MICROBIAL ECOLOGY

Microb Ecol (2001) 41:281–288
DOI: 10.1007/s002480000040

Expression of the ACC Deaminase Gene from *Enterobacter cloacae* UW4 in *Azospirillum brasilense*

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

[1] Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1
[2] Department of Microbiology, Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, A.P. 128, B.C.S., 23000, México

Received: 21 January 2000; Accepted: 13 April 2000; Online Publication: 7 November 2000

ABSTRACT

The ACC deaminase structural gene (*acd*S) from *Enterobacter cloacae* UW4 was cloned in the broad host range plasmid pRK415 under the control of the *lac* promoter and transferred into *Azospirillum brasilense* Cd and Sp245. *A. brasilense* Cd and Sp245 transformants showed high ACC deaminase activity, similar to that observed in *Enterobacter cloacae* UW4. The expression of ACC deaminase improved the existing growth promoting activity of *Azospirillum.* The roots of tomato and canola seedlings were significantly longer in plants inoculated with *A. brasilense* Cd transformants than those in plants inoculated with the nontransformed strains of the same bacterium. In the case of wheat seedlings, inoculation with *A. brasilense* Cd transformants did not promote root growth. The difference in plant response (canola and tomato versus wheat) is attributed to the greater sensitivity of canola and tomato plants to ethylene as compared to wheat plants.

Introduction

In addition to the well-characterized mechanisms that plant growth promoting bacteria use to stimulate the growth of plants, including solubilization of phosphorus, producing siderophores to sequester iron, fixation of nitrogen, synthesis of phytohormones, and decreasing the damage to plants from phytopathogens [9, 16], it was proposed that many plant growth promoting bacteria may promote plant growth through the activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme hydrolyzes ACC, the immediate precursor of ethylene in plant tissues 9, 15].

Ethylene is essential for growth and development of plants, but when it is present in high levels it can be damaging for plants, leading to epinasty, shorter roots, and premature senescence, for example. Bacteria that have ACC deaminase can cleave ACC to ammonia and α-ketobutyrate, and thus prevent the effects of high levels of ethylene [15, 19]. In nature, ACC deaminase is commonly found in rhizosphere bacteria [12–14].

Azospirillum is a plant growth promoting bacterium that colonizes and promotes the growth of numerous plant species, including cereals, grasses, weeds, annuals, perennials,

Correspondence to: B.R. Glick; Fax: (519) 746-0614 E-mail: glick@sciborg.uwaterloo.ca

and even plants that have no previous record of colonization by *Azospirillum* [4, 30]. Although many *Azospirillum* species stimulate plant growth, members of this genus do not produce ACC deaminase and thus cannot lower ethylene levels in plants that may result from stimulation of the synthesis of the plant enzyme ACC synthase [24] by bacterial IAA as well as a response to environmental stresses such as flooding, drought, temperature stress, high salt, and phytopathogens [1].

We postulate that transfer of the ACC deaminase gene into *Azospirillum brasilense* will result in an improved plant growth promoting bacterium. Here we report the expression of ACC deaminase in *A. brasilense*after transformation with a plasmid containing an ACC deaminase gene from *Enterobacter cloacae* under the control of the *Escherichia coli lac* promoter.

Methods

Bacterial Strains and Growth Conditions

The *Azospirillum* strains used in this study were *A. brasilense* Cd ATCC 29710 and *A. brasilense* Sp245 [2]. *Escherichia coli* DH5α [20] is the host strain for the ACC deaminase gene from *Enterobacter cloacae* UW4 in pUC18 [32]. The plant growth promoting bacteria *Enterobacter cloacae* CAL2 and *Enterobacter cloacae* UW4 have ACC deaminase activity and were isolated from a tomato rhizosphere soil from King City, California, USA, and from a reed rhizosphere sample from Waterloo, Ontario, Canada, respectively [14].

The *Enterobacter cloacae* strains were grown on either solid or liquid tryptic soybean broth (TSB) medium (Difco Laboratories, Detroit, MI) at 30°C, or on M9 minimal medium [26] supplemented with either 0.2% w/v $(NH_4)_2SO_4$ or 3.0 mM ACC. Bacteria grown on minimal media plus ACC produce higher levels of ACC deaminase activity than do bacteria grown on more complete media. *Escherichia coli* DH5α was grown in Luria broth (Difco) at 37°C or in M9 minimal medium. *A. brasilense* Cd and Sp245 were grown in nutrient broth (NB) medium (Sigma Laboratories) or in OAB medium [27] at 30°C. For the ACC deaminase assays, the growth medium was supplemented with 3.0 mM ACC, and when indicated with isopropylthio-β-D-thiogalactoside (IPTG).

