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PLANT MICROBE INTERACTIONS

Plant Growth-Promoting Rhizobacteria Associated with Ancient Clones of Creosote Bush (*Larrea tridentata*)

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Abstract Plant growth-promoting rhizobacteria (PGPR) are common components of the rhizosphere, but their role in adaptation of plants to extreme environments is not yet understood. Here, we examined rhizobacteria associated with ancient clones of *Larrea tridentata* in the Mohave desert, including the 11,700-year-old King Clone, which is oldest known specimen of this species. Analysis of unculturable and culturable bacterial community by PCR-DGGE revealed taxa that have previously been described on agricultural plants. These taxa included species of *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* that commonly carry traits associated with plant growth promotion, including genes encoding aminocyclopropane carboxylate deaminase and β–propeller phytase. The PGPR activities of three representative isolates from *L. tridentata* were further confirmed using cucumber plants to screen for plant growth promotion. This study provides an intriguing first view of the mutualistic bacteria that are associated with some of the world's oldest living plants and suggests that PGPR likely contribute to the adaptation of *L. tridentata* and other plant species to harsh environmental conditions in desert habitats.

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Introduction

The ability of some desert plant species to survive for thousands of years under extreme conditions suggests that such plants harbor plant growth-promoting rhizobacteria (PGPR) that have contributed to their fitness. In the southwestern United States, one of the predominant plant species inhabiting the Mohave, Sonora, and Chihuahua deserts is creosote bush (*Larrea tridentata*). Some of the oldest specimens are known to be several thousand years old and includes one of the oldest plants on Earth, known as the King Clone (KC), which is estimated to be 11,700 years old [18] (Fig. 1). Soils of the Mohave desert are typically coarse-textured, excessively drained, and contain almost no organic matter, and the area occupied by the KC sometimes goes without rain for over a year, which demonstrates this plant's ability to withstand extreme drought. To access water and nutrients, the root system of *L. tridentata* consist of a shallow tap root that extends 2−5 m in depth, along with coarse superficial roots that occupy the top meter of the soil. In adapting to these soils, it is also reasonable to hypothesize that this plant species may harbor plant beneficial microorganisms that colonize the root surfaces and that contribute in part to its ability of the roots to withstand stress. Nonetheless, very little is known about the microflora that colonizes the roots of desert plants and the populations and characteristics of PGPR that are associated with ancient plants in desert environments.

Studies with agricultural plants show that the rhizosphere commonly contains diverse bacteria that can suppress diseases and that assist in nutrient cycling and stress tolerance through the production of enzymes and hormones. In this research, we were particularly interested in characterization of bacteria that carry the ability to suppress the production of stress ethylene and to mineralize organic P from phytate. Under stress conditions (drought, heat, and/or salinity), high

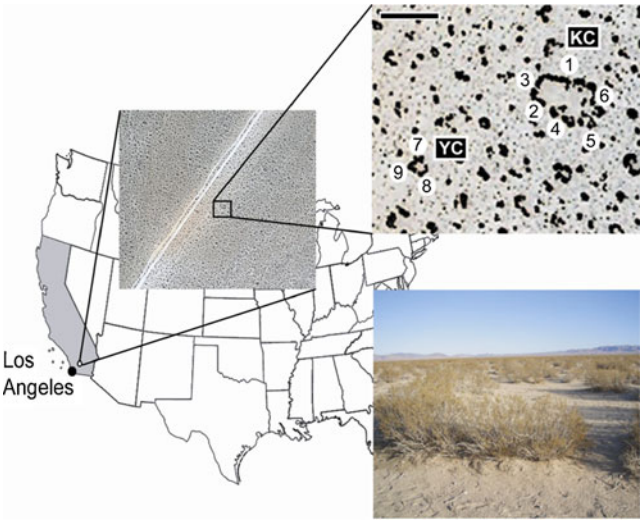

Figure 1 Map of California showing the location of ancient clones of *L. tridentata* and the points (*1* to *9*) where the rhizosphere samples were taken. Satellite images were acquired by using software Google™ Earth. *KC*: King Clone; *YC*: younger clone. *Bar* represents 15 m

rates of ethylene production cause suppression of root growth [4]. The fine-tuning of this response is controlled by an interactive process in which plants secrete aminocyclopropane carboxylate (ACC) into the rhizosphere. ACC is then readsorbed by the plant roots and converted to ethylene, or else it may be degraded by certain bacteria that produce the enzyme ACC deaminase (ACCD), which hydrolyzes the cleavage of ACC to α−ketobutyrate and ammonia [5]. Many studies have shown that inoculation of crop plants with PGPR that express ACCD can enhance root growth and general tolerance of plants to a variety of environmental stresses [6, 19, 20].

Another PGPR function of interest is the ability to mineralize phytate, which is the predominant form of organic phosphate that is stored in plants and that is released into the soil as plant tissues undergo decomposition. In order to be re-utilized by plants, phytate must be mineralized by phytase enzymes that are produced by microorganisms in the plant rhizosphere. Many different rhizobacteria carry genes encoding different types of phytases, including the endospore-forming bacteria, *Bacillus*, which have been widely studied for their potential use as phosphorus-solubilizing biofertilizers [13, 16]. In this context, recent studies by our group have shown that some rhizopshere bacilli are able to degrade phytate by means of β−propeller phytase (BPP) enzymes [1, 10].

