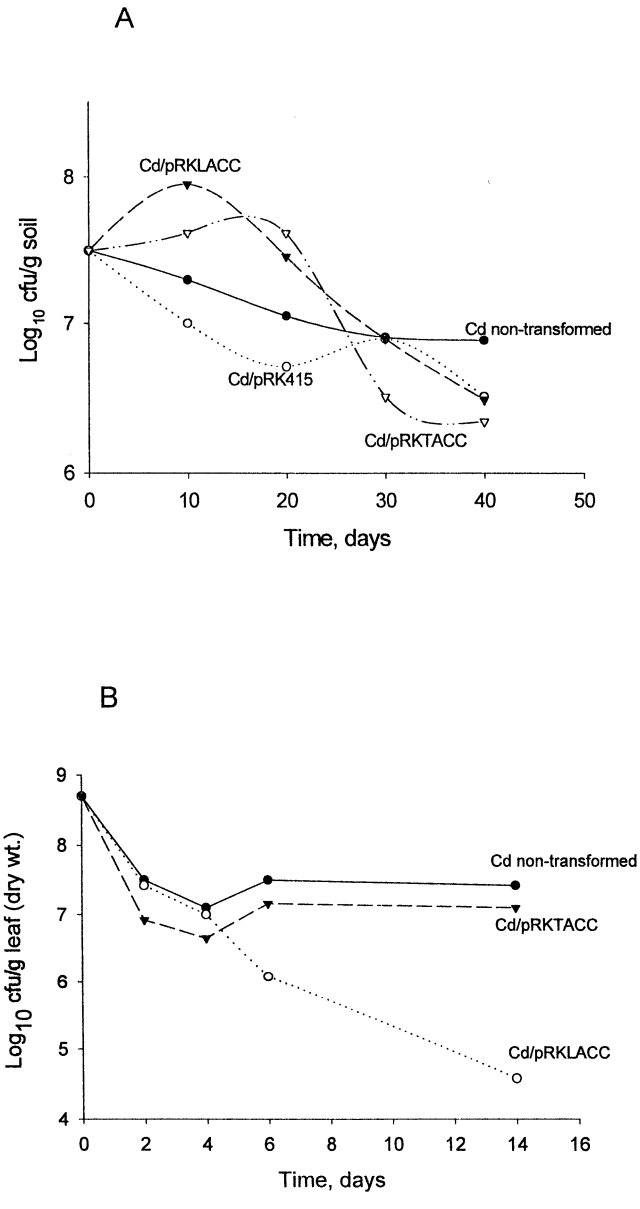


Fig. 7. (A) Survival of *A. brasilense* Cd, Cd/pRKTACC, Cd/pRKLACC, and Cd/pRK415 in soil during a period of 40 days. (B) Survival of *A. brasilense* Cd, *A. brasilense* Cd/pRKLACC, and *A. brasilense* Cd/pRKTACC on the surface of tomato leaves. The results are the average values from two experiments.

## Discussion

The *E. coli lac* promoter is considered to be relatively strong, allowing high levels of transcription of a downstream cloned gene [35]. Because cloning of the ACC deaminase gene under the control of a constitutive promoter that is weaker than the *lac* promoter might impose less metabolic load on *Azospirillum,* it was decided to clone *acdS* under the control of a *Tet*$^r$ promoter. Previous work showed that *Azospirillum* recognized the latter [36]. As expected, plasmid pRKTACC, which included the *acdS* gene under the control of the *Tet*$^r$ promoter, did not affect the growth rate and IAA synthesis of *A. brasilense* Cd as compared to pRKLACC, which decreased the cell growth rate and the ability of *Azospirillum* to synthesize IAA (Fig. 6A, B).

Persistence of *Azospirillum* spp. in the soil is crucial for plant growth promotion because seed inoculation is impractical in many situations [2]. The survival in soil of nontransformed *A. brasilense* Cd remained relatively stable during the duration of the experiment (Fig. 7A). Comparison of the survival of *A. brasilense* Cd/pRKLACC with *A. brasilense* Cd/pRKTACC showed that the population of the latter stabilized after 30 days, but the population of Cd/pRKLACC showed a continuous decline. These results might be a reflection of a lower metabolic load imposed on *Azospirillum* with *acdS* under the control of the *Tet*$^r$ gene promoter.