ACC Deaminase Assay

Enterobacter cloacae CAL2, nontransformed *A. brasilense* Cd and Sp245, and the *Azospirillum* strains transformed with plasmid pRKACC (carrying the ACC deaminase gene of *Enterobacter cloacae* UW4 cloned in the plasmid pRK415; [32]) were grown in rich medium (TSB for *Enterobacter cloacae* and NB for the *Azospirillum* strains) for 18 h. The cells were then 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. The bacterial cells were collected by centrifugation and resuspended in 0.1 M Tris-HCl (pH 8.5). Bacteria were disrupted by either sonication with a Branson Sonifier Cell Disruptor or vigorous vortexing with 5% (v/v) toluene. The lysate was assayed for ACC deaminase activity as described by Honma and Shimomura [21]. This assay measures the formation of α-ketobutyrate following the cleavage of ACC. The protein concentration in the cell lysates was determined by the method of Bradford [5].

IPTG (0.8 mM) was added to some of the cultures to determine the requirement for this compound to induce the *lac* promoter.

IAA Assay

IAA was detected colorimetrically using Salkowski's reagent [17] in the supernatants of*A. brasilense* Cd and *A. brasilense* Cd/pRKLACC (the ACC deaminase gene controlled by the *lac* promoter) cultures, grown at 30°C in NB or OAB with or without 0.5 g L^{-1} tryptophan, and 20 mg mL^{-1} tetracycline in the case of the transformants.

Plasmid Isolation and Transformation of Escherichia coli Cells

Plasmid DNA isolation and transformation of *Escherichia coli* DH5α cells were performed as described [31].

Triparental Mating

The broad-host-range plasmids pRK415 [23], pRKACC [32], and pRKLACC were transferred from *Escherichia coli* DH5α to *A. brasilense* Cd and Sp245 by triparental mating using pRK2013 in *Escherichia coli* HB101 as the helper plasmid [8].

Cloning of the ACC Deaminase Gene with the lac Promoter

A 3.8-kb DNA fragment from *Enterobacter cloacae* UW4 that includes the ACC deaminase gene (*acd*S) and an 855 bp upstream region containing the *acd*S promoter region was previously cloned in plasmid pUC18 and named p4U2 [32].

The ACC deaminase gene (*acd*S) was excised from plasmid p4U2 without its native promoter by digestion with *Eco*RV (Fig. 1). The 1174-p fragment containing *acd*S was inserted into the *Sma*I site within the multiple cloning site of pUC19, and the resulting plasmid, pUCLACC, was introduced into *Escherichia coli* DH5α by transformation. The size and orientation of the insert was determined by restriction enzyme digestion and agarose gel electrophoresis. Analysis of transformants with ACC deaminase activity showed that, as expected, they contained *acd*S immediately downstream from the *lac* promoter.

After demonstrating that *acd*S could be expressed in *Escherichia coli* under the control of the *lac* promoter, *acd*S was cloned into plasmid pRK415 (Fig. 1) by ligating a*Hin*dIII-*Sac*I fragment from pUCLACC to pRK415 digested with the same enzymes. The plasmid from a clone with high ACC deaminase activity was named pRKLACC and transferred from *Escherichia coli* DH5α into *A. brasilense* Cd and Sp245 by triparental mating.

Gnotobiotic Root Elongation Assay

The effect of various bacterial strains on the elongation of plant seedling roots was determined as described by Lifshitz et al. [25].

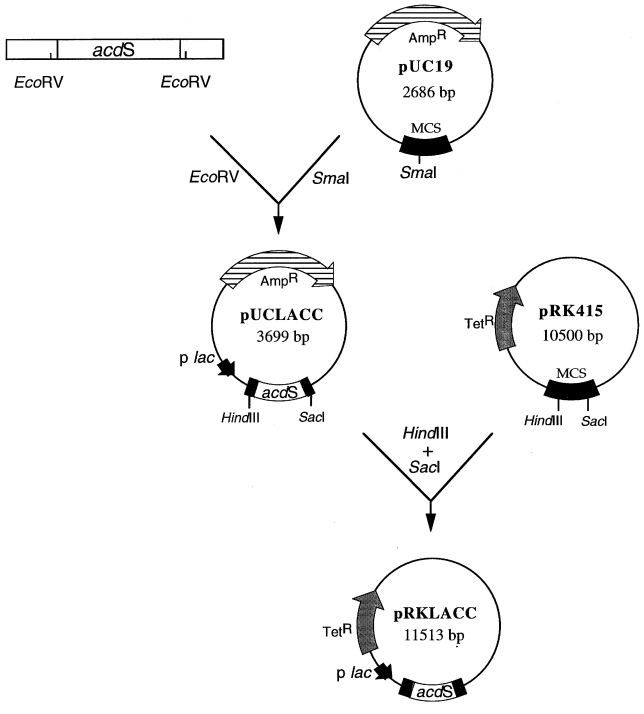

Fig. 1. Cloning of the *Enterobacter cloacae* UW4 ACC deaminase gene, *acd*S, in the broad host range plasmid pRK415 under the control of the *Escherichia coli lac* promoter. MCS stands for multiple cloning site.

