Applied and Environmental Microbiology, Oct. 2009, p. 6581–6590 Vol. 75, No. 20
0099-2240/09/$08.00+0 doi:10.1128/AEM.01240-09

ACC (1-Aminocyclopropane-1-Carboxylate) Deaminase Activity, a Widespread Trait in *Burkholderia* Species, and Its Growth-Promoting Effect on Tomato Plants[▽]

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Received 30 May 2009/Accepted 13 August 2009

The genus *Burkholderia* includes pathogens of plants and animals and some human opportunistic pathogens, such as the *Burkholderia cepacia* complex (Bcc), but most species are nonpathogenic, plant associated, and rhizospheric or endophytic. Since rhizobacteria expressing ACC (1-aminocyclopropane-1-carboxylate) deaminase may enhance plant growth by lowering plant ethylene levels, in this work we investigated the presence of ACC deaminase activity and the *acdS* gene in 45 strains, most of which are plant associated, representing 20 well-known *Burkholderia* species. The results demonstrated that ACC deaminase activity is a widespread feature in the genus *Burkholderia*, since 18 species exhibited ACC deaminase activities in the range from 2 to 15 μmol of α-ketobutyrate/h/mg protein, which suggests that these species may be able to modulate ethylene levels and enhance plant growth. In these 18 *Burkholderia* species the *acdS* gene sequences were highly conserved (76 to 99% identity). Phylogenetic analysis of *acdS* gene sequences in *Burkholderia* showed tight clustering of the Bcc species, which were clearly distinct from diazotrophic plant-associated *Burkholderia* species. In addition, an *acdS* knockout mutant of the N_2-fixing bacterium *Burkholderia unamae* MTl-641^T and a transcriptional *acdSp-gusA* fusion constructed in this strain showed that ACC deaminase could play an important role in promotion of the growth of tomato plants. The widespread ACC deaminase activity in *Burkholderia* species and the common association of these species with plants suggest that this genus could be a major contributor to plant growth under natural conditions.

Burkholderia is a common genus in the bacterial communities present in agricultural and polluted soils (24, 56, 63) and includes over 40 properly described species (18). While some of these species are opportunistic pathogens of humans (for example, species in the *Burkholderia cepacia* complex [Bcc]) and others are phytopathogenic (17), most species have neutral or beneficial interactions with plants (18). For a long time, the ability of bacteria belonging to the genus *Burkholderia* to fix N_2 was found only in the species *B. vietnamiensis* (36), a member of the Bcc (17). At present, several diazotrophic plant-associated *Burkholderia* species have been validly described, including *B. unamae* (6), *B. xenovorans* (41), *B. tropica* (62), and *B. silvatlantica* (60), all of which are able to colonize the rhizosphere and/or the endophytic environment of a wide range of host plants (6, 7, 36, 59, 60, 62). In addition, legume-nodulating N_2-fixing strains have formally been classified as novel species, including *B. phymatum*, *B. tuberum* (68), *B. mimosarum*, and *B. nodosa* (12, 13). It is worth noting that most of the plant-associated *Burkholderia* species are phylogenetically distant from the Bcc species and exhibit potential activities of interest in agrobiotechnology (7). Among the plant-associated *Burkholderia*, *B. unamae* has relevant features, such as colonization of the rhizosphere and internal tissues of taxonomically unrelated host plants, including maize, coffee, sugarcane, and tomato, has a wide distribution in different geographical regions, and exhibits several potential activities involved in plant growth promotion, bioremediation, or biological control (6, 7, 59).

The bacterial enzyme ACC (1-aminocyclopropane-1-carboxylate) deaminase promotes plant growth by lowering plant ethylene levels (37, 39). This enzyme catalyzes the conversion of ACC, the immediate precursor of ethylene synthesis in plants, to ammonia and α-ketobutyrate (α-KB). ACC is exuded from seeds or plant roots and then metabolized by bacteria expressing ACC deaminase activity, which stimulates plant ACC efflux, decreasing the root ACC concentration and root ethylene evolution and increasing root growth (39). Moreover, it has been reported that some ACC deaminase-producing bacteria promote plant growth under a variety of stressful conditions, such as flooding (42), saline conditions (54), and drought (55). In addition, this enzyme has been implicated in enhancing nodulation in pea (51) and alfalfa (50) plants.

Growth on a minimal medium with ACC as the sole N source is indicative of ACC deaminase-containing bacteria (38). Based on this criterion, it has been postulated that soil bacteria capable of degrading ACC are relatively common (2, 38).

The *acdS* gene, encoding ACC deaminase, has been isolated from different species and strains of genera belonging to the *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*, as well as the *Firmicutes* and *Actinobacteria* (3, 25, 28, 44, 64). However, the identification of most *acdS* genes has been based only on partial sequences, and some of the strains analyzed have been poorly characterized taxonomically. Moreover, it is notewor-

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▽ Published ahead of print on 21 August 2009.

TABLE 1. Sources of *Burkholderia* species and strains analyzed and ACC deaminase activities

Species or strain	Relevant characteristics of species	Reference(s)	ACC deaminase activity (μmol α-KB/h/mg protein)[a]
B. unamae[b]	Rhizospheric; endophyte; N_2 fixer	6	3.78 ± 0.66[h]
B. xenovorans[c]	Rhizospheric; biphenyl degrader; N_2 fixer	41	8.62 ± 2.26[h]
B. tropica[d]	Rhizospheric; endophyte; N_2 fixer	62	ND
B. silvatlantica[e]	Rhizospheric; endophyte; N_2 fixer	60	12.40 ± 2.34[h]
B. phymatum STM815^T	Legume nodulating	68	3.55 ± 0.78
B. tuberum STM678^T	Legume nodulating	68	4.63 ± 0.59
B. vietnamiensis[f]	Rhizospheric; endophyte; opportunistic human pathogen; N_2 fixer	32, 36	5.10 ± 1.62[h]
B. caribensis MWAP64^T	Soil; exopolysaccharide producer	1	3.91 ± 1.03
B. phenoliruptrix LMG 22037^T	Soil; 2,4,5-trichlorophenoxyacetic acid degrader	14	2.13 ± 0.71
B. fungorum LMG 16225^T	Fungus; aromatic compound degrader	15	2.46 ± 0.13
B. graminis C4D1 M^T	Maize roots; environmental grasses	70	10.19 ± 0.51
B. caledonica LMG 19076^T	Rhizosphere soil	15	12.11 ± 3.25
B. terricola LMG 20594^T	Agricultural soil	40	12.59 ± 0.46
B. phytofirmans PsJN^T	Endophyte; plant growth-promoting rhizobacterium	65	12.03 ± 0.43
B. stabilis LMG 14294^T	Opportunistic human pathogen; rhizospheric	18, 69	11.85 ± 1.58
B. cenocepacia J2315^T	Rhizospheric; opportunistic human pathogen (cystic fibrosis)	17, 24	2.63 ± 0.23
B. cepacia ATCC 25416^T	Rhizospheric; opportunistic human pathogen (cystic fibrosis)	5, 18	4.85 ± 0.41
B. caryophylli LMG 2155^T	Plant pathogen (carnation)	74	5.53 ± 1.05
B. ambifaria 6991	Soil, maize roots; opportunistic human pathogen (cystic fibrosis)	16	ND
B. kururiensis[g]	Trichloroethylene degrader; endophyte; N_2 fixer	7, 32, 75	5.17 ± 0.31