All *Azospirillum brasilense* Cd strains, both nontransformed and transformed strains, showed the capacity to attach to tomato leaves (Figs. 7B, 8a–f). There is evidence suggesting that bacteria can modify their environment to enhance colonization of plants, such as by increasing local nutrient concentrations or by producing a layer of extracellular polysaccharides. This habitat modification may occur on the surface of leaves, as well as in the leaf interior [7].

The SEM micrographs (Fig. 8a–f) show that all of the *Azospirillum* strains examined can colonize leaves forming aggregates, a prominent feature of the colonization of leaf surfaces [4, 7, 30].

The micrographs in this work reveal the presence of fibrillar material that emanates from and between bacterial cells on leaves. Other studies on root colonization by *Azospirillum* have revealed the presence of fibrillar material that anchors the bacterial cells to the root surface and establishes connections between cells within bacterial aggregates [3, 4, 30]. In these images, bacteria are closely packed but clearly embedded in an amorphous material that is presumably composed of exopolysaccharides of bacterial origin. Exopolysaccharides may anchor cells to the leaf surface and prevent cells from being desiccated; retention of water in the highly hygroscopic polysaccharide matrix should increase the water available to the bacteria. For example, purified exopolysaccharide from several phytopathogens induced persistent water soaking after introduction by infiltration into leaves [7].