Canola seeds (*Brassica campestris* cv. Reward), tomato seeds (*Lycopersicum esculentum* Mill. cv. Heinz 1439 VF, and hard red spring wheat seeds (*Triticum aestivum* cv. Quantum), stored at 4°C, were surface disinfected by immersion in 1% sodium hypochlorite for 5 min, and then thoroughly rinsed with sterile distilled water immediately prior to use. Inoculum levels ranging from 10^7 to 10^9 cfu mL^{-1} were used since plants treated with lower levels did not respond to *A. brasilense* Cd or *A. brasilense* Cd/pRKLACC.

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

Results

ACC Deaminase Activity in Nontransformed A. brasilense Cd and Sp245

Nontransformed *A. brasilense* Cd and Sp 245 cells, cultured in nutrient broth or OAB minimal medium, showed no measurable ACC deaminase activity in either log or stationary phase.

Expression of the ACC Deaminase Gene in A. brasilense

Escherichia coli strains harboring pRKACC showed high ACC deaminase activity (1.74 ± 0.06 μmol α-ketobutyrate/mg protein/h), similar to that in *Enterobacter cloacae* UW4 (2.43 ± 0.04 μmol α-ketobutyrate/mg protein/h). However, when pRKACC was transferred to *A. brasilense* Cd and Sp245, the transformants did not have any measurable ACC deaminase activity. To test for the presence of an inhibitor of ACC deaminase activity, the cell lysate of *A. brasilense* Cd was concentrated and added to a lysate prepared from the cells of *Enterobacter cloacae* CAL2, previously shown to have a high level of ACC deaminase activity. The addition of various amounts of *A. brasilense*lysate to the CAL2 lysate did not alter the ACC deaminase activity of this bacterium Table 1).

Table 1. Effect of a lysate from *Azospirillum brasilense* CD pKACC cells on the ACC deaminase activity of *Enterobacter cloacae* CAL2

Enterobacter clocae CAL2	*Enterobacter clocae* CAL2 + *A. brasilense* Cd/pRKACC lysate** 9 : 1	*Enterobacter clocae* CAL2 + *A. brasilense* Cd/pRKACC lysate 2 : 3	*Enterobacter clocae* CAL2 + H_2O 2 : 3
4:13 ± 0.08 b	4.11 ± 0.07 b	2.58 ± 0.08 a	2.53 + 0.05 a

ACC deaminase activity is expressed as μmoles of α-ketobutyrate/mg protein/h. The cell lysates were prepared by breaking down the cells with sonication as described in Methods. Numbers followed by different letters denote statistically significant differences at $P \leq 0.05$ in one way ANOVA.
* Protein concentration = 3.14 mg/ml
** Protein concentration = 8.72 mg/ml

To overcome the lack of expression of ACC deaminase in *A. brasilense* strains, the *acd*S native promoter was replaced with the *Escherichia coli lac* promoter (Fig. 1). We inferred that in *A. brasilense* the *lac* promoter would not be regulated as it is in *Escherichia coli,* since *A. brasilense* does not grow on lactose [33] and therefore is unlikely to encode a *lac* repressor protein. The ACC deaminase gene was cloned in plasmid pRK415 under the control of the *lac* promoter and was transferred from *Escherichia coli*DH5α to *A. brasilense* Cd and *A. brasilense* Sp245. The transconjugants, *A. brasilense*Cd/pRKLACC and *A. brasilense* Sp245/pRKLACC, showed high ACC deaminase activity similar to that observed in *Enterobacter cloacae* UW4 (Fig. 2). As expected, in contrast to *Escherichia coli* transformants, ACC deaminase expression in *A. brasilense*transformants was not regulated by IPTG.

IAA Synthesis in A. brasilense Transformants

Cultures of *A. brasilense* Cd/pRKLACC in OAB minimal medium supplemented with tryptophan showed a reduction

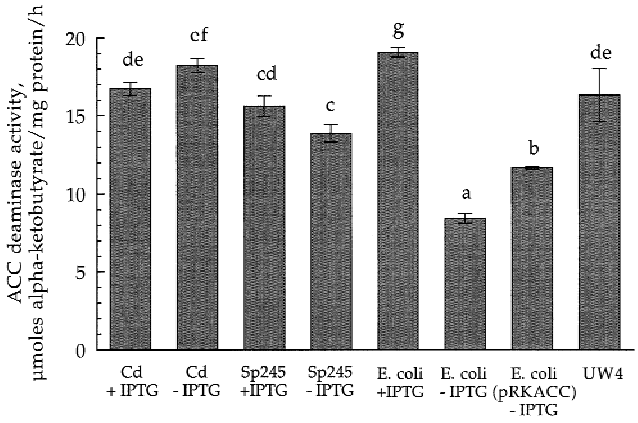

Fig. 2. ACC deaminase activity in *A. brasilense* Cd, *A. brasilense* Sp245 and *Escherichia coli*DH5α transformed with pRKLACC. *Escherichia coli* DH5α/pRKACC carries *acd*S under the control of its native promoter. Columns marked with different letters differ significantly at $P \leq 0.05$ in a one-way ANOVA. Bars represent standard error values.

in IAA concentration compared to cultures of nontransformed *A. brasilense* Cd (Fig. 3B). Similarly, the cell density in cultures of *A. brasilense* Cd/pRKLACC was lower compared to cultures of *A. brasilense* Cd (Fig. 3A). However, for cells grown in NB medium containing tryptophan, no differences in IAA concentration or cell density was found between *A. brasilense* Cd/pRKLACC and *A. brasilense* Cd (Figs. 3C, 3D).