[a] The data are the means ± standard deviations of three independent assays performed in duplicate. ND, not detected.
[b] The strains analyzed were *B. unamae* MTl-641^T, SCCu-23, CGC-72, SCZa-211, M2Cy-71, CAC-98, SCZa-39, CAC-382, CGC-321, and MEd-9573 (6).
[c] The strains analyzed were *B. xenovorans* LB400^T, CAC-124, and LMG16224 (41).
[d] The strains analyzed were *B. tropica* Ppe8^T, MTo-293, MOc-725, MXo-435 (62), BM16. and BM273 (31).
[e] The strains analyzed were *B. silvatlantica* SRMrh-20^T, AB48, and PPCR-2 (60).
[f] The strains analyzed were *B. vietnamiensis* TVV75^T, TVV69, MMi-324, CCE-101, SXo-702, and MMi-344 (32, 36).
[g] The strains analyzed were *B. kururiensis* KP23^T and M130 (7, 32, 75).
[h] Average of the activities obtained for the strains analyzed.

thy that the beneficial effect of ACC deaminase on plant growth has usually been tested using wild-type isolates with ACC deaminase activity; only a few studies have used ACC deaminase-negative mutants (49, 51, 67).

Although the presence and activity of ACC deaminase in few nonpathogenic *Burkholderia* strains (65, 7) and in some Bcc strains (3) have been analyzed, nothing is known about the expression of this enzyme in association with plants. Similarly, the effect of ACC deaminase-negative mutants of plant-associated *Burkholderia* strains on plant growth is unknown.

In this work, the ACC deaminase activities and *acdS* gene sequences of most of the novel rhizospheric, endophytic diazotrophic, and legume-nodulating *Burkholderia* species, as well as of non-N_2-fixing *Burkholderia* strains (mainly plant associated), were analyzed. In addition, we analyzed the effect of an *acdS* knockout mutant of *B. unamae* and the expression of the ACC deaminase gene by use of a *gusA* (*uidA*) reporter gene fusion on tomato plants grown under different conditions. The colonization patterns of a constitutive *gusA*-marked strain in tomato roots were examined as well.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and mating. Bacterial strains and plasmids used in this work are listed in Table 1 and Table 2, respectively. Since most of the *Burkholderia* species listed in Table 1 were proposed on the basis of one to three isolates analyzed (59), in this work only the type strain of the majority of the species was examined. In addition to type strains, several previously characterized strains of *B. unamae* (6), *B. xenovorans* (41), *B. tropica* (62), *B. silvatlantica* (60), *B. vietnamiensis* (32, 36), and *B. kururiensis* (7) were included in the analysis (Table 1). For phenotypic identification of the *acdS* gene, *Burkholderia* isolates were grown on salts medium (SM) plates (7) supplemented with 3.0 mM ACC; the plates were incubated for 5 to 6 days at 29°C. *Burkholderia* strains able to grow on ACC as a sole nitrogen source were analyzed for the presence of the *acdS* gene. For triparental mating, *B. unamae* strains, *Escherichia coli* DH5α (donor), and *E. coli* M607 (with helper plasmid pRK600) were cultured in Luria-Bertani medium, and antibiotics were added as required at the following concentrations: kanamycin, 50 μg/ml; tetracycline, 15 μg/ml; gentamicin, 30 μg/ml; and chloramphenicol, 20 μg/ml. These experiments were performed as described previously (11). Growth kinetics for wild-type and derivative strains were determined at least three times. Strains were grown in liquid BSE medium or SM as described previously (7). Cells were collected and washed, and the initial optical density at 600 nm (OD_{600}) was adjusted to 0.02. The cultures were incubated at 29°C with reciprocal shaking (250 rpm), and the growth over time was determined by quantifying the number of cells.

ACC deaminase activity assay. Cultures of *Burkholderia* strains used for ACC deaminase assays were grown as described previously (7). ACC deaminase activity was measured by measuring the production of α-KB as described by Honma and Shimomura (43). The protein concentration in cell extracts was determined by the Bradford method (4).

PCR amplification and sequencing of *acdS* genes in *Burkholderia*. For PCR amplification of partial *acdS* genes, primers 5′ACC and 3′ACC were used, as described previously (7). To obtain the complete *acdS* gene sequences, the following two sets of degenerate primers were designed: primers F-*acdS* (5′ATGAAYCTSCARCGHTTY3′) and R-*acdS* (5′TYARCCGTYSCGRAARRT3′); and primers NF-*acdS* (5′ATGAAYCTSCARMRHTTYC3′) and NR-*acdS* (5′TYARCCGTYGCGRAARATV3′). PCR assays were performed using 50-μl reaction mixtures with PFX polymerase (Invitrogen) under the following conditions: initial denaturation for 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C and then a final 5-min elongation at 72°C. The amplified products were cloned into the vector pCR 2.1 (Invitrogen), and the *acdS* gene sequences were determined at the Biotechnology Institute, UNAM (Mexico). Multiple-sequence alignments of *acdS* sequences were constructed with CLUSTALW2 software (48). The tree topology was inferred by the neighbor-joining method, and a distance matrix analysis was performed as described by Jukes and Cantor using the program MEGA, version 3.1 (47).

Isolation of a genomic region harboring the regulatory region and the *acdS* structural gene of *B. unamae* MTl-641^T. A 785-bp segment containing the central region of the *acdS* gene was amplified by PCR (using primers 5′ACC and 3′ACC) and cloned into the Km^r suicide vector pUX19 (71). The resulting