In the present study, we used a combination of culture-based and molecular approaches to characterize the rhizobacterial community associated with ancient clones of *L. tridentata* and to identify putative PGPR strains having ACC and/or phytase genes. Additional studies were conducted to confirm that representative strains identified from the roots of *L. tridentata* in fact function as PGPR when inoculated on a test plant species. Our results provide initial insight into the species composition of the rhizosphere of *L. tridentata* and provide evidence in support the hypothesis that desert plants harbor large populations of PGPR that may contribute to the fitness this plant species in its native habitat.

Materials and Methods

Sampling

Rhizosphere samples were collected from both the KC and from an adjacent younger clone (YC) located in the Mohave Desert of southern California (34°25'13"N, 116°42'17"W) (Fig. 1). Samples were taken at nine random locations around the perimeter of the King Clone by excavating the soil with a sterile trowel and removing live superficial roots (1−2 mm in diameter), including the soil that adhered to the roots. The roots were clipped with sterile tools, placed into sterile plastic Falcon tubes, and were transported on ice to the laboratory for microbiological analyses.

The bacterial community structure and ACCD and phytase activity of bacteria from the rhizosphere were examined using both culture-dependent methods to isolate bacteria on agar media for further study and culture-independent methods using PCR−DGGE to profile the entire community and communities generated by selective enrichment on ACC or phytate. To profile the predominant members of the indigenous communities, whole-community DNA was extracted from 0.5 g sample of rhizosphere soil using a FastDNA® SPIN Kit (Qbiogene, Inc.). Fragments of bacterial 16S rRNA genes were amplified by touchdown polymerase chain reaction (PCR) as described by Jorquera et al. [9] with GoTaq® Flexi DNA polymerase (Promega, Inc.) and the primer set EUBf933−GC/EUBr1387 designed by Iwamoto et al. [8]. The PCR−DGGE profiles were generated using a DCode system (Bio-Rad Laboratories, Inc.) to separate the 16S rRNA fragments. The polyacrylamide gels (6 % *w/v*) were prepared using a 25 % to 70 % gradient (urea and formamide). After electrophoresis for 10 h at 100 V, the gels were stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min and photographed on a UV−transilluminator (Bio-Rad Laboratories, Inc.). Dominant bands in the DGGE gels were carefully excised, re−amplified by PCR, and run again on another DGGE gel to further separate out any similarly migrating DNA fragments. The purified DNA fragments were then sequenced in both directions (sense and antisense) by Macrogen, Inc. (Korea). More than one band was sequenced to provide replicate evidence of taxonomic assignment, and the consensus nucleotide sequences were deposited (accession no. from HQ396633 to HQ396640) and compared with those in the GenBank database from the National Center for Biotechnology Information

(NCBI) using blastn algorithms (http://blast.ncbi.nlm.nih.gov/). Comparison of rhizobacterial community structures between KC and YC was performed by clustering of DGGE banding profiles using Phoretix 1D analysis software (TotalLab Ltd.).

Characterization of Culturable ACCD Bacteria

The occurrence of rhizobacteria with ACCD activity was evaluated according to the protocol described by Penrose and Glick [15]. Bacteria were isolated from 0.5 g of root samples with adhering soil were suspended in 15 mL of sterile saline solution (0.85 % NaCl) and shaken vigorously for 10 min. Sterile tubes containing 5 ml of PAF liquid medium (10 g L^{-1} proteose peptone, 10 g L^{-1} casein hydrolysate, 1.5 g L^{-1} anhydrous $MgSO_4$, 1.5 g L^{-1} K_2HPO_4, and 10 % glycerol) were inoculated with 200 μL of the rhizosphere suspension and incubated at 30°C for 48 h. After growth of culturable bacteria, 100 μL of the bacterial suspensions were transferred to sterile tubes containing 5 ml of DF minimal medium (4.0 g L^{-1} KH_2PO_4, 6.0 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} MgSO4 × $7H_2O$, 2.0 g L^{-1} gluconic acid, 2.0 g L^{-1} citric acid, and trace elements—1 mg L^{-1} $FeSO_4 \times 7H_2O$, 10 mg L^{-1} H_3BO_3, 11.19 mg L^{-1} $MnSO_4 \times H_2O$, 124.6 mg L^{-1} $ZnSO_4 \times 7H_2O$, 78.22 mg L^{-1} $CuSO_4 \times 5H_2O$, 10 mg MoO_3). Three millimolars of ACC (Calbiochem®) was added as source of N. The culturable bacterial communities that grew in both PAF and DF media were then analyzed using the same PCR−DGGE methods described above; dominant bands in the DGGE gels were identified as described above, and sequences were deposited in GenBank database under accession no. from HQ396641to HQ396658, for PAF medium, and accession no. from HQ396708 to HQ396687, for DF medium. Cluster analysis of DNA band profiles for both DGGE gels was carried to compare rhizobacterial communities for KC and YC cultivated on PAF and DF media.