Elongation of Seedling Roots Following Inoculation with A. brasilense Transformants

Canola seeds inoculated with *A. brasilense* Cd/pRKLACC cells developed longer roots than did noninoculated seeds or seeds inoculated with nontransformed *A. brasilense* Cd (Fig. 4). *Escherichia coli* DH5α/pRKACC induced root elongation in canola seeds to the same extent as *Enterobacter cloacae* CAL2 or *A. brasilense* Cd/pRKLACC; however, although ACC deaminase-containing *Escherichia coli* can promote root elongation under laboratory conditions, it is not a soil bacterium and is not useful in the field.

Tomato plants inoculated with *A. brasilense* Cd/pRKLACC at an inoculum concentration of 10^7 cfu mL^{-1} had significantly longer roots compared to nontreated plants or plants inoculated with the nontransformed strain (Fig. 5). Nontransformed *A. brasilense* induced root elongation in tomato only when the inoculum level was increased to 10^8 cfu mL^{-1}. At this inoculum density, the length of tomato roots inoculated with *A. brasilense* Cd/pRKLACC was still significantly greater than plants inoculated with the nontransformed strain.

In the case of wheat, no significant difference in shoot or root fresh weight (Figs. 6A, B), shoot dry weight (Fig. 6C), or total plant water content (Fig. 6E) was observed between plants treated with *A. brasilense* Cd/pRKLACC and plants treated with nontransformed *A. brasilense* Cd. Nevertheless, plants treated with *A. brasilense* Cd/pRKLACC at an inoculation level of 10^7 cfu mL^{-1} promoted the dry weight of roots compared to plants inoculated with nontransformed *A. brasilense* Cd at a concentration of 10^8 cfu mL^{-1} (Fig. 6D).

Discussion

When the DNA fragment from *Enterobacter cloacae* UW4, which included *acd*S and its regulatory region, was transferred into *A. brasilense* Cd and *A. brasilense* Sp245, the transconjugants did not show any ACC deaminase activity. This was surprising since the *A. brasilense* transformants contained the entire upstream region of *acd*S and this construct was readily expressed in *Escherichia coli* (Table 1). However, it is possible that the *A. brasilense* strains may be incapable of recognizing the promoter of the*Enterobacter cloacae acd*S gene.

In a number of different plants, ethylene stimulates germination and breaks the dormancy of the seeds [7]; however, if the level of ethylene following germination is too high, root elongation is inhibited [22]. Plant growth promoting bacteria with ACC deaminase activity lower the levels of ACC, and hence ethylene, in plants and thereby prevent impairment of root growth [15]. This bacterial stimulation of root elongation could enhance the survival of seedlings, especially during the early stages of growth [9].

To determine if the expression of an ACC deaminase gene in *A. brasilense* could contribute to lowering the levels of ethylene in the early development of plants, canola, tomato, and wheat seeds were inoculated with *A. brasilense* Cd transformed with an ACC deaminase gene. The transfer of *acd*S gave both *A. brasilense* Cd and *Escherichia coli*the ability to induce root elongation in canola plants (Fig. 4). This presumably occurs as a result of the acquired ability of these bacteria to break down ACC. Root elongation in tomato seedlings inoculated with nontransformed *A. brasilense* Cd was enhanced only when inoculated with 10^8 cfu mL^{-1} (Fig. 5); lower inoculum densities appeared to have no effect on tomato roots. Similar results were found by Hadas and Okon