TABLE 2. Strains and plasmids used in this work

Strain/genotype or plasmid	Relevant characteristics[a]	Reference or source
Strains		
MTl-641^T	*B. unamae* wild-type strain	6
acdS::ΩKm	MTl-641^T ACC deaminase-negative mutant, Km^r	This study
acdS::ΩKm(pF*acdS*$^+$)	MTl-641^T ACC deaminase-negative mutant complemented with wild-type *acdS* gene, Km^r Tc^r	This study
acdSp-gusA	MTl-641^T containing pF*acdSp-gusA*, GUS tagged	This study
s7p-gusA	MTl-641^T containing pF*s7p-gusA,* GUS tagged	This study
gusA negative	MTl-641^T containing pF*gusA*$^-$, GUS tagged	This study
E. coli DH5α	*recA1* ϕ80d*lac*ZΔM15 *gyrA96*	Gibco BRL
Plasmids		
pRK600	Helper plasmid, Mob Cm^r	35
pCR2.1	TA cloning vector for PCR products, Amp^r Km^r	Invitrogen
pUX19	Suicide vector, Km^r	71
pJQ200mp18	Suicide vector for gene replacement, *sacB* Mob Gm^r	61
pHP45Ω-Km	ΩKm^r vector, Ap^r	34
pVO155	Suicide vector containing the *uidA* gene, Km^r	57
pACYC184	Cloning vector, Cm^r Tc^r	10
pUX-UD	pUX19 derivative containing genome sequences flanking the *acdS* gene from strain MTl-641^T, Km^r	This study
pUX19*acdS*	pUX19 derivative containing a partial *acdS* gene sequence (785 bp) of *B. unamae* MTl-641^T, Km^r	This study
pACY*lrpL-acdS*	pACYC184 derivative containing a 2.2-kb region with the *lrpL* and *acdS* genes from MTl-641^T, Cm^r Tc^r	This study
pACY*acdS*::ΩKm	pACYC184 derivative containing the *acdS* gene interrupted by a kanamycin resistance cassette, Cm^r Tc^r Km^r	This study
pJQ*acdS*::ΩKm	pJQ200mp18 derivative containing the *acdS* gene interrupted by a kanamycin resistance cassette Gm^r Km^r	This study
pACY*lrpL-acdSp-gusA*	pACYC184*lrpL-acdS* derivative containing the *gusA* gene cloned in the coding region of *acdS* gene, Cm^r Tc^r Km^r	This study
pFAJ1700	RK2 broad-host-range vector, Tc^r	27
pF*acdSp-gusA*	pFAJ1700 derivative containing the upstream *acdS* regulatory sequence fused to the *uidA* gene, Tc^r	This study
pF*gusA*$^-$	pFAJ1700 derivative containing promoterless *gusA* gene, Tc^r	This study
pF*acdS*$^+$	pFAJ1700 derivative containing the structural and regulatory region of the *acdS* gene, Tc^r	This study
pF*s7p-gusA*	pFAJ1700 derivative containing a constitutive promoter fused to the *gusA* gene, Tc^r	This study

[a] GUS, β-glucuronidase.

plasmid (pUX19*acdS*) was transferred by triparental mating into *B. unamae* MTl-641^T, and recombinant strains were selected in the presence of kanamycin. The total DNA of a single-recombinant strain was digested with restriction enzyme BclI (Invitrogen); the BclI restriction site was absent in the pUX*acdS* vector, which allowed incorporation of sequences upstream and downstream of the *acdS* gene into the vector. The digested DNA was religated and transformed into *E. coli* DH5α. Ten Km^r colonies were purified, and the clones were digested with BclI. One of these clones (pUX-UD) was sequenced by primer walking to verify that sequences surrounding the *acdS* gene were cloned. In silico analysis of the resulting sequence was performed with the programs FGENESB, BPROM (http://linux1.softberry.com/berry.phtml), and virtual footprint promoter analysis version 3.0 (http://www.prodoric.de/vfp/vfp_promoter.php).

Construction of *B. unamae* MTl-641^T *acdS* mutant and complementation experiments. A DNA fragment containing the structural and regulatory region of the *acdS* gene was amplified by PCR using primers *Xba*-404 (5′GCTCTAGAC CAGGCCACACCATCATC-3′) and *Xba*-2631 (5′GCTCTAGAAGTTACCAG TTGCCAGTT3′); the 2,261-bp PCR product was cloned in pACYC184, and the *acdS* gene was then disrupted with the Km^r gene of pHP45Ω-Km (34). The interrupted gene was cloned in the suicide vector pJQ200mp18 (61). The suicide plasmid harboring the interrupted gene was introduced into strain MTl-641^T, and double crossover, Km^r sucrose-resistant clones were selected. The gene replacement was confirmed by Southern blot hybridization. To complement the *acdS* mutant, an 1,864-bp fragment harboring the complete *acdS* gene and its 5′ regulatory sequence was cloned in the broad-host-range vector pFAJ1700 (27), and the resulting plasmid (pF*acdS*$^+$) was used for complementation experiments.

Construction of transcriptional *gusA* reporter fusions. To determine bacterial *acdS* gene expression in plants, a transcriptional fusion was constructed. A 653-bp fragment from MTl-641^T containing the upstream *acdS* regulatory region, as well as 150 bp of the *acdS* coding sequence, was fused to *gusA* as follows. The *gusA* gene from pVO155 (57) was inserted 102 nucleotides downstream of the start codon of the *acdS* gene to obtain a transcriptional fusion, which was subsequently cloned into pFAJ1700 (pF*acdSp-gusA*) and introduced into strain MTl-641^T (*acdSp-gusA*). To obtain a constitutive *gusA*-marked strain, the promoter region (839 bp) of the *s7* ribosomal gene from *B. xenovorans* LB400^T was cloned into the promoterless pF*gusA* vector, resulting in pF*s7p-gusA*. To generate a negative control for *gusA* expression, the *gusA* gene was cloned into the promoterless vector pFAJ1700, resulting in the pF*gusA*$^-$ plasmid. Fusions were introduced by conjugation into *B. unamae* MTl-641^T, and the strains were designated the *s7p-gusA*, *acdSp-gusA*, and *gusA* strains (Table 2). Transcriptional gene fusions were sequenced to verify the correct DNA sequences.

Plasmid stability. Stationary-phase cultures of *B. unamae* were diluted to obtain an OD_{600} of 0.02 in 6 ml of fresh BSE liquid medium without antibiotics and cultivated for 8 h, and 100-μl aliquots of these cultures were inoculated into fresh BSE liquid medium and incubated for 24 h; this procedure was repeated once, but the culture was incubated for 48 h, and then samples were diluted and plated on BAc agar (32) without antibiotics. Two hundred colonies were picked onto plates with tetracycline or without an antibiotic, and the pFAJ1700 stability frequency in *B. unamae* derivatives was based on the total number of recovered colonies on medium without an antibiotic compared to the number of colonies resistant to tetracycline.

β-Glucuronidase activity measurement. The *Burkholderia acdSp-gusA* and *gusA* strains were grown for 18 h at 29°C in BSE liquid medium with reciprocal shaking (200 rpm); the initial OD_{600} of the cultures were adjusted to 0.02 (approximately 6.30 log CFU/ml) in SM broth supplemented with ACC (100 nM to 3 mM) or NH_4Cl (3 mM) as a single nitrogen source, and the cultures were

TABLE 3. Effect of *B. unamae* wild-type strain MTl-641^T and an ACC deaminase-negative mutant (*acdS*::ΩKm) on tomato plants grown under different conditions[a]

Treatment	Inoculated strain	Shoot dry wt (mg)	Root dry wt (mg)	Chlorophyll concn (mg/g [fresh wt])	Log CFU/g root (fresh wt)
Control	Wild type	88 ± 7.8 a	48 ± 4.4 a	0.55 ± 0.01 a	6.81 ± 0.13 a
	acdS::ΩKm mutant	61 ± 8.1 b	38 ± 4.1 b	0.56 ± 0.06 a	6.43 ± 0.51 a
	Not inoculated	65 ± 7.5 b	40 ± 4.7 b	0.35 ± 0.05 b	ND
100 mM NaCl	Wild type	77 ± 9.3 a	49 ± 2.5 a	0.48 ± 0.05 a	6.51 ± 0.45 a
	acdS::ΩKm mutant	61 ± 10.8 b	41 ± 1.7 b	0.49 ± 0.03 a	6.41 ± 0.39 a
	Not inoculated	60 ± 2.6 b	40 ± 3.5 b	0.35 ± 0.02 b	ND
Water saturation	Wild type	70 ± 7.3 a	50 ± 5.0 a	0.57 ± 0.03 a	6.88 ± 0.13 a
	acdS::ΩKm mutant	53 ± 7.0 b	35 ± 6.2 b	0.55 ± 0.009 a	6.90 ± 0.05 a
	Not inoculated	57 ± 3.6 b	37 ± 6.0 b	0.41 ± 0.05 b	

