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Diversity and Isolation of Culturable ACC Deaminase-Producing Rhizobacteria from the Red-crowned Crane National Nature Reserve, China

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This study aimed to assess the richness and diversity of ACC deaminase-producing bacteria (ADPB) in native plant rhizospheres, and also to screen ADPB isolates for further development. In September 2013 (summer) and February 2014 (winter), we sampled three types of plants *Saudeasalva* (SS), *Imperata koenigii* (IK), and *Phragmites australis* (PA) with distinct salt-tolerant abilities from the reserve. Using enrichment culture method together with amplifed ribosomal DNA restriction analysis technology, an average of 23.25 (20.5), 23.75 (13.75), and 21.50 (11.75) ADPB were screened out corresponding to SS, IK, and PA in summer (winter). 16S rRNA gene sequence data showed that there were 5 and 8 OTUs in summer and winter respectively, in which, *Klebsiella* was the absolutely dominant genus, while before enrichment, *Pseudomonas* was the dominant genus as revealed by PCR-DGGE. The comparison of ADPB communities among three plants reflected that there is no correlation between ADPB population in rhizosphere and salt-tolerant abilities. Two representative isolates of each OUT were determined for the possible plant growth-promoting traits, among which, *Sphingobacterium* sp. is the first reported isolate with the ability of producing ACC deaminase. Surprisingly, all isolates were gram-negative, suggesting that most ADPB in nature may belong to Gram-negative group.

Key words: ACC deaminase-producing bacteria (ADPB); Amplifed ribosomal DNA restriction analysis (ARDRA); Enrichment culture; The Red-crowned Crane National Nature Reserve.

Bacteria encoding the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase is widely known to be capable of protecting plants against abiotic stresses, such as heavy metals[1], salts[2, 3], organics[4], flooding[5, 6], drought[7, 8] and so on, by metabolizing ACC (an immediate precursor of ethylene in plants) into α-ketobutyric acid and ammonia and thus lowering plant ethylene levels. To date, *Bacillus* sp., *Agrobacterium* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Klebsiella* sp., *Enterobacter* sp., *Ralstonia* sp., *Pantoea* sp., *Burkholderia* sp., *Micrococcus* sp. are the main reported ACC deaminase-producing bacterial strains[1, 9-13]. Those relatively harsh environments always have more ACC deaminase-producing bacteria (ADPB) and thus can be easily isolated[14, 15].

The Red-crowned Crane National Nature Reserve is located in Sheyang China with a thirty-year history. It has undergone unusual geological and ecological successions by deposition of abundant sediment called longshore drift, as a result, the intertidal zone were changed to land gradually[16, 17], where the moderate climate, abundant vegetation, as well as rare anthropogenic disturbance are important and essential conditions to be a perfect habitat of the red-crowned crane. Such a unique geographical condition, as well as the migration of the red-crowned crane each year[18],

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may have diverse and special organisms especially the microorganisms, for many new isolates were isolated from different special environment including some nature reserve[19-22]. To date, little is known about the topic in Sheyang reserve. Owing to possession of environmental change, we believe that there must be diverse ADPB in native plant rhizospheres, especially in those have strong abilities of salt tolerance. Thus, in this present work, we collect three salt tolerant native plants rhizosphere soil from the Red-crowned Crane National Nature Reserve, compare the culturable ADPB population in both summer and winter season, and isolate ADPB with traditional culture method. We also analyze the variation of dominant bacterial genera by denaturing gradient gel electrophoresis (PCR-DGGE) technology. The aims of this study are (i) to reveal the diversity of culturable ADPB in Sheyang reserve; (ii) to isolate ADPB from the reserve as a resource for further development; and (iii) to discuss if plants with higher abilities of salt resistance have more abundant and diverse rhizobacteria with ACC deaminase activities.

MATERIALS AND METHODS

Sampling soils and characteristics

In September 2013 (summer) and February 2014 (winter), 24 rhizosphere soil samples containing three types of plants (*Saudeasalva*, *Imperata koenigii* and *Phragmites australis*) were collected from the Red-crowned Crane National Nature Reserve that was about ten kilometers from the Yellow Sea, China. For each plant, four repeats were sampled. Rhizosphere samples were kept temporarily in an ice box before analysis, and a fraction in which was stored at -80°C for molecular analysis.