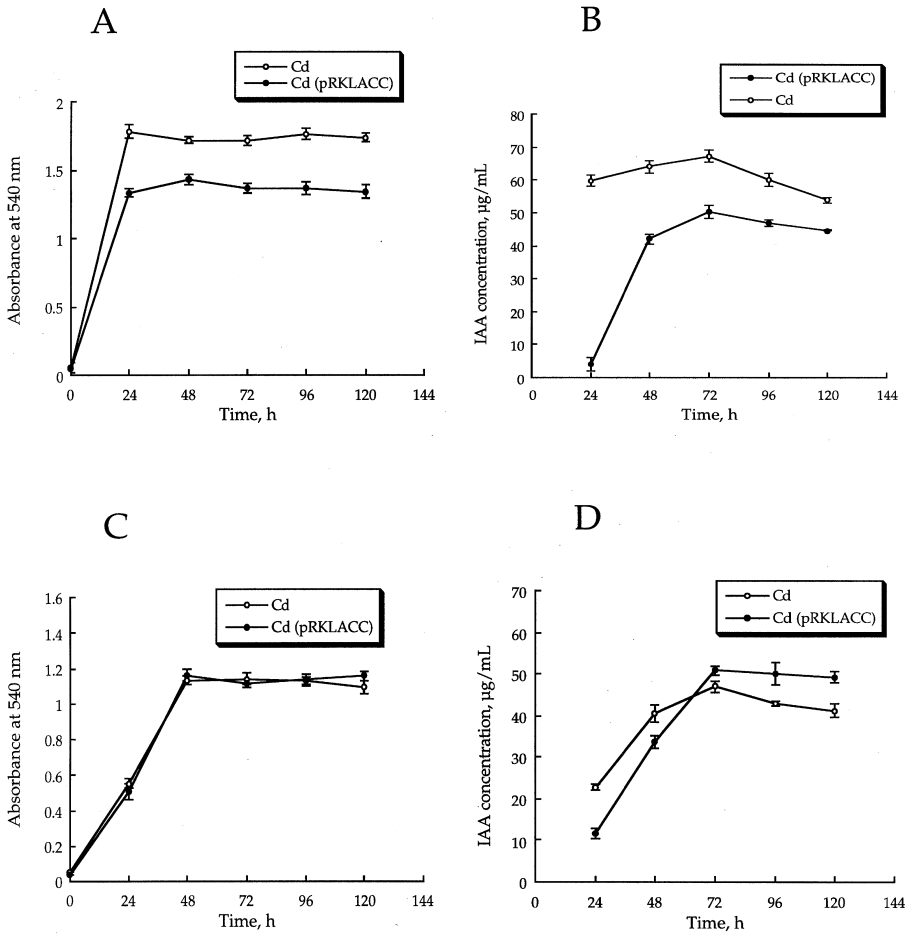

Fig. 3. IAA concentration and cell density in *A. brasilense* Cd and *A. brasilense*Cd/pRKLACC cultures grown in OAB medium (A and B) or in NB medium (C and D), both with tryptophan. Bars represent standard error values.

[18], who reported that the optimal *A. brasilense* Cd inoculum level for tomato was $>10^8$ cfu mL^{-1}. However, seedlings treated with *A. brasilense* Cd/pRKLACC responded to an inoculum level of 10^7 cfu mL^{-1}, indicating that root elongation of tomato seedlings was probably induced by the ACC deaminase activity in *A. brasilense* Cd transconjugants. When inoculated with 10^8 cfu mL^{-1} *A. brasilense* Cd/pRKLACC, the length of seedling roots increased significantly compared to seeds inoculated with the same density of nontransformed cells. This suggests that *A. brasilense* Cd possesses inherent mechanisms for plant growth promotion, presumably related to the synthesis of IAA, and that the acquisition of ACC deaminase activity, and hence the ability to break down ACC, further enhances its ability to promote root elongation.

In minimal medium, the presence of pRKLACC in *A. brasilense* Cd can reduce the growth rate and IAA synthesis of the bacterium by approximately 25%. However, in nutrient broth, *A. brasilense* Cd/pRKLACC cells showed the same ability to produce IAA as the nontransformed bacterium. This result is consistent with the possibility that synthesis of ACC deaminase imposes a metabolic load on *A. brasilense* Cd only when nutrients are scarce, and energy sources are limited [10]. To infer that pRKLACC interferes with the ability of *A. brasilense* Cd to synthesize IAA when associated with the root system would be premature, considering that several pathways for IAA synthesis are found in *Azospirillum brasilense,* and that scant data is available regarding the regulation of IAA synthesis in this bacterium [28, 29, 34,].

Wheat plants inoculated with *A. brasilense* Cd/pRKLACC responded similarly to plants inoculated with nontransformed bacterial cells. This probably reflects the lower sensitivity of wheat to ethylene [19]. However, these results do not necessarily imply that wheat plants grown under different conditions or wheat plants in different growth stages will not respond to inoculation with *A. brasilense* Cd/pRKLACC.