[a] The shoot and root dry weight data are the averages ± standard deviations of five pot replicates with three seedlings per pot for each treatment. The chlorophyll content data are the averages ± standard deviations of three samples, which were obtained from different plants and independently processed. The CFU data are the averages ± standard deviations of three replicates, where two seedlings served as a replicate, for each treatment. Plant data followed by the same letter in a column for each treatment do not differ significantly at $P \leq 0.05$, as determined using one-way analysis of variance, followed by Tukey's analysis. CFU values followed by the same letter for each treatment do not differ significantly at $P \leq 0.05$, as determined using Student's *t* test. Statistical differences ($P \leq 0.05$) among control plants without stress and plants grown in the presence of NaCl or water saturation treatments were not found using one-way analysis of variance, followed by Tukey's analysis. ND, not detected.

grown for 6, 18, and 24 h at 29°C. The cultures were centrifuged and resuspended in a salt wash solution supplemented with 100 μg/ml chloramphenicol. Quantitative β-glucuronidase assays were performed with the *p*-nitrophenyl glucuronide substrate as described previously (73). The protein concentration was determined by the Bradford method (4). Three independent experiments were performed in duplicate.

Inoculation of tomato plants with *B. unamae* wild-type and mutant strains. *B. unamae* MTl-641^T and derivative strains were grown in BSE liquid medium (supplemented with tetracycline or kanamycin when necessary) and incubated at 29°C for 18 h. The cells were collected and resuspended in sterile 10 mM $MgSO_4 \cdot 7H_2O$, and the concentration was adjusted to 7 log CFU/ml. Germinated seeds were sown after immersion in the bacterial suspension for 1 h.

Tomato gnotobiotic assay. Seeds of tomato variety saladet were surface disinfected by soaking them in 1.5% sodium hypochlorite for 15 min, thoroughly rinsed in sterile distilled water, and germinated on agar plates. Germinated seeds were soaked for 1 h in a 10 mM $MgSO_4 \cdot 7H_2O$ solution (noninoculated) or in a bacterial suspension (wild-type strain or the *acdS*::ΩKm mutant). After inoculation, three germinated seeds were aseptically transferred to 500-ml plastic pots containing sterilized river sand (approximately 1 kg) moistened with 140 ml Fahraeus solution (33) supplemented with NH_4Cl (7.5 mg N/pot), and 1 ml of a bacterial suspension (7 log CFU/ml) was added to each seed. The plants were maintained under greenhouse conditions, and 80 ml of sterile water was applied every 3 days. After 25 days of growth, the plants were treated either with 80 ml of a sterile saline solution (100 mM NaCl) every 3 days or with 80 ml of sterile water (water saturation) daily; 80 ml of sterile water applied every 3 days was used for the control treatment. The plants were harvested 15 days after each treatment, and the root length, chlorophyll content, and dry weight were analyzed. The chlorophyll content was determined as described by Wellburn (72).

Root elongation assay. Tomato seeds were surface disinfected as described above and transferred to petri dishes containing filter paper moistened with 5 ml of a Farheus-bacterium suspension containing wild-type, *acdS*::ΩKm, or *acdS*::ΩKm(p*acdS*$^+$) bacteria or with Farheus solution without bacteria. After 5 days of incubation in the dark at 29°C, root lengths were measured.

Histochemical localization of β-glucuronidase activity. The expression of the *gusA* reporter gene was monitored in plants inoculated with *gusA*-marked strains (the *acdSp-gusA*, *s7p-gusA*, and *gusA* strains). One assay was carried out as described above for the root elongation test, but β-glucuronidase activity was analyzed instead of root length. In addition, inoculated germinated tomato seeds were sown aseptically in assay tubes containing sand and Fahraeus solution with NH_4Cl (1.0 mg N/tube). The seedlings were grown in a greenhouse for 3 weeks. Localization of bacterial β-glucuronidase activity was determined using X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) as the substrate, as described by Jefferson et al. (46). After the samples were washed in phosphate buffer, the plant tissues were examined and photographed using a light microscope (Zeiss).

Enumeration of bacteria associated with roots. Enumeration of root-associated bacteria was performed in all of the plant-inoculation assays. Roots of seedlings were weighed and homogenized in a sterile 10 mM $MgSO_4$ solution with a mortar and pestle. Homogenates were serially diluted and plated in selective BAc medium. After incubation for 3 days at 29°C, the number of colonies of each strain was determined. In addition, amplified 16S rRNA gene restriction profiles for five colonies grown on the selective medium inoculated with the highest dilution were determined as previously described (32) in order to confirm the identity of the inoculated strain.

Statistical data analysis. Unless otherwise indicated, plant and bacterial population data were analyzed by one-way analysis of variance, followed by Tukey's analysis, using the Minitab 15 statistical software. Bacterial population data were log transformed before statistical analysis. All analyses were performed using a *P* value of ≤0.05. The numbers of replicates and seedlings or plants sampled in each experiment for statistical data analysis are indicated in Tables 3 and 4.

Nucleotide sequence accession numbers. Twenty-one *acdS* gene sequences (15 complete sequences and 6 partial sequences) have been deposited in the EMBL/GenBank database under accession numbers EU886299 through EU886320. Specific accession numbers of each strain are shown in Fig. 1.

RESULTS

Growth on ACC as a nitrogen source and ACC deaminase activity. Of 45 *Burkholderia* strains belonging to 20 species (Table 1), 38 were able to grow on ACC as a sole nitrogen source. These 38 strains, belonging to 18 species, expressed ACC deaminase activity at levels ranging from 2 to 15 μmol α-KB/h/mg protein (Table 1). All of the *B. tropica* strains and *B. ambifaria* 6991 were not able to grow on ACC, and neither of these species expressed ACC deaminase activity.

TABLE 4. Root lengths in 5-day-old tomato seedlings inoculated with *B. unamae* MTl-641^T and derivatives[a]

Strain inoculated	Root length (cm)	Root length difference (%)	Log CFU/g (fresh wt) root
None	3.97 ± 0.08 a		ND
Wild type	5.32 ± 0.05 c	34	6.49 ± 0.16 a
acdS::ΩKm(p*acdS*$^+$)	5.28 ± 0.02 c	33	6.11 ± 0.17 a
acdS::ΩKm mutant	4.87 ± 0.06 b	23	6.27 ± 0.29 a

[a] Root length assays were performed with five replicates; one petri dish containing 20 seeds served as a replicate. The root lengths are averages ± standard deviations of five replicates. The CFU data are averages ± standard deviations of three replicates, where five seedlings served as a replicate, for each strain. Values followed by the same letter in a column do not differ significantly at $P \leq 0.05$, as determined using one-way analysis of variance, followed by Tukey's analysis. ND, not detected.