Enumeration of culturable ADPB populations

Each five-gram air-dried sample was added to a 200 mL flask with 45 mL of sterile ddH_2O, followed by a gradient dilution, one hundred-microliter of a proper diluted suspension for each sample was spread evenly on a 1/10 nitrogen-free agar (pH=8.0) containing 3 mM of ACC, and cultured at 28°C for seven days. Colonies were numerated and the numbers of ADPB in soil samples were deduced.

Diversity and abundance of culturable ADPB in rhizosphere of different plants by enrichment culture method

Five-gram of rhizosphere soils were cultured in 25 mL of DF minimal medium with 3 mM ACC at 30°C and 150 rpm, every other day, 5 mL of culture solution was transferred to another fresh medium and thus cultured for 7 days. After enrichment, fifty-microliter of the soil suspension was spread on 1/10 nitrogen-free agar containing 3 mM of ACC, and cultured at 28°C for seven days. The colonies were picked separately and streaked on LB medium for purification, and the purified strains were stored in freezer under -80°C with 20% glycerine.

Identification and classification of isolates

Each strain was grown on LB agar plate for 12-48 h depending on growth rates, then three loops of bacterial lawns were scraped into 200 µL sterile ddH_2O, followed by a water bath at 100°C for 10 mins and a centrifugation at 8000 rpm for 3 mins, supernatant was kept at -20°C as DNA template.

Nearly full length 16S rRNA gene was amplified with primer 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') (23). The reaction was carried out in 50 µL mixture system with 10 µL DNA template, 0.2 mM of each dNTP, 0.4 µL of each primer, 1.25 U PrimeSTAR® HS DNA Polymerase (TaKaRa, Dalian, China), and 1 × buffer (including Mg^{2+} at 1.5 mM final concentration). Amplification was performed in an Eppendorf Mastercyclers (Perkin-Elmer, Inc., Waltham, MA) under the following conditions: initial denaturation at 94°C for 5 mins; 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 mins; and terminated by a final extension at 72°C for 10 mins. The amplification products were checked by agarose gel electrophoresis (1%, w/v, agarose in Tris-Borate-EDTA buffer). The PCR products were digested with the restriction enzyme *Hinf*I (TaKaRa, Dalian, China) separately, and distinguished according to different patterns mirrored by agarose gel electrophoresis at 0.8% concentration.

Only one randomly selected PCR product within the same *Hinf*I-digested fingerprint pattern was sequenced by Sangon Biotech. Co., Ltd., Shanghai, China. After comparing with the GenBank reference sequences, the sequences of

representative strains from different operational taxonomic units (OTUs) were deposited in GenBank using a submission tool Sequin.

Method for measurement of soil bacterial diversity before enrichment

Total bacterial DNA from each rhizosphere soil was extracted according to the protocol of the Power-Soil™ DNA Isolation Kit and UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The quality of DNA extracts was routinely checked by agarose gel electrophoresis at 1% concentration, and the yield of purified genomic DNA was estimated by UV absorbance at 260 nm.

PCR was performed in a 50 µL reaction mixture as mentioned above, except for 2.5 ng of DNA template and another primer pair 338f (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3'), 518r (5'-ATTACCGCGGCTGCTGG-3'). Amplification was carried out under the following conditions: initial denaturation at 94°C for 5 mins, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min (decreasing 0.1°C per cycle to 52°C), 72°C for 1.5 mins, and terminated by a final extension at 72°C for 10 mins. The amplification products were checked by agarose gel electrophoresis as described above.

DGGE analysis was performed on 8% (w/v) polyacrylamide gel with a denaturing gradient of 30-60% in 1× Tris-Acetic acid-EDTA buffer using a D-Code Mutation Detection System (Bio-Rad Laboratories, Inc., Her-cules, CA) at 150 V and 60°C for 7 h. Following electrophoresis, the gel was stained with silver nitrate (1 g L^{-1}) for 25 mins and photographed under UV light with a Gel Doc XR system (Bio-Rad Laboratories, Inc.). Images were documented with the Umax PowerLook 1000 and analyzed with Quantity One software (Bio-Rad Laboratories, Inc.). In order to check the reproducibility of DGGE results of replications of the same plant type, three PCR products were selected to run on DGGE. To compare the patterns of all different plant types on a single denaturing gel, only one PCR product from one plant type was finally loaded on the gel.