To isolate culturable ACC degrading bacteria, culture samples produced on DF minimal medium were spread on DF agar, and the presence of the genes encoding ACCD (*acd*S) was screened by colony PCR [11] using the primer set F1936/F1939 (Table 1). PCR reactions were carried out as previously described [2] using a hotstart at 95°C for 5 min. The first cycle used a denaturation step at 95°C for 30 s, followed by annealing at 50°C for 30 s, and extension at 72°C for 30 s. PCR was then continued for another 35 cycles following the published procedures. A final extension was performed at 72°C for 7 min. Five representative isolates that yielded PCR products were purified by streaking on LB (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 5 g L^{-1} NaCl, and15 g L^{-1} agar) agar and stored in LB broth/glycerol (7:3) at −80°C for further study. The PCR fragments were sequenced in both directions (sense and antisense) and deposited in GenBank database (accession no. from HQ396688 to HQ396692), and the nucleotide sequences were translated to their corresponding protein sequences and compared using the blastx tool (http://blast.ncbi.nlm.nih.gov/).

Table 1 Primers used in this study for characterization of bacterial domains based on 16S rRNA, ACC deaminase (*acd*S), and β−propeller phytase (BPP) genes

Primer name	Sequence (5′−3′)	Reference
16S rRNA		
EUBf933−GC[a]	GCACAAGCGGTGGAGCATGTGG	[8]
EUBr1387	GCCCGGGAACGTATTCACCG	
27f	AGAGTTTGATCCTGGCTCAG	[14]
1492r	TACGGYTACCTTGTTACGACTT	
acdS		
F1936	GHGAMGACTGCAAYWSYGGC	[2]
F1939	GARGCRTCGAYVCCRATCAC	
BPP		
BPPf	GACGCAGCCGAYGAYCCNG CNITNTGG	[7]
BPPr	CAGGSCGCANRTCIACRTTRTT	
DP1	GAYGCIGCIGAYGAYCCIGC	[17]
DP2	TCRTAYTGYTCRAAYTCIC	

[a] GC-clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGG) was attached to the 5′-end of the primer

Culturable Endospore-Forming Bacteria

The occurrence of endospore-forming bacteria (EFB) carrying BPP genes were evaluated according to the protocol described by Jorquera et al. [10]. The rhizosphere suspension described above was treated at 80°C for 10 min and then inoculated into glass test tubes containing PAF medium. The cultures were incubated at 30°C for 48 h, after which the culturable EFB community was analyzed by PCR−DGGE, and dominant bands in gels were identified as described above. The sequences were deposited in GenBank database (accession no. from HQ396659 to HQ396677) and comparison of rhizobacterial communities by clustering of DGGE banding profiles was also carried out. Samples of the PAF culture broth containing EFB were then used to inoculate tubes containing DF minimal medium (containing 3 mM of ACC) which were then spread on PAF agar to isolate individual colonies. The presence of putative phytase-encoding genes was screened by colony PCR using specific primer sets for phytases: BPPf/BPPr and DP1/DP2 (Table 1). Touchdown PCR for detection of putative phytases was carried out according to previously described methods [7] using a hotstart at 95°C for 4 min, followed by touchdown PCR. The annealing temperature was initially at 57°C for the first cycle followed by stepwise decreases of 1°C until 48°C and then continued at this temperature for an additional 27 cycles, followed by a final extension at 72°C

for 5 min. The PCR using DP1/DP2 was performed according to described by Jorquera et al. [10]. The nucleotide sequences were deposited in GenBank database under accession no. from HQ396693 to HQ396699 and translated to their corresponding protein sequences and compared with GenBank database using the blastx tool.

Characterization of Putative PGPR

Bacteria carrying *acd*S− and β−propeller phytase (BPP)-encoding genes were identified to the genus level by partial sequencing of their 16S rRNA genes. The 16S rRNA gene fragments were amplified by PCR with the primers 27f/1492r (Table 1). After a hotstart at 94°C for 5 min, PCR amplification was carried out for 35 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min. The PCR products were purified and sequenced, after which the sequences were deposited under accession no. from HQ396625 to HQ396632 and compared with those in the GenBank database. Three representative bacteria carrying ACCD-encoding genes were selected to confirm the ability of the putative PGPR strains to promote plant growth in a greenhouse experiment.

Evaluation of Putative PGPR Strain Abilities to Promote Plant Growth

The ability of the three selected PGPR to promote plant growth was evaluated in a greenhouse experiment using cucumber as a model plant. These strains, identified as *Pseudomonas* sp., were designated as strains KC−A5, YC−A8, and YC−A18. The greenhouse experiment was performed using intact soil cores taken from the same area around the KC. The soil cores were sterilized by autoclaving to remove the background population before inoculation with the test strains. Intact soil cores were obtained using a slide hammer tool that encased the cores in metal sleeves (15 cm length, 4.5 cm diameter), which were then closed on the bottom using aluminum foil. The cores were transported to the lab, and the foil was replaced with sterilized Whatman 2 filter paper 2 and a fine mesh cotton cloth to allow drainage.