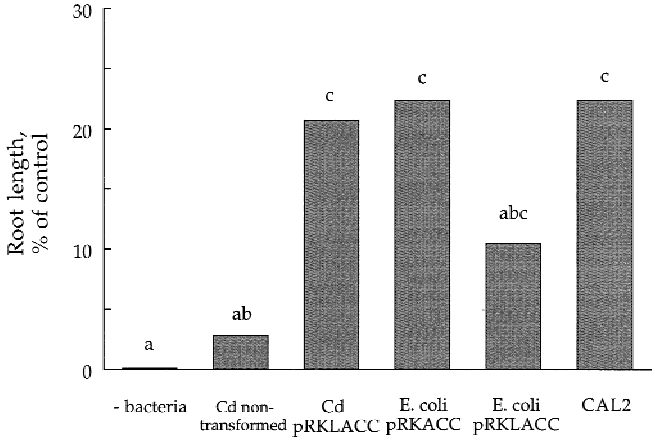

Fig. 4. Root length of canola seedlings, nontreated or inoculated with *A. brasilense* Cd,*A. brasilense* Cd/pRKLACC, *Escherichia coli* DH5α/pRKACC, *Escherichia coli*DH5α/pRKLACC, or *Enterobacter cloacae* CAL2. The inoculum concentration of all strains was 10^7 cfu/mL. The values on the *y* axis represent the percentage of increase from the control, which was considered as 0%. Columns marked with different letters differ significantly at $P \leq 0.05$ in a one-way ANOVA. $N = 80$ for each treatment.

Interestingly, inoculation of wheat plants with *A. brasilense* Cd/pRKLACC at a concentration of 10^7 cfu mL^{-1} promoted root dry weight, whereas inoculation of wheat with nontransformed *A. brasilense* Cd at 10^8 cfu mL^{-1} inhibited root dry weight. Reports of wheat growth inhibition by high

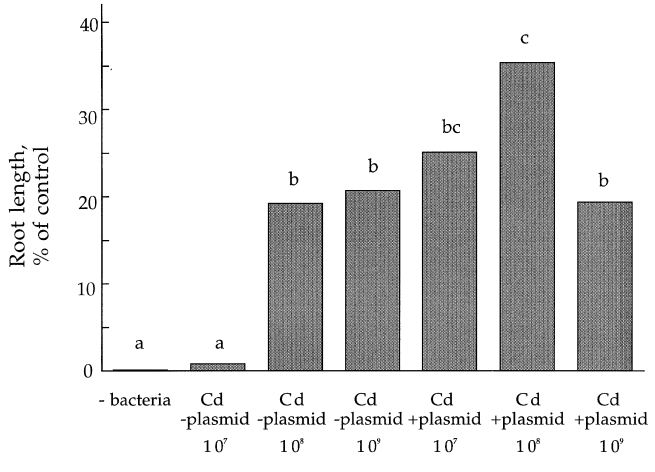

Fig. 5. Root length of tomato seedlings, nontreated or inoculated with *A. brasilense* Cd or *A. brasilense* Cd/pRKLACC, at different concentrations of inoculum. "Cd - plasmid" stands for nontransformed cells and "Cd + plasmid" stands for cells transformed with pRKLACC. The numbers represent cfu mL^{-1}. The values on the *y*axis represent the percentage of increase from the control, which was considered as 0%. Columns marked with different letters differ significantly at $P \leq 0.05$ in a one-way ANOVA. $N = 80$ for each treatment.

levels of *A. brasilense* Cd inoculum are widespread in the literature (e.g., [4]). In this work, the differences in root dry weight between nontreated plants and plants inoculated with high inoculum levels of *A. brasilense* Cd are not statistically significant. However, inhibition of plant growth by high inoculum levels of *A. brasilense* Cd is usually observed in longer term experiments [3], and it is possible that these differences will be more pronounced at later stages of plant growth.

Different types of perturbations in plants can be environmentally generated and can create stress conditions that make the plants increasingly responsive to ethylene already in the tissue, thereby inducing ripening, senescence, or abscission [1]. Inoculation of plants with plant growth-promoting bacteria that have ACC deaminase activity may ameliorate some of the effects of these stresses on plants. In this regard, the ACC deaminase-containing bacterium *Pseudomonas putida* GR12-2 promotes the development of canola seedlings grown in saline soil or exposed to cold night temperatures [11], and the ACC deaminase-containing bacterium *Kluyvera ascorbata* SUD165 promotes plant growth in the presence of high levels of nickel in the soil by decreasing the level of ethylene produced by the plant [6]. Moreover, *Enterobacter cloacae* UW and CAL2 have been shown to act as a sink for ACC, thereby lowering the ethylene level, and the concomitant loss of yield, in flooded tomato plants (Grichko and Glick, submitted for publication).

In conclusion, the transfer of the ACC deaminase gene into *A. brasilense* Cd gave this bacterium the ability to induce root elongation of canola and tomato seedlings, presumably as a result of the ability of the transformants to break down ACC, a precursor of ethylene. It is envisioned that these transformants will help to ameliorate some of the deleterious effects of a variety of environmental stresses on plants. The ability of *A. brasilense* to produce ACC deaminase, together with its inherent mechanisms to promote plant growth, may render this bacterium very useful in an agricultural setting.

Acknowledgments

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to Bernard R. Glick. Gina Holguin participated in this work thanks to the support of the director of the CIBNOR, Dr. Mario Martinez Garcia, who is supporting her temporary stay at the University of Waterloo. We thank Ms. Cheryl Patten and Dr. Trevor Charles for critically reading the manuscript.