DGGE bands of interest were excised with a clean razor blade and eluted into 40 mL of sterile ddH_2O, and incubated in a water bath at 50°C for 30 mins, followed by centrifugation at 12,000 rpm for 5 mins, and the supernatant was used for PCR amplification with 338f and 518r primers without the GC clamp. The purified PCR products were cloned into the pMD® 18-T Vector (TaKaRa, Dalian, China) and transformed into sensitive *Escherichia coli* DH5α cells using a calcium chloride/heat shock method. Three positive clones randomly selected from each plate were sequenced by Sangon Biotech. Co., Ltd., and the sequencing results were also compared to the GenBank reference sequences.

Assay for ACC deaminase activities of typical isolates

The ACC deaminase activity of cell-free extracts of each selected strain was determined by estimating the amount of α-ketobutyrate (α-KB) generated by the enzymatic hydrolysis of ACC according to the procedure of Honma and Shimomura[24]. The amount of α-KB was measured by comparing the absorbance at 540 nm of a sample to a standard curve of α-KB. The protein concentrations of cell suspensions and in cells disrupted by toluene were determined by the method of Bradford[25]. The enzyme activity was expressed as µM α-KB $mg^{-1}h^{-1}$.

Evaluation of plant growth-promoting (PGP) abilities

Besides ACC deaminas activity, some other recognized PGP activities were also determined for 20 randomly selected strains to further explore the multiple PGP activities of isolates.

Indoleacetic acid (IAA) production was detected by the modified method as described by Loper and Schroth[26]. Bacterial cultures were grown for 48-72 h at 28°C in nutrient broth containing an inductor tryptophan at a final concentration of 0.5 g L^{-1}, followed by a centrifugation at 3000 rpm for 30 mins. The 2mL of supernatant was mixed with 2 mL of the Salkowski reagent (50 mL, 0.225 g $FeCl_3$ in 10.8 M H_2SO_4). Optical density of development of pink colour was taken at 530 nm with the help of spectrophotometer. Concentration of IAA produced by cultures was obtained according to the standard curve of IAA (Sigma-Aldrich).

Protease activity was determined based on the method of Kembhavi *et al.*,[27] with minor modifications. Cell-free culture supernatant was added to a tube containing 0.3 mL of 1% (w/v) casein (dissolved in 20 mM Tris-HCl buffer, pH

7.4) and incubated at 37°C for 30 mins. The proteolytic reaction was stopped with 0.45 ml of a 10% (w/v) tri-chloroacetic acid solution at a final concentration of 5%. The mixture was incubated at room temperature for 1 h followed by a centrifugation at 12000×*g* for 5 mins, and the absorbance of the supernatant was measured at 275 nm. One unit of protease is defined as the amount of enzyme that hydrolyses casein to produce equivalent absorbance to 1 µg of tyrosine per min with tyrosine as standard.

Quantitative analysis of solubilization of tricalcium phosphate in liquid medium was determinated after qualitative screening on Pikovskaya's agar plate according to the presence or absence of halo zones. Bacterial strains were inoculated in 25mL Pikovskaya's broth and incubated for 4 days at 28°C with the rotating rate of 200 rpm. Then, cultures were centrifuged at 12000 rpm for 30 mins. Five-milliliter of supernatant was transferred to a 50 mL volumetric flask, and added with ddH_2O to the volume of about 30 mL, then 5 mL of the colour developing agent (1.5 g ascorbic acid, 0.05 g antimony potassium tartrate, 1.0 g ammonium molybdate, and 12.6 mL sulfuric acid in 100 mL) were added, the volume was made up to 50 mL with ddH_2O. After keeping for 30 mins under room temperature, the developed blue colour was measured at 700 nm, and the amount of soluble phosphorus was detected from the standard curve of KH_2PO_4.

The almost same method was adopted to determine organophosphorus-solubilizing abilities of isolates, except that the insoluble phosphorus source was replaced with lecithin.

The potassium-solubilizing bacteria were screened on Aleksandrov medium plate with insoluble aluminosilicate[28]. The selected strains were cultured in potassium-free broth containing 1.0% (w/v) of insoluble aluminosilicate for 5 days at 30°C, followed by a centrifugation at 12000 rpm for 30mins. The soluble potassium in supernatant was measured using atomic absorption method by comparison to KCl standard curve.

The nitrogen-fixation abilities of isolates were determined after qualitative screening on Ashby nitrogen-free agar plates. For quantitative determination, isolates were cultured in N-free broth at 30°C for 7 days, total N in both culture solution and cell-free supernatants were estimated using Kjeldahl method, and the amounts of nitrogen fixed by isolates represented the N-fixation ability.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan µs test at