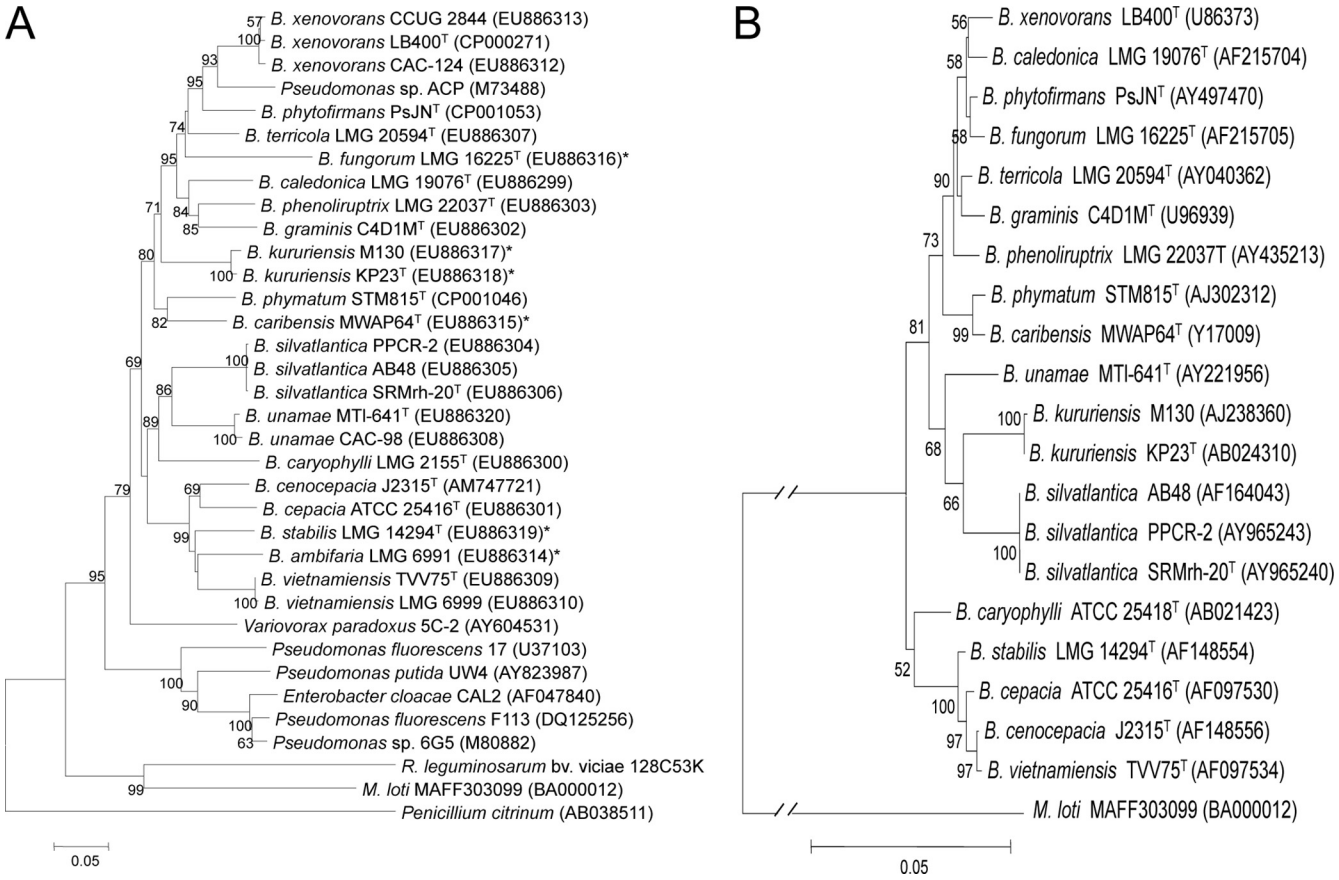

FIG. 1. (A) Phylogenetic tree based on complete *acdS* gene sequences (1,017 bp) of *Burkholderia* species and *acdS* gene partial sequences (785 bp) (indicated by an asterisk). (B) Phylogenetic tree based on 16S rRNA gene sequences (1,310 bp) of *Burkholderia* species. The trees were constructed using the neighbor-joining method. The nodal robustness of the trees was assessed using 1,000 bootstrap replicates. For both phylogenetic trees the bar indicates 5 nucleotide substitutions per 100 nucleotides. The NCBI GenBank accession number for each strain is shown in parentheses.

PCR amplification and sequencing of *acdS* genes in *Burkholderia*. PCR amplification of *acdS* genes encoding ACC deaminase from 25 representative strains analyzed belonging to 18 *Burkholderia* species was performed using three sets of primers. Although ACC deaminase activity was found in *B. tuberum* STM678^T, the *acdS* gene sequence was not obtained with any of the primer sets tested. The sequences of *Burkholderia* species were highly conserved, with levels of identity between 76 and 99% at the nucleotide level. Phylogenetic analysis of the *acdS* gene sequences showed that there were robust clusters containing strains having different origins and from different sources, including plant-associated diazotrophic, non-N_2-fixing species and opportunistic pathogens (Fig. 1). Phylogenetic trees based on *acdS* and 16S rRNA gene sequences of *Burkholderia* species showed similar topologies (Fig. 1).

ACC deaminase gene and regulatory sequences in *B. unamae* MTl-641^T. Analysis of the sequence of 2.2-kb pUX-UD revealed two putative open reading frames, one of which is 1,017 bp long and encodes an ACC deaminase with high levels of identity (69 to 87%) with other complete ACC deaminases (8, 66, 67). The second open reading frame (501 bp), transcribed in the opposite direction from *acdS*, encodes a putative polypeptide that showed 74% identity to Lrp (leucine-responsive regulatory protein) of *Pseudomonas putida* UW4. In silico analysis of the *acdS-lrpL* intergenic region showed that these genes contain putative σ^{70}-dependent promoters and that the transcriptional start sites of *acdS* and *lrpL* were located 75 and 42 bp upstream of the corresponding ATG initiation codon. Two putative Lrp-binding sites were located upstream of *acdS*. L-B1 overlapped the −10 box, and L-B2 was localized 84 bp upstream of the ATG initiation codon (Fig. 2); these sites had 11 and 10 identical nucleotides compared to the consensus sequence (YAGHAWATTWTDCTR) in *E. coli* (22).

Characterization of *B. unamae* MTl-641^T, an *acdS* mutant, and a complemented strain. ACC deaminase activity was not detected in the *acdS* mutant (*acdS*::ΩKm). However, in a liquid rich medium, the growth of the *acdS* mutant and the growth of wild-type strain MTl-641^T were not significantly different ($P \leq 0.05$, Student's *t* test) after 12 h of incubation, reaching concentrations of 8.61 ± 0.02 and 8.74 ± 0.09 log CFU/ml, respectively. In the complemented *acdS*::ΩKm(pF*acdS*$^+$) strain the ACC deaminase activity was restored (4.32 ± 0.73 μmol α-KB/h/mg protein) to a level similar to that in the wild-type strain (4.87 ± 0.34).