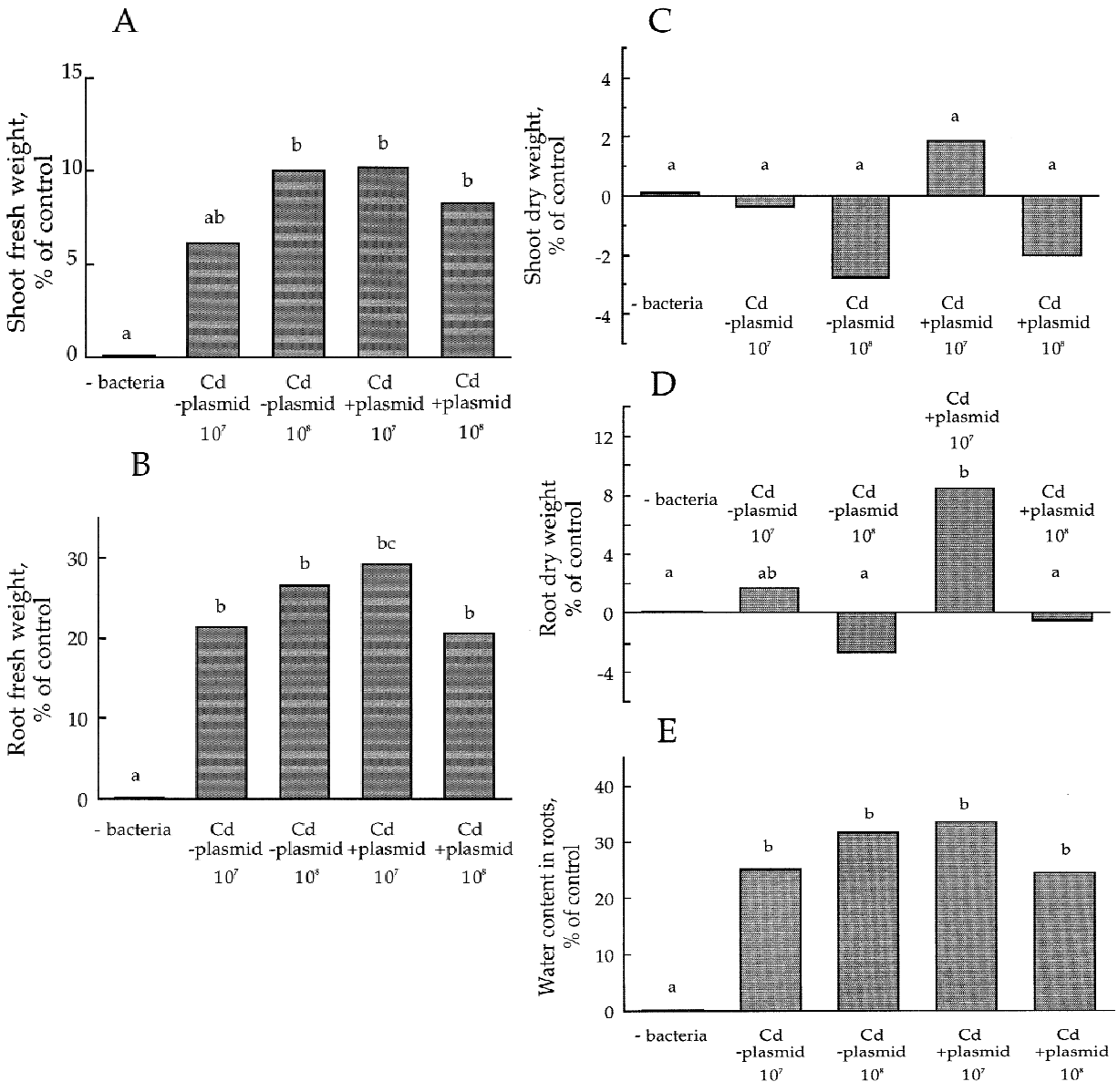

Fig. 6. Shoot and root fresh weight (A and B), dry weight (C and D), and water content (E) of wheat plants inoculated with *A. brasilense* Cd (Cd - plasmid) *A. brasilense*Cd/pRKLACC (Cd + plasmid) at different concentrations of inoculum. The values on the *y* axis represent the percentage of increase from the control, which was considered as 0%. Columns marked with different letters differ significantly at $P \leq 0.05$ in a one-way ANOVA. N = 80 for each treatment.