The experiment compared six treatments using autoclaved soil cores, autoclaved soil cores that were re−inoculated with a soil−water suspension from nonsterilized soil, and autoclaved soil cores that were inoculated with one of the three strains. An additional treatment included autoclaved soil cores that were inoculated with a soil−water suspension from a garden soil to evaluate whether the inoculants provided any greater plant growth response that what might be obtained with microflora from an organically managed soil with a similar texture (sandy loam) but that had received regular organic amendments for the past 20 years. The cores that were inoculated were autoclaved twice, with a 24-h interval between each autoclaving to eliminate gram-positive bacteria. As plants of *L. tridentata* are difficult and slow to produce from seed, cucumber was selected as a test plant species to evaluate the efficacy of the PGPR strains for promoting plant growth. Cucumber seeds were surface-sterilized by dipping in a 95 % ethanol solution for 2 min, followed by immersion in a 0.2 % $HgCl_2$ solution for 3 min. The seeds were then rinsed five times with sterile DI water. For seed inoculation, an inoculum of each strain was prepared in liquid DF minimal medium containing ACC as sole N source. After 24 h, the cell density in the liquid broth was adjusted to an OD_{600} of 0.8 for each strain. The seeds were placed in 250-ml Erlenmeyer flasks containing 50 ml of inoculum and placed on an orbital shaking incubator (150 rpm, 28°C) for 45 min. Seeds that were used to produce uninoculated control plants were treated identically using sterile DF medium. The seeds were placed on moist filter paper in sterile Petri dishes for 2 days. Uniformly germinated seedlings were then transferred to the intact soil cores. There were ten replications for each treatment. The cores were placed in a growth chamber (Conviron model CMP 3244, Controlled Environment Ltd. Manitoba, Canada) equipped with HEPA filtration. The plants were grown using a 16/8-h day and night cycle with temperatures set at 25°C and 18°C. The growth chamber provided a maximum light intensity of 80 μmol $m^{-2}S^{-1}$. The plants were fertilized with 0.5× sterile Hoagland solution at the first irrigation, after which the plants were watered with sterile distilled water as needed.

After 25 days, the plants were harvested, and root and shoot growth data were recorded. To quantify the population density of ACC−deaminase bacteria on the roots, root samples with adhering soil were agitated in a sterile saline solution (0.85 % NaCl) with vigorous shaking for 1 h. The roots were then removed from the solution, and the suspension was centrifuged for 10 min at 8,000 rpm. The supernatant was discarded, and 1 g of the suspension was resuspended into 10 ml sterile water. Serial dilutions of the soil suspension (10^{-1} to 10^{-5}) were prepared, and 100 μL of each dilution was spread on to plates containing solidified DF minimal medium with ACC as sole nitrogen source. The numbers of colonies were counted from each plate, and colony-forming units (cfu) per gram soil were calculated.

Results

Rhizosphere Bacterial Community Composition

The 16S rRNA gene sequences produced by PCR−DGGE analysis of the indigenous bacterial community revealed

members of the *Proteobacteria* (Bradyrhizobiaceae, Rhodospirillaceae, Pseudomonadaceae, and Aurantimonadaceae) and *Bacteoridetes* (Chitinophagaceae and Flexibacteraceae) (Table 2). Similarly, excised DNA bands from DGGE gels of rhizosphere soil enriched on PAF medium showed that the majority of the predominant DNA bands represented *Pseudomonas* sp, with the exception of two bands that represented species of *Actinobacteria* (Micrococcaceae) and *Firmicutes* (Bacillaceae). As expected, DNA bands from gels used to profile EFB that were cultured after heat treatment were associated with *Firmicutes*, with the majority of bands representing Bacillaceae. Community profiles and DNA sequences for bacteria cultured on DF medium showed that the majority of ACC-degrading bacteria were mainly members of the Pseudomonaceae. Other ACC-degrading bacteria revealed by DNA sequencing of the DGGE bands revealed members of Xanthomonaceae and Alcanigenacea. No EFB that were enriched using the heat treatment were cultured on DF medium, suggesting that this medium was not suitable for their culture or the absence of ACC-degrading EFB.

Comparison of the DNA band profiles generated by PCR−DGGE by cluster analysis did not reveal any consistent differences between the rhizobacterial communities of the KC and YC plants (Fig. 2).

Detection and Characterization of ACCD− and BPP −Encoding Genes

The use of specific primer sets allowed the detection of a number of *Pseudomonas* isolates that carried the *acd*S gene and various *Bacillus* isolates that carried genes encoding for β−propeller phytases (Table 3). The translated nucleotide sequences derived from this study showed a high similarity (95−98%) to ACCD genes reported in *Pseudomonas* and *Burkholderia* and a high similarity to 3−phytase from *Bacillus*. Among the isolates that were examined here, YC−A18 was noteworthy in that it carried genes encoding both ACCD and BPP activity. In contrast to results obtained using the degenerate primer set BPPf/BPPr, the primer set DP1/DP2 yielded a PCR product only for *Bacillus* sp. YC−B8.

Plant Growth Responses to Inoculation

Inoculation of cucumber seeds with putative PGPR-carrying ACCD genes significantly increased the biomass of the test plants as compared with plants cultivated in sterile soil (Table 4). Cell densities of ACC-degrading bacteria ranged from 10 cfu g^{-1} soil for sterilized, uninoculated soils to 10^3 for sterilized soil inoculated with bacteria from a garden soil to 5×10^4 for plants grown in nonsterilized desert soil. Inoculation with the test PGPR strains resulted in cell densities of approximately 5×10^6 cfu g rhizosphere soil. Both root biomass and total plant biomass values were approximately threefold greater for plants inoculated with the test strains. In comparison, intermediate plant biomass was produced in nonsterilized soil and in sterilized soil that was reinoculated with a soil−water suspension from the desert soil.