Vector stability of *gusA*-marked strains. Plasmid frequency analysis showed that there was 100% stability of pFAJ1700 in

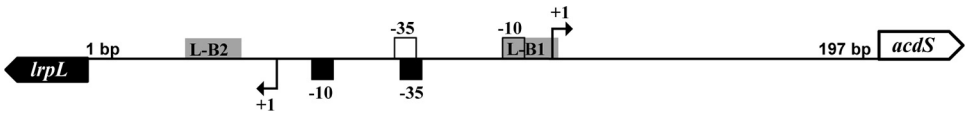

FIG. 2. In silico analysis of the upstream region (197 bp) of the *acdS* gene of *B. unamae* MTl-641^T. The putative *acdS* and *lrpL* −10 and −35 sequences (TACCATN$_{23}$TTGGCA and TACGTTN$_{17}$TTGCCA) are indicated by open and filled boxes, respectively. The transcriptional start sites are indicated by bent arrows. Putative Lrp-binding sites L-B1 (TACCATCTTTTGCAT) and L-B2 (CTGAAAATGTTCGAC) are indicated by gray boxes.

B. unamae without selective pressure; the results were virtually identical for all of the cultures analyzed. The growth patterns of the *acdSp-gusA* strain in liquid SM medium with 3 mM ACC after 48 h of incubation were similar to those of wild-type strain MTl-641^T. While the *acdSp-gusA* strain reached concentrations of 8.25 ± 0.03 log CFU/ml, the wild-type strain concentrations were on the order of 8.18 ± 0.08 log CFU/ml; the concentrations were not statistically different ($P \leq 0.05$, Student's *t* test).

Expression profile of the *acdS* promoter region and enzymatic ACC deaminase activity. Transcriptional fusions were evaluated in the presence of ACC and NH_4Cl. In the presence of ACC, transcriptional *acdS* activity was detected at 6, 18, and 24 h; in contrast, no activity was detected with NH_4Cl (Fig. 3A). These results were in agreement with the ACC deaminase activity of wild-type strain MTl-641^T, since the ACC deaminase did not produce α-KB when the bacterium was grown with 3 mM NH_4Cl as the sole nitrogen source (Fig. 3B). Transcriptional assays with different ACC concentrations (100 nM to 1,000 μM) showed that the maximal *acdS* activity (1,030 ± 107.61 nmol *p*-nitrophenyl/min/mg protein) was obtained with 1,000 μM ACC; 1,000 μM NH_4Cl and ACC concentrations lower than 0.5 μM did not induce *acdS* transcription (Fig. 3C). At the highest concentration (1,000 μM) and at 1 μM ACC, no transcriptional activity was detected in the promoterless *gusA* strain (Fig. 3C).

Inoculation of tomato plants with *B. unamae* wild-type and mutant strains. The *B. unamae* MTl-641^T (wild type) and *acdS*::ΩKm mutant populations on tomato plants grown under different conditions were not significantly different (Table 3). The shoot and root dry weights of tomato plants inoculated with strain MTl-641^T were significantly higher ($P \leq 0.05$) than those of plants inoculated with the *acdS*::ΩKm mutant and of noninoculated plants for all the treatments (Table 3). In addition, the chlorophyll contents of plants inoculated with both strains were statistically increased compared to those of noninoculated plants (Table 3). Moreover, statistical differences ($P \leq 0.05$) were not found among control plants without stress and plants grown either in the presence of NaCl or with water saturation treatments (Table 3).

Effect of *acdS* gene encoding ACC deaminase on tomato root elongation. Root growth was enhanced by all bacterial treatments compared to the growth of noninoculated seeds (Table 4). However, root length was significantly greater with the wild-type and *acdS*::ΩKm(pF*acdS*$^+$) treatments than with the *acdS*::ΩKm mutant treatment, although the bacterial populations associated with the roots from inoculated seedlings were not significantly different (Table 4).

Histochemical localization of β-glucuronidase activity. For the seedlings grown on filter paper and inoculated with the

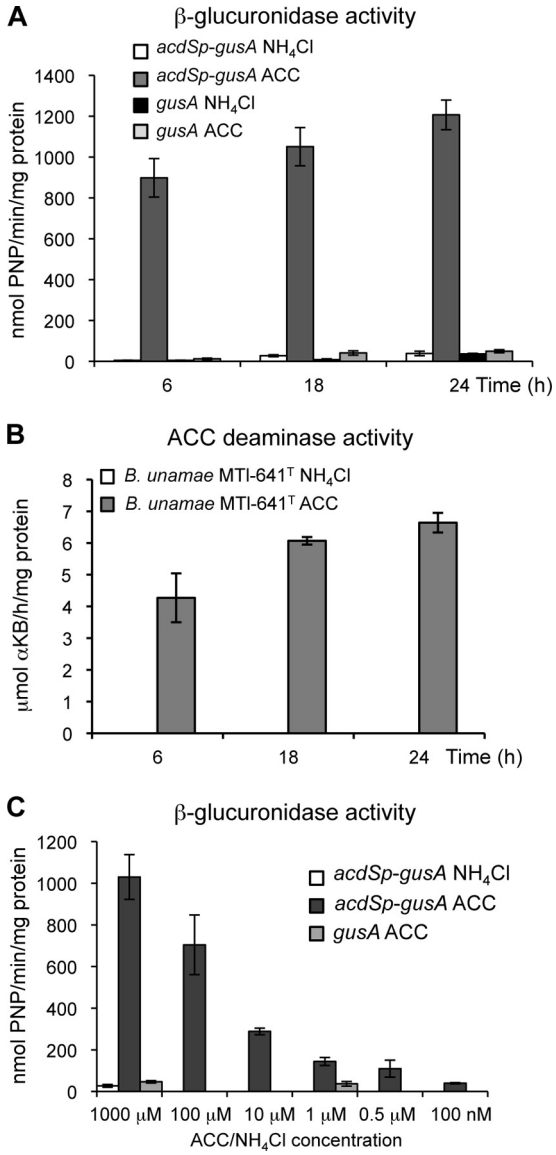

FIG. 3. *acdS* promoter expression and ACC deaminase activity. (A) Transcriptional activities of the *B. unamae acdSp-gusA* and promoterless *gusA*-negative strains. (B) ACC deaminase activities of *B. unamae* strain MTl-641^T. For panels A and B, cultures were grown in SM broth supplemented with 3 mM ACC or 3 mM NH_4Cl as a nitrogen source. (C) Expression profile for the *B. unamae acdSp-gusA* strain grown in SM broth supplemented with ACC at different concentrations or 1 mM NH_4Cl. The promoterless *gusA*-negative strain was tested using 1,000 μM and 1 μM ACC, and no activity was detected. The cultures were grown for 18 h before transcriptional activities were analyzed. PNP, *p*-nitrophenyl. For all of the experiments, the bars indicate the means of three independent assays performed in duplicate.