References

1. Abeles FB, Morgan PW, Saltveit ME Jr. (1992) The biosynthesis of ethylene. Ethylene in Plant Biology, 2nd ed. Academic Press, San Diego, pp 414
2. Baldani VLD, Alvarez de B MA, Baldani JL, Döbereiner J (1986) Establishment of inoculated *Azospirillum* spp in the rhizosphere and in roots of field grown wheat and sorghum. Plant Soil 90:35–46
3. Bashan Y (1986) Significance of timing and level of inoculation with rhizosphere bacteria on wheat plants. Soil Biol Biochem 18:297–301
4. Bashan Y, Levanony H (1990) Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture. Can J Microbiol 36:591–608
5. Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
6. Burd GI, Dixon DG, Glick BR (1998) A plant growth-promoting bacterium that decreases nickel toxicity in seedlings. Appl Environ Microbiol 64:3663–3668
7. Esashi Y (1991) Ethylene and seed germination. In: Matto AK, Suttle JC (eds) The Plant Hormone Ethylene. CRC Press, Boca Raton, FL, pp 133–157

8. Figurski DH, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 76:1648–1652
9. Glick BR (1995a) The enhancement of plant growth by free-living bacteria. Can J Microbiol 41:109–117
10. Glick BR (1995b) Metabolic load and heterologous gene expression. Biotechnol Adv 13:247–261
11. Glick BR, Liu C, Ghosh S, Dumbroff EB (1997) Early development of canola seedlings in the presence of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2. Soil Biol Biochem 29:1233–1239
12. Glick BR, Jacobson CB, Schwarze MMK, Pasternak JJ (1994a) Does the enzyme 1-aminocyclopropane-1carboxylate deaminase play a role in plant growth promotion by *Pseudomonas putida* GR12-2? In: Ryder MH, Stephens PM, Bowen GD (eds) Improving Plant Productivity with Rhizosphere Bacteria. CSIRO, Adelaide, pp 150–152
13. Glick BR, Jacobson CB, Schwarze MMK, Pasternak JJ (1994b) 1-Aminocyclopropane-1-carboxylic acid deaminase mutants of the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2 do not stimulate canola root elongation. Can J Microbiol 40:911–915
14. Glick BR, Karaturovíc DM, Newell PC (1995) A novel procedure for rapid isolation of plant growth-promoting pseudomonads. Can J Microbiol 41:533–536
15. Glick BR, Penrose DM, Li J (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J Theor Biol 190:63–68
16. Glick BR, Patten CL, Holguin G, Penrose DM (1999) Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria Imperial College Press, London, pp
17. Gordon SA, Weber RP (1951) Colorimetric estimation of indoleacetic acid. Plant Physiol 26:192–195
18. Hadas R, Okon Y (1987) Effect of *Azospirillum brasilense* inoculation on root morphology and respiration in tomato seedlings. Biol Fertil Soils 5:241–247
19. Hall JA, Peirson DG, Ghosh S, Glick BR (1996) Root elongation in various agronomic crops by the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. Isr J Plant Sci 44:37–42
20. Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557–580
21. Honma M, Shimomura T (1978) Metabolism of 1-aminocyclopropane-1-carboxylic acid. Agric Biol Chem 42:1825–1831
22. Jackson MB (1991) Ethylene in root growth and development. In: Matoo AK, Suttle JC (eds) The Plant Hormone Ethylene. CRC Press, Boca Raton, FL, pp 159–181
23. Keen NT, Tamaki S, Kobayashi D, Trollinger D (1988) Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene 70:191–197
24. Kende H (1993) Ethylene biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 44:283–307
25. Lifshitz R, Kloepper JW, Kozlowski M, Simonson C, Carlson J, Tipping EM, Zaleska I (1987) Growth promotion of canola (rapeseed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. Can J Microbiol 33:390–395
26. Miller JH (1972) Experiments in Molecular Genetics Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp
27. Okon Y, Albrecht SL, Burris RH (1977) Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Appl Environ Microbiol 33:85–88
28. Patten CL, Glick BR (1996) Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol 42:207–220
29. Prinsen E, Costacurta A, Michiels K, Vanderleyden J, Van Onckelen H (1993) *Azospirillum brasilense* indole-3-acetic acid biosynthesis: evidence for a non-tryptophan dependent pathway. Molec Plant Microbe Interact 6:609–615
30. Puente ME, Bashan Y (1993) Effect of inoculation with *Azospirillum brasilense* strains on the germination and seedlings growth of the giant columnar cardon cactus (*Pachycereus pringlei*) Symbiosis 15:49–60
31. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp
32. Shah S, Li J, Moffatt BA, Glick BR (1998) Isolation and characterization of ACC deaminase genes from two different plant growth-promoting rhizobacteria. Can J Microbiol 44:833–843
33. Tarrand JJ, Krieg NR, Döbereiner J (1978) A taxonomic study of the *Spirillum lipoferum* group, with description of a new genus, *Azospirillum* gen nov, and two species, *Azospirillum lipoferum* (Beijerinck) comb nov and *Azospirillum brasilense* sp nov. Can J Microbiol 24:967–980
34. Vande Broek A, Lambrech M, Eggermont K, Vanderleyden J (1999) Auxins upregulate expression of the indole-3-pyruvate decarboxylase gene in *Azospirillum brasilense.* J Bacteriol 181:1338–1342