Discussion

Plant growth-promoting bacteria associated with native plants in extreme environments have not yet been well studied but are common components of the rhizosphere in agricultural soils where they have been shown to increase the growth, yields, and stress tolerance of crop plants. In this research, we examined the rhizobacterial composition of the ancient clones of *L. tridentata* (KC and YC), with the hypothesis that such rhizobacteria might play an important role in the adaptation of this plant to the desert. Screening for plant growth-promoting rhizobacteria on the basis of ACCD activity revealed that the predominant PGPR were pseudomonads that degraded ACC as a nitrogen source. A large number of these strains were cultivated, among which three representative ACC-degrading strains were shown to have significant effects in stimulating the growth of cucumber, which was used as a test plant species to confirm putative PGPR activity.

Analysis by PCR−DGGE did not reveal consistent differences in the rhizobacterial communities between KC and YC. Predominant bacteria associated with *L. tridentata* belonged to the *Proteobacteria* (Bradyrhizobiaceae, Rhodospirillaceae, Pseudomonadaceae, Aurantimonadaceae, Enterobacteriaceae, Xanthomonadaceae, and Alacaligenaceae), *Bacteoridetes* (Chitinophagaceae and Flexibacteraceae), *Firmicutes* (Bacillaceae), and *Actinobacteria* (Micrococcaceae). These bacterial groups have been previously isolated from the rhizosphere of plants grown in agricultural soils and thus appear to be cosmopolitan taxa for both native and agricultural plants in many soils. The diversity of pseudomonads in the rhizosphere was further studied by enrichment culture on PAF medium, which revealed a wide range of pseudomonads with a high similarity (>98 %) to *Pseudomonas fluorescens* and *Pseudomonas putida*. This same medium also served for enrichment of various EFB including strains related to *Bacillus megaterium* and *Bacillus cereus*, which have been studied previously with respect to plant-growth promotion and suppression of root diseases [3]. This result provides strong support for the hypothesis that PGPR may contribute to the adapation of *L. tridentata* and other plants to desert soils. Many of the bacteria with ACCD activity that were identified were taxonomically similar to PGPR strains that have been previously isolated from the rhizosphere of agricultural plants [5]. Two others identified by PCR−DGGE

Table 2 Phylogenetic assignment of DGGE bands

Band	Taxonomic group[a]	Closest relatives or cloned sequences (accession no.)	Similarity (%)[b]	Accession no.
Total DNA (unculturable bacteria)				
T1	Bacteroidetes, Sphingobacteria, Chitinophagaceae	Unidentified bacterium from arable bulk soil (EF606667)	83	HQ396633
T3	Proteobacteria, Alphaproteobacteria, Bradyrhizobiaceae	Uncultured soil bacterium (GQ425756)	86	HQ396634
T4	Proteobacteria, Alphaproteobacteria, Rhodospirillaceae	Uncultured soil bacterium (AB497746)	84	HQ396704
T5	Bacteroidetes, Sphingobacteria, Chitinophagaceae	Uncultured Bacteroidetes from unvegetated soil (EF220064)	81	HQ396702
T6	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*Pseudomonas vancouverensis* from rhizosphere (GU784934)	97	HQ396635
T7	Proteobacteria, Alphaproteobacteria, Aurantimonadaceae	*Aurantimonas* sp. from legume (GQ871210)	78	HQ396710
T9	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*P. fluorescens* from roots (GU391475)	97	HQ396636
T10	Bacteroidetes, Sphingobacteria, Chitinophagaceae	Uncultured *Chitinophaga* sp. from roots (FJ527527)	94	HQ396637
T14	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	Uncultured soil bacterium (FJ154997)	77	HQ396638
T15	Proteobacteria, Gammaproteobacteria	Uncultured Gamma proteobacterium from soil (AM936521)	75	HQ396711
T16	Bacteroidetes, Sphingobacteria, Flexibacteraceae	Uncultured soil bacterium from abandoned mine (EU141784)	83	HQ396706
T17	Bacteroidetes, Sphingobacteria, Chitinophagaceae	Uncultured bacterium from undisturbed grass (FJ479213)	89	HQ396639
T18	Bacteroidetes, Sphingobacteria, Flexibacteraceae	Uncultured soil bacterium from abandoned mine (EU141871)	80	HQ396709
T19	Bacteroidetes, Sphingobacteria, Flexibacteraceae	Uncultured *Flexibacter* sp. from plant (DQ279363)	91	HQ396640
PAF medium (culturable bacteria)				
C1	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*Pseudomonas* sp. from unflooded rice paddy soil (DQ910438)	98	HQ396641
C3	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*Pseudomonas* sp. from legume nodule (FJ527669)	98	HQ396642
C4	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	Uncultured bacterium from biological soil crusts (GU564701)	98	HQ396643
C6	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*P. fluorescens* from roots (GU391475)	98	HQ396644
C7	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*Pseudomonas* sp. from soil (GU459175)	96	HQ396645
C8	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*P. vancouverensis* from rhizosphere (GU784934)	97	HQ396646
C9	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*P. fluorescens* from soil (FJ226759)	90	HQ396647
C10	Firmicutes, Bacillales, Bacillaceae	Uncultured soil bacterium (AF423227)	87	HQ396648
C11	Actinobacteria, Actinomycetales, Micrococcaceae	*Arthrobacter* sp. from vegetable soil (EU882856)	81	HQ396649
C12	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*Pseudomonas* sp. from paddy soil (AY303259)	96	HQ396650
C13	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*P. putida* from rhizosphere (GU396284)	95	HQ396651
C14	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*P. putida* from mine soil (GU971728)	96	HQ396652
C15	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	Uncultured bacterium from phyllosphere (FN421636)	94	HQ396653
C16	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	Uncultured bacterium from leaves (GU722246)	94	HQ396654
C17	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*P. putida* from roots (FJ639236)	98	HQ396655
C18	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	Uncultured *Pseudomonas* sp. from sediment (GU000498)	98	HQ396656