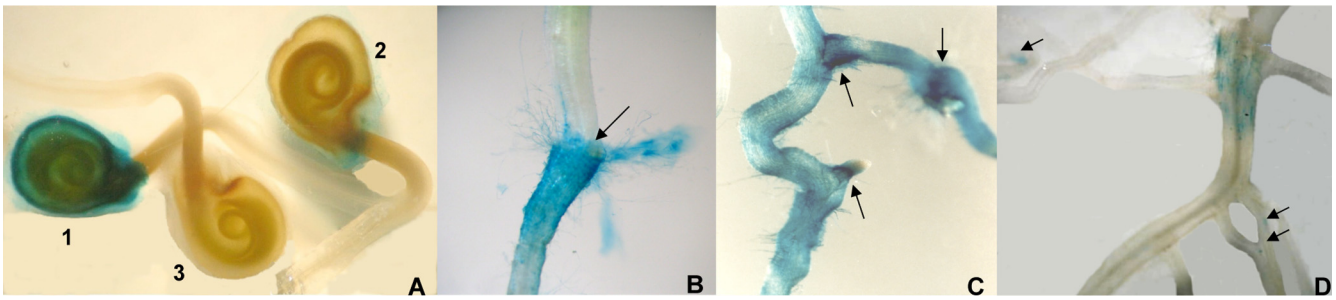

FIG. 4. Histochemical β-glucuronidase assays for localization of bacterial β-glucuronidase activity. (A) Germinated seeds grown on filter paper. Seedling 1, seed inoculated with the *s7p-gusA* strain (constitutive *gusA* strain); seedling 2, seed inoculated with the *acdSp-gusA* strain; seedling 3, seed inoculated with a promoterless *gusA* strain. (B and C) Seedlings grown in sand-filled tubes inoculated with the *s7p-gusA* strain. The arrows indicate the strongest β-glucuronidase activity at the stem base and points of emergence of lateral roots. (D) Inoculation of seedlings with the *acdSp-gusA* strain. The arrows indicate *gusA* expression on a reduced number of root sections.

s7p-gusA strain (constitutive *gusA* strain) there was an intense blue color on the whole surface of germinated tomato seeds and at the base of the radicle (Fig. 4A, seedling 1), while for the seedlings inoculated with the *acdSp-gusA* strain there was a very light blue color on the seed surface and a blue color at the base of radicle that was more intense than the color on the seed surface (Fig. 4A, seedling 2) but less intense than the color observed with the *s7p-gusA* strain. As expected, no blue color was detected on seedlings inoculated with a promoterless *gusA*-marked strain (Fig. 4A, seedling 3). Similar numbers of the three *gusA*-marked strains colonized the seedlings (7.37 ± 0.08, 7.32 ± 0.24, and 7.41 ± 0.25 log CFU/g root for the *acdSp-gusA*, *s7p-gusA*, and *gusA* strains, respectively). The root systems of seedlings grown on sand and inoculated with the *s7p-gusA* strain were well colonized (6.44 ± 0.12 log CFU/g roots), as revealed by intense blue staining of the tissue. The blue staining was most intense at the stem base and where lateral roots emerged (Fig. 4B and C). In contrast, the root systems of seedlings treated with the *acdSp-gusA* strain appeared to have less intense blue staining on fewer root sections (Fig. 4D). However, the root colonization by this strain (6.59 ± 0.14 log CFU/g root) was not statistically different from the root colonization of the plants inoculated with the *s7p-gusA* and *gusA* (6.59 ± 0.21 log CFU/g roots) strains used as positive and negative *gusA* expression controls, respectively. No blue color was detected on seedlings inoculated with the *gusA* strain (data not shown).

DISCUSSION

Considering the limited availability of *Burkholderia* strains, and since the genomic and phenotypic characteristics of strains of a particular bacterial species are the same or highly similar (1, 6, 12), only the type strains of most *Burkholderia* species were analyzed in this work. The results showed that ACC deaminase activity and the *acdS* gene are widely distributed in *Burkholderia* species. Since an activity of 20 nmol α-KB/h/mg protein has been reported (58) to be sufficient to cause plant growth promotion and activities of 2 to 15 μmol α-KB/h/mg protein were detected for the strains tested, it appears that most of the plant-associated *Burkholderia* strains analyzed in this study, either rhizospheric (e.g., *B. xenovorans*, *B. phenoliruptrix*, and *B. graminis*) or endophytic (*B. unamae*, *B. silvatlantica*, and *B. kururiensis*), could be considered potential plant growth-promoting bacteria. Similarly, the legume-nodulating species *B. phymatum* and *B. tuberum* could contribute to enhanced nodulation through ACC deaminase activity, as observed in pea plants inoculated with *Rhizobium leguminosarum* bv. viciae (51) and in alfalfa plants inoculated with a *Sinorhizobium meliloti* strain expressing an introduced *acdS* gene (50). Homologs of *acdS* genes have been found in whole-genome sequences of *B. xenovorans* LB400^T (accession no. CP000271), *B. phymatum* STM815^T (accession no. CP001046), and *B. cenocepacia* J2315^T (accession no. AM747721), and their ACC deaminase activities were demonstrated in this work.

Interestingly, the topology of the *acdS* phylogenetic tree was fairly congruent with that of the bacterial 16S rRNA tree. Phylogenetic trees based on 16S rRNA and *acdS* sequences show separation of the genus *Burkholderia* into two major clusters, one of which is represented mainly by human-pathogenic species, including the opportunistic Bcc species (17). The second major cluster, phylogenetically distant from the Bcc, is formed solely by environmental nonpathogenic species, including mainly plant-associated, rhizospheric and/or endophytic bacteria, many diazotrophic organisms (53), and/or legume-nodulating species (13, 30, 68). Phylogenetic analysis indicated that *acdS* gene sequences of *Burkholderia* strains were highly conserved, even though the strains were isolated from different sources and from distant geographic regions. It is worth noting that there is tight clustering of the Bcc species analyzed in this work; their *acdS* gene sequences were clearly distinct from those of the plant-associated *Burkholderia* species, even *B. unamae* and *B. silvatlantica*. Although the cluster formed by *Burkholderia* species (*Betaproteobacteria*) was well separated from clusters containing a variety of *Alphaproteobacteria* (*R. leguminosarum* bv. viciae and *Mesorhizobium loti*) and *Gammaproteobacteria* (*Pseudomonas* spp. and *Enterobacter cloacae*), *Pseudomonas* sp. strain ACP (accession no. M73488) formed a cluster with *B. xenovorans* strains, and the *acdS* sequences of these strains were closely related to those of *B. phytofirmans* and *B. terricola*. Analysis based on nucleotide and protein sequences revealed high levels of identity (90 to 91% and 94 to 96%, respectively) between *B. xenovorans* strains CAC-124 and CCUG 2844 obtained in this work and *B. xenovorans* LB400^T (accession no. CP000271.1), TCo-382, and TCo-26 described previously (7). In contrast, alignment of the