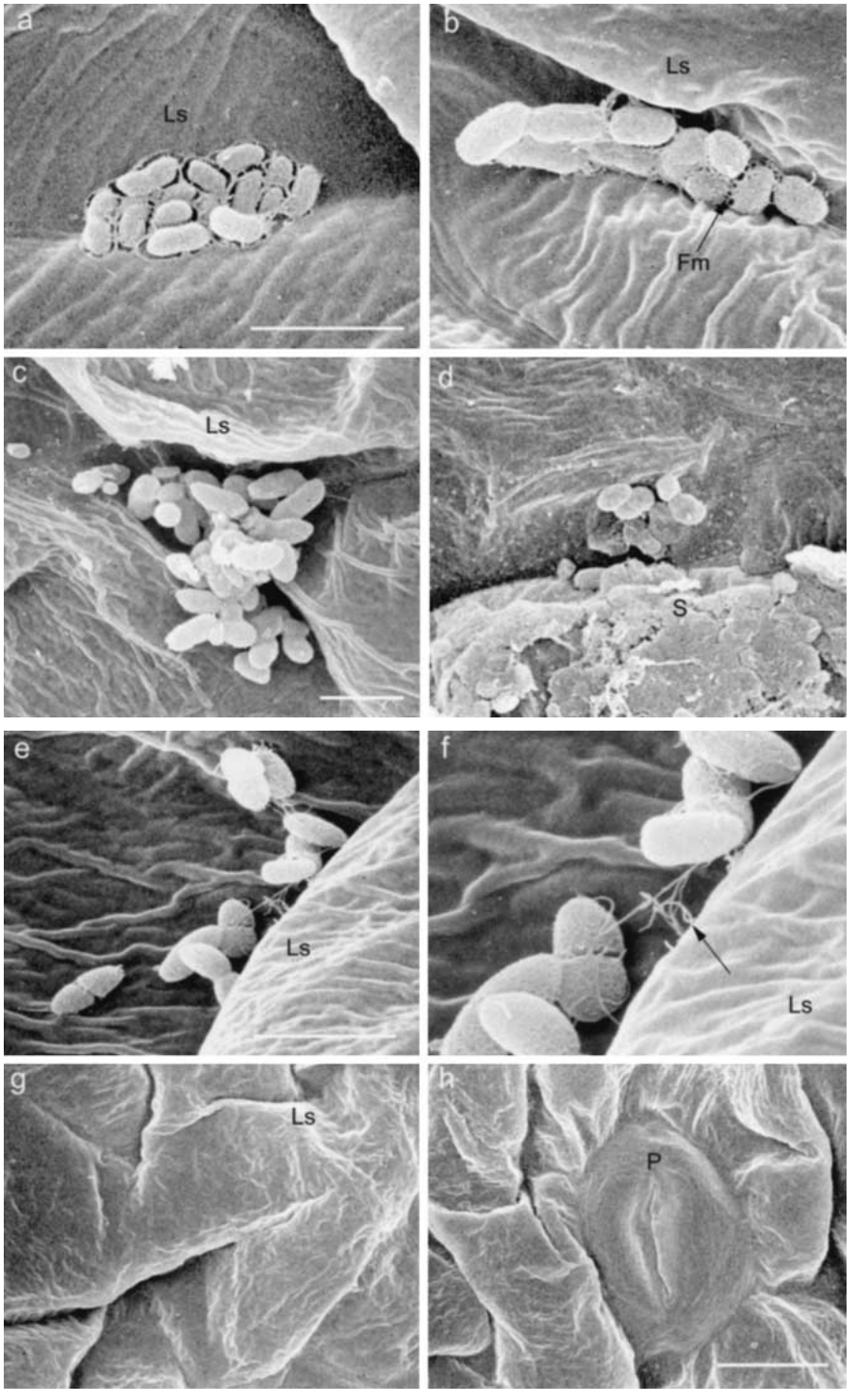


Fig. 8. (a) and (b) Colonization of tomato leaves by nontransformed *A. brasilense* Cd 2 days after inoculation. Bacterial aggregates show fibrillar material connecting the cells to eachother and to the leaf surface. Bar on (a) represents 6 μm. (c) and (d) Colonization of tomato leaves by *A. brasilense* Cd/pRKLACC. (c) Cell aggregates colonizing orifices on the leaf surface 1 day after inoculation. (d) After four days of inoculation a sheath covered the cells. Bar on (c) represents 3.75 μm. (e) and (f) Colonization of tomato leaves by *A. brasilense* Cd/pRKTACC 1 day after inoculation. (f) magnification of (e) showing the cells attaching to each other by fibrils (arrow). Bar on (e) represents 4.3 μm. (g) and (h) Leaf surface of noninoculated tomato seedlings. (h) Leaf surface showing a closed pore. Bar on (h) represents 12 μm. Abbreviations: Ls, leaf surface; Fm, fibrillar material; p, pore; s, sheath.

It is possible that in our work, the use of a minimal medium with fructose and ammonium chloride as carbon and nitrogen sources, respectively, contributed to the ability of the cells to attach to the leaf surface. Burdman et al. [13] reported that growth of *A. brasilense* Cd in such a medium resulted in flocculation (formation of visible aggregates) after 24 h; aggregated cells were rich in poly-β-hydroxybutyrate that can contribute to survival of the cells under nutrient and water stress conditions [32].

One day after inoculation, the cell aggregates of *A. brasilense* Cd/pRKLACC were visible by SEM. However, 4 days following inoculation the cells were covered by a sheath and were difficult to visualize (Fig. 8d).