Table 2 (continued)

Band	Taxonomic group[a]	Closest relatives or cloned sequences (accession no.)	Similarity (%)[b]	Accession no.
C19	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*Pseudomonas* sp. from soil (AJ512403)	87	HQ396657
C20	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*Pseudomonas* sp. (GU784939)	96	HQ396658
PAF medium (culturable endospore-forming bacteria)				
B1	Firmicutes, Bacillales, Bacillaceae	*Bacillus* sp. from pesticide contaminated soil (GU384331)	94	HQ396659
B2	Firmicutes, Bacillales, Bacillaceae	*Bacillus anthracis* from soil (GU826153)	97	HQ396660
B3	Firmicutes, Bacillales, Bacillaceae	*B. megaterium* from cassava residue (HM104233)	99	HQ396661
B4	Firmicutes, Bacillales, Bacillaceae	*Bacillus endophyticus* from bean (EU867383)	90	HQ396662
B5	Firmicutes, Bacillales, Bacillaceae	*Bacillus simplex* from soil (FN298320)	99	HQ396663
B6	Proteobacteria, Gammaproteobacteria, Enterobacteriaceae	*Erwinia rhapontici* from rhizosphere (HM008951)	84	HQ396664
B7	Firmicutes, Bacillales, Bacillaceae	*Bacillus* sp. from vegetable field soil (AY176766)	91	HQ396665
B8	Firmicutes, Bacillales, Bacillaceae	*Bacillus* sp. from soil (AY572480)	100	HQ396666
B9	Firmicutes, Bacillales, Bacillaceae	*B. megaterium* from roots (FJ639202)	99	HQ396667
B10	Firmicutes, Bacillales, Bacillaceae	*Bacillus* sp. from natural reserve (GU321095)	99	HQ396668
B11	Firmicutes, Bacillales, Bacillaceae	*B. megaterium* from soil (FJ973534)	98	HQ396669
B12	Firmicutes, Bacillales, Bacillaceae	*Bacillus* sp. from plant thorns (FJ943256)	91	HQ396670
B13	Firmicutes, Bacillales, Bacillaceae	*B. cereus* from mud volcano (DQ289059)	99	HQ396671
B14	Firmicutes, Bacillales, Bacillaceae	*Bacillus* sp. from soil (FJ373035)	98	HQ396672
B15	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*Pseudomonas* sp. from rhizosphere (GU124698)	86	HQ396673
B16	Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	Uncultured *Pseudomonas* sp. from cultivated soil (HM011997)	100	HQ396674
B18	Firmicutes, Bacillales, Bacillaceae	*B. megaterium* from cassava residue (HM104232)	100	HQ396675
B19	Firmicutes, Bacillales, Bacillaceae	*Bacillus* sp. from plant (GU471201)	100	HQ396676
B20	Firmicutes, Bacillales, Bacillaceae	*Bacillus thuringiensis* (CP000485)	98	HQ396677
DF medium (culturable ACC-degrading bacteria)				
A1	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*Pseudomonas* sp. from roots (GU391471)	75	HQ396708
A2	Proteobacteria, Xanthomonadales, Xanthomonadaceae	*Stenotrophomonas rhizophila* from wheat (GQ130132)	90	HQ396678
A3	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	Uncultured *Pseudomonas* sp. from sediment (GU000313)	79	HQ396707
A4	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*Pseudomonas thivervalensis* from roots (FJ639178)	99	HQ396679
A7	Proteobacteria, Betaproteobacteria, Alcaligenaceae	*A. faecalis* from bean processing soil (GQ497151)	93	HQ396680
A8	Proteobacteria, Betaproteobacteria, Alcaligenaceae	Uncultured bacterium from velvetleaf seed (EU769162)	79	HQ396705
A9	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*Pseudomonas* sp. from roots (GU391471)	87	HQ396681
A10	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*Pseudomonas syringae* from leave (GU722244)	89	HQ396714
A11	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*Pseudomonas* sp. from rhizosphere (EF102851)	93	HQ396682
A12	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*P. putida* from soil (FN298299)	74	HQ396703
A14	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*Pseudomonas* sp. from unflooded rice paddy soil (DQ910438)	96	HQ396683
A15	Proteobacteria, Xanthomonadales, Xanthomonadaceae	*Stenotrophomonas rhizophila* from rhizosphere (GU186108)	87	HQ396684
A16	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	Uncultured *Pseudomonas* sp. from soil (EU449581)	77	HQ396713

Table 2 (continued)

Band	Taxonomic group[a]	Closest relatives or cloned sequences (accession no.)	Similarity (%)[b]	Accession no.
A17	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*Pseudomonas* sp. from unflooded rice paddy soil (DQ910473)	77	HQ396712
A18	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*Pseudomonas* sp. from soil (AJ512403)	87	HQ396685
A19	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	*Pseudomonas* sp. from paddy soil (AY303259)	94	HQ396686
A20	Proteobacteria, Gammaproteobacteria, Pseudomonadacea	Uncultured *Pseudomonas* sp. from agricultural soil (EU449579)	99	HQ396687