acdS sequence of *Pseudomonas* sp. strain ACP and reported sequences of *P. putida* UW4 (accession no. AF0477710), *Pseudomonas fluorescens* 17 (accession no. UFU37103), *P. fluorescens* F113 (accession no. DQ125256), and *Pseudomonas* sp. strain 6G5 (accession no. M80882) showed levels of identity of 73 to 74% and 81 to 82% for nucleotide and protein sequences, respectively. Based on these data, lateral transfer of the *acdS* gene between *Betaproteobacteria* (*Burkholderia*) and *Gammaproteobacteria* (*Pseudomonas*) has been suggested (3). Nevertheless, although the *acdS* gene sequence of strain ACP has been well characterized, we cannot exclude the possibility that this strain belongs to the genus *Burkholderia*, since strain ACP was tentatively classified as *Pseudomonas* sp. using a very limited set of phenotypic tests (43); to our knowledge, the exact taxonomic status of this strain has never been defined. Recently, a partial *acdS* gene sequence (797 nucleotides; accession no. DQ125247) of *B. caledonica* LMG 19076^T was reported (3). However, our results show that *B. caledonica* forms a tight cluster with *B. phenoliruptrix* and *B. graminis* (Fig. 1), but not with several *Pseudomonas* species described by Blaha et al. (3). Moreover, in a previous study (7) and in this study, we were unable to detect ACC deaminase activity and presence of the *acdS* gene in our original strain *B. tropica* BM-273 (31), as previously reported (3). The absence of ACC deaminase activity and of the *acdS* gene was confirmed using other six *B. tropica* strains (62). Our analyses based on 15 complete and 6 partial *acdS* sequences from *Burkholderia* strains belonging to 15 species and on the genomes of four other species (*B. xenovorans* LB400^T, *B. phytofirmans* PsJN^T, *B. phymatum* STM815^T, and *B. cenocepacia* J2315^T) do not support the lateral transfer hypothesis for such a gene in this genus, as previously suggested (3), although lateral transfer appears to have happened between bacteria belonging to other genera (44).

Although the N_2-fixing species *B. unamae* exhibited low ACC deaminase activity (however, the activity was high enough to promote plant growth), this species was chosen to assess the effect of the enzyme on tomato plant growth due to its phylogenetically distant relationship with the Bcc species and relevant features (6, 7, 59) compared to other *Burkholderia* species analyzed in this work. *B. unamae* wild-type strain MTl-641^T and derivative strains had very similar growth patterns in culture media and very similar abilities to colonize tomato roots. On this basis, the higher shoot and root dry weights of tomato plants inoculated with wild-type strain MTl-641^T could be attributed to the ACC deaminase activity, since the values for these parameters were significantly lower for plants inoculated with the ACC deaminase-negative mutant (*acdS*::ΩKm) and for noninoculated plants. A beneficial effect on tomato root elongation was consistently observed in plants inoculated with the wild-type strain and the complemented *acdS* mutant [*acdS*::ΩKm(pF*acdS*$^+$)], as these two strains were similarly able to promote root elongation compared to the noninoculated plants and plants inoculated with the *acdS* mutant (*acdS*::ΩKm) strain. However, an additional plant growth-promoting mechanism seems to be expressed by *B. unamae* MTl-641^T, since the presence of the *acdS*::ΩKm mutant also increased, although at a lower rate than the wild type and the complemented strain, the root length of inoculated plants compared to noninoculated plants. A similar effect on the chlorophyll content of the plants inoculated with the *acdS*::ΩKm mutant compared to noninoculated plants was observed as well. Such an additional beneficial mechanism could be related to the ability of *B. unamae* MTl-641^T to synthesize auxins like indoleacetic acid (IAA), which is produced in culture media (unpublished results). The participation of bacterial IAA in plant growth promotion has been demonstrated in plant-rhizobacterium interactions in many studies (26). Thus, whether IAA synthesized by *B. unamae* MTl-641^T partially masked the positive effect of ACC deaminase on tomato plant growth could be demonstrated by engineering an *acdS* and IAA-negative mutant in further studies. Although it has been reported that some ACC deaminase-producing bacteria promote plant growth under stressful conditions, such as flooding (42) and saline conditions (54), in this work these stress conditions caused no significant differences in shoot or root dry weights between controls and stress treatments. It is possible that the method used to apply stress treatments was not strong enough to cause a significant reduction in the growth parameters analyzed and, as a consequence, plants could gradually undergo an adaptive process in response to mild or moderate stress without major changes in their growth (9, 21).

Tomato inoculation assays with the constitutive *gusA* strain (*s7p-gusA*) confirmed the ability of *B. unamae* to colonize seeds during germination and seedling roots, mainly at the radicle and stem base, as well as at the points of emergence of lateral roots. It has long been known that increased exudation of many organic compounds occurs in natural wounds, such as cracking of seed coats and damage by radicle and lateral root emergence (23). The colonization pattern and infection sites observed with *B. unamae* seem to be common features in plant-bacterium interactions; for instance, they occur with the endophytic bacteria *G. diazotrophicus* (45) and *Azoarcus* sp. strain BH72 (29) in association with gramineous plants and with *Burkholderia* sp. (currently *B. phytofirmans*) strain PsJN associated with *Vitis vinifera* (19). Although compared to the constitutive *gusA* strain a lower level of *gusA* expression was observed for the *acdSp-gusA* strain on germinating seeds and at the base of the radicle, the results presented here suggest that the concentration of ACC exuded was at least 0.5 μM (the minimum concentration required for induction of the *acdS* gene) just after the seed germinated, as well as in natural wound sites. Micromolar levels of ACC (range, 1 to 100 μM) were sufficient to induce expression of AcdS in *R. leguminosarum* bv. viciae 128C53K (52). Similarly, while *gusA* expression was observed along the root system (plants grown in sand) with the constitutive *gusA* strain, a reduced number of root sites showing *gusA* expression (which notably was highest at the base of the radicle and stem) were observed with the *acdSp-gusA* strain. It is known that root exudates differ in chemical composition and quantity, which vary at specific sites on roots (23). In addition, the impact of AcdS apparently is modest, and AcdS probably specifically affects local regulatory mechanisms, such as the mechanisms controlling root hair elongation in *Arabidopsis* inoculated with *Rhizobium* and *Pseudomonas* (20). Accordingly, this could explain the limited number of root sites where *gusA* expression was observed with the *acdSp-gusA* strain in a quantity of ACC sufficient to induce the *acdS* promoter induction at localized root sites. Even so, the localized effect produced by *B. unamae* MTl-641^T was enough to im-

prove seedling growth, as described above for the tomato inoculation experiments with wild-type strain MTl-641^T, and the root length observed with the *acdS*::ΩKm(pF*acdS*$^+$) strain.

Although the beneficial effect of ACC-expressing bacteria on plant growth, illustrated in this study with *B. unamae*, needs to be confirmed in field experiments, the widespread ACC deaminase activity in *Burkholderia* species and the common association of these species with plants suggest that this genus could be a major contributor to plant growth under natural conditions.

ACKNOWLEDGMENTS

We thank L. Martínez-Aguilar for technical assistance and V. M. Hernández-Velázquez (CEIB-UAEM) for his valuable advice on statistical data analysis. We are grateful to Michael Dunn (CCG-UNAM) for reading the manuscript.

J.O.-L. is a Ph.D. student at the Doctorado en Ciencias Biomédicas-UNAM and acknowledges fellowships from the CONACYT and the DGEP-UNAM.

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