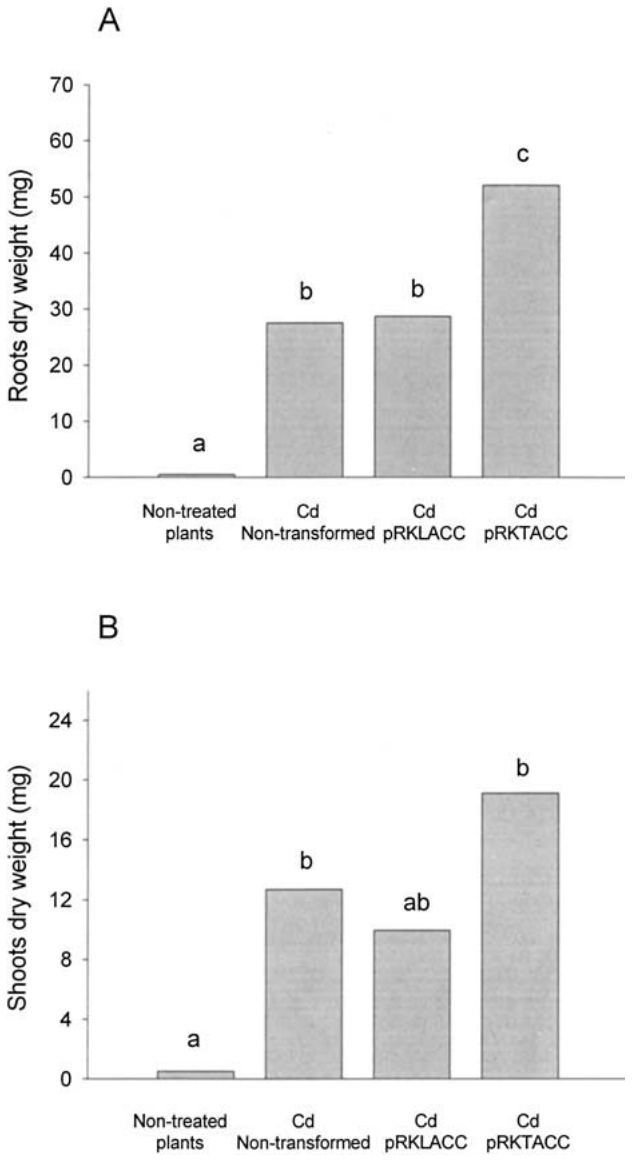


Fig. 9. (A) Dry weight of roots and (B) shoots of 15 days postgermination tomato seedlings treated with nontransformed *A. brasilense* Cd, Cd/pRKLACC, and Cd/pRKTACC. The values on the *y*-axis represent the percentage of increase from the control, which was considered as 0%. Bars marked with different letters show statistically significant differences.

In addition, IAA may be involved in the epiphytic fitness of some plant growth-promoting bacteria. The secretion of IAA by the bacterium may modify the microhabitat of epiphytic bacteria by increasing nutrient leakage from plant cells; enhanced nutrient availability may better enable IAA-producing bacteria to colonize the phyllosphere [7]. In our work, the survival of *A. brasilense* Cd/pRKLACC on the surface of tomato leaves steadily decreased from the start of the inoculation. The population of *A. brasilense* Cd/pRKTACC, however, remained constant throughout the experiment. It is possible that the decreased ability of *A. brasilense* Cd/pRKLACC to synthesize IAA *in vitro* (Fig. 6B), probably as a result of the imposition of a metabolic load on the transformed cells, was reproduced on the leaf surface, thus reducing the ability of the bacterium to survive. Brandl and Lindow [11] showed that when wild-type *Erwinia herbicola* and an IAA-deficient mutant were inoculated simultaneously onto bean plants and pear flowers, the IAA deficient mutant could not compete against the wild type and declined to a significantly lower population.

We previously reported that inoculation of tomato seedlings with *A. brasilense* Cd transformed with pRKLACC enhanced root elongation of 1-week-old canola and tomato seedlings [25]. However, the persistence of these beneficial effects provided by the transformants was not tested in longer-term experiments. Here we observed that *A. brasilense* Cd/pRKLACC does not show an improved ability over nontransformed *A. brasilense* Cd to promote the growth of 15-day-old tomato seedlings (Fig. 9). In contrast, inoculation with Cd/pRKTACC promotes the growth of the same seedlings. These results suggest that transformation of *A. brasilense* Cd with *acdS* under the control of the *lac* promoter may impose a metabolic load on the cells that reduces the ability of the bacterium to promote plant growth. Transformation with *acdS* under the control of the weaker $Tet^r$ gene promoter, however, decreases the ability of the bacterium to synthesize ACC deaminase but does not debilitate the bacterium that is able to promote plant growth for longer periods of time. Previous studies [16, 27] have shown that expression in a bacterium of a foreign protein at high levels can debilitate the bacterium physiologically, hampering its plant growth promoting abilities. In this regard, when the broad host range plasmid vector pGSS15 was used to genetically transform the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2, the transformants were physiologically debilitated, probably due to the β-lactamase gene that is expressed at high levels in transformed bacteria [27]. Deletion of the ampicillin resistance gene in plasmid pGSS15 and the subsequent transformation of *Pseudomonas putida* GR12-2 with the modified plasmid overcame the metabolic load imposed by the expression at high levels of the protein and restored the plant growth promoting abilities of the bacterium [27].

Transformation of *Azospirillum brasilense* Cd with *acdS*, under the control of either the $Tet^r$ gene promoter or

the *lac* promoter pRKLACC, significantly reduced the ability of the bacterium to reduce nitrogen. Nevertheless, *A. brasilense* Cd/pRKTACC enhanced the growth of tomato seedlings. Previous work [5] showed that when inoculated onto tomato seeds, the significant positive effects of an *A. brasilense* Cd *nif* mutant (totally deficient in $N_2$-fixation capability) on seedling growth under greenhouse conditions were similar to that of the wild-type strain. Thus, nitrogen fixation by *A. brasilense* may only be an important mechanism of plant growth promotion under conditions where soil nitrogen is extremely limited.

In summary, expression of the ACC deaminase gene in *A. brasilense* Cd under the control of a constitutive but weaker promoter than the *lac* promoter allows the bacteria to conserve its plant growth promoting properties with the additional benefit of expressing ACC deaminase. The ability of *A. brasilense* Cd/pRKTACC to benefit other ethylene-sensitive plants besides tomato still has to be evaluated.

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