[a] The phylogenetic assignment is based on sequence analysis by blastn of GenBank database from NCBI (http://www.ncbi.nlm.nih.gov). It is given the phylum as well as the lowest predictable phylogenetic rank

[b] Based on partial sequencing of 16S rRNA gene and comparison with those present in GenBank by using Blastn

included ACC-degrading bacteria with relatively low (87 % and 90 %) similarities to *Stenotrophomonas* and another with 93 % similarity to *Alcaligenes faecalis*. Both genera have also been reported as ACC degraders. In addition to these taxa, some taxa that were identified here from 16S rRNA gene analysis of the indigenous community and culturable ACC-degrading bacteria showed very low sequence similarities (75 % and 77 %) to DNA sequences in the BLAST database (i.e., bands T14, T15, A1, A16, and A17) suggesting the presence of novel bacterial taxa that remain to be better characterized.

The culturable ACC-degrading bacteria taxa that were identified on agar media were largely the same as those identified by sequencing of DNA bands from the PCR–DGGE gels of the enrichment cultures produced on ACC. The ACC degraders included representatives from three families, Pseudomonadaceae, Xanthomonadaceae, and Alcaligenaceae and carried *acdS* gene sequences with high similarity to those reported for *Pseudomonas* and *Burkholderia* spp. None of the EFB was capable of growth on ACC, but five were shown to contain genes encoding BPP. The presence of *Bacilli* encoding BPP in the rhizosphere of plants has recently been reported [10]. The presence of both ACCD and BPP genes in the isolate *Pseudomonas* sp. YC−A18 is noteworthy. To our knowledge, this is first report that describes the detection of two genes related with plant-growth promotion in a single bacterial strain by gene analysis.

To confirm that the putative PGPR identified here were in fact capable of promoting plant growth, experiments were conducted to evaluate the effects of inoculation with

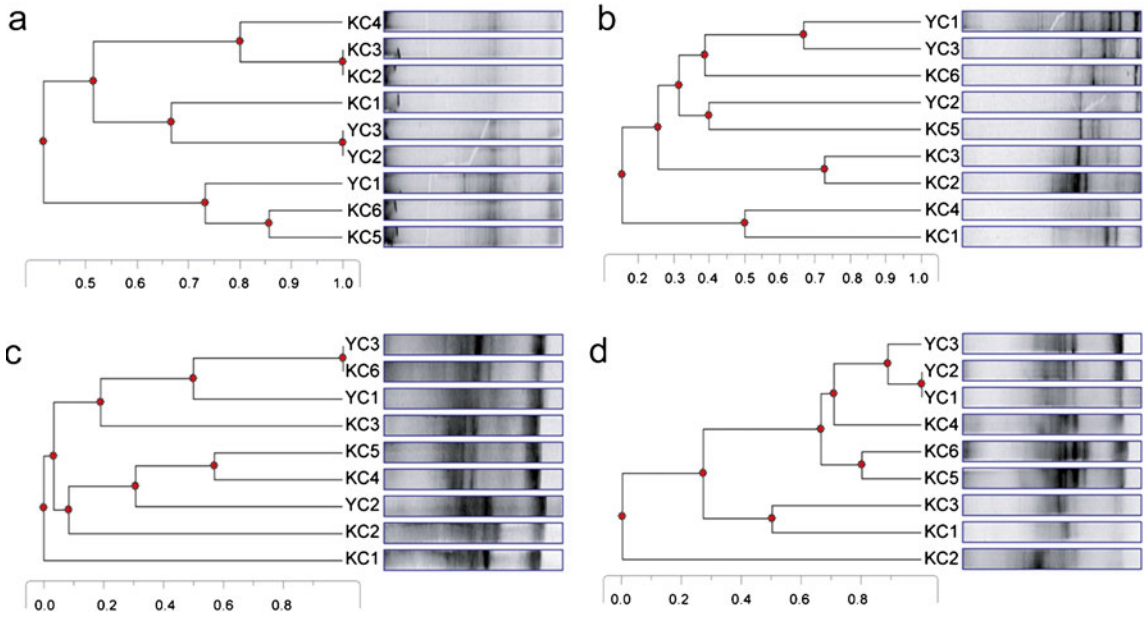

Figure 2 Dendrograms of DGGE profiles of rhizobacterial communities of both the King Clone (KC1, KC2, KC3, KC4, KC5, and KC6) and from an adjacent younger clone (YC1, YC2, and YC3) of *L. tridentata*. **a** Unculturable bacteria; **b** culturable bacteria; **c** culturable endospore-forming bacteria; **d** culturable aminocyclopropane carboxylate-degrading bacteria

Table 3 Genetic characterization of isolates by sequencing of 16S rRNA, ACC deaminase (*acdS*), and β−propeller phytase (*BPP*) genes

Isolate	Closest relatives or cloned sequences (accession no.)[a]	Similarity (%)	Accession no.
16S rRNA			
KC-A2	Uncultured *Pseudomonas* sp. from unfertilized soil (HM011621)	99	HQ396625
KC-A5	Uncultured *Pseudomonas* sp. from unfertilized soil (HM011621)	99	HQ396626
KC-A6	Uncultured *Pseudomonas* sp. from unfertilized soil (HM011621)	95	HQ396627
YC-A8	Uncultured *Pseudomonas* sp. from unfertilized soil (HM011621)	99	HQ396628
YC-A18	Pseudomonad from mulberry rhizosphere (EF462378)	99	HQ396629
KC-B4	*B. cereus* from rice field soil (HM026606)	99	HQ396630
KC-B5	*Bacillus pseudomycoides* from roots (GU391527)	94	HQ396700
KC-B6	*B. cereus* from roots (EF035137)	91	HQ396701
YC-B7	*B. pseudomycoides* from roots (GU391527)	94	HQ396631
YC-B8	*Bacillus amyloliquefaciens* from roots (HM016080)	98	HQ396632
acdS			
KC-A2	ACCD from *P. fluorescens* (ABE66285)	95	HQ396688
KC-A5	ACCD from *Burkholderia caledonica* (ABE66287)	95	HQ396689
KC-A6	ACCD from *B. caledonica* (ABE66287)	95	HQ396690
YC-A8	ACCD from *P. fluorescens* (ABE66285)	96	HQ396691
YC-A18	ACCD from *P. fluorescens* (ABE66285)	98	HQ396692
BPP			
YC-A18	Phytase precursor from *Bacillus* sp. (ABP52059)	86	HQ396693
KC-B4	3-phytase from *Bacillus pseudomycoides* (ZP_04154570)	72	HQ396694
KC-B5	3-phytase from *Bacillus pseudomycoides* (ZP_04154570)	84	HQ396695
KC-B6	3-phytase from *Bacillus pseudomycoides* (ZP_04154570)	85	HQ396696
YC-B7	3-phytase from *Bacillus pseudomycoides* (ZP_04154570)	68	HQ396697
YC-B8	Phytase precursor from *Bacillus* sp. (ABP52059)	81	HQ396698
YC-B8	Phytase precursor from *Bacillus coagulans* (ABC75078) [b]	94	HQ396699

[a]Based on partial sequencing of genes and comparison with those present in GenBank (http://www.ncbi.nlm.nih.gov) by using blastn or blastx

[b]From amplified DNA fragment with primer (DP1/DP2) set described by Tye et al. (2002)

representative strains from *L. tridentata*. Our results showed that the inoculation with selected ACC-degrading bacteria significantly improved the growth of cucumber plants that were used as test plants (Table 4), resulting in approximate threefold increases in root and total plant biomass as compared with plants grown in sterilized soils, even with the addition of fertilizer nutrients at levels that would support normal plant growth. Plant growth responses were intermediate in nonsterilized desert soil and in sterilized desert soils that were re-inoculated with a soil suspension following autoclaving. Restoration of the soil microflora by reintroducing a bacterial suspension from the desert soil or from a garden soil resulted in recovery of plant growth to the same level as plants grown in nonsterilized desert soil.

Although, the inoculation experiment was performed mainly to confirm PGPR activity, an interesting finding from

Table 4 Plant growth responses to inoculation with selected putative PGPR carrying ACCD genes isolated from *L. tridentata*

Treatment	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g)	Plant biomass (g)	PGPR colonization (CFU g^{-1})
Sterile soil	6.19	1.19	5.88	0.45	1.64	1.0×10^{1}
Sterile soil reinoculated	7.25	2.16	6.31	1.37	3.53	1.4×10^{4}
Nonsterilized soil	7.88	3.61	12.75	1.42	5.03	5.4×10^{4}
Sterile soil + KC-A5	7.94	2.84	14.38	3.11	5.95	4.6×10^{6}
Sterile soil + YC-A8	7.81	2.78	12.75	2.35	5.13	3.9×10^{6}
Sterile soil + YC-A18	8.31	2.80	13.56	2.73	5.53	3.4×10^{6}
Sterile soil + garden soil	7.13	2.71	13.06	2.00	4.71	1.0×10^{3}
LSD (α=5 %)	0.97	1.2	0.31	1.26		

this experiment was the relationship between population densities of ACC-degrading bacteria in the rhizosphere and growth of the test plants (Table 4). Population densities of ACC-degrading bacteria in the rhizosphere were approximately 100-fold greater for inoculated plants than for plants grown in nonsterilized soil or re-inoculated soils. Although there were no significant differences in plant growth responses among plants inoculated with the three putative PGPR, strain YC−A8 gave the lowest growth response at the same population density. Plant ethylene production is controlled both by auxin production and ACC degradation. Further evaluation of strains with different levels of PGPR activities is needed to determine whether measurements of particular activities are relevant to selection of the most effective strains.

The present study provides an intriguing first look into the rhizosphere of one of the Earth's oldest plants and other members of this plant species that today occupies a large region of the desert southwest in North America. The predominant bacteria were related to many bacteria that have been identified on agricultural plants, but the degree to which these bacteria have undergone specific selection for the King Clone and this particular soil habitat is still unknown. The taxonomic composition of the rhizosphere of this plant comprises pseudomonads and bacillus, many of which carry PGPR traits for ACC and phytate degradation. Our results lay the foundation for future studies on the role of rhizosphere bacteria in contributing to plant adaptation to arid environments. It will be interesting to extend this research to other ancient plants in this region, including the 5,000-year-old bristlecone pines and the recently discovered 13,000-year-old Jurupa oak clone [12]. The extent to which the rhizosphere microflora and functional groups identified here are shared by ancient plants on other continents also remains as an open research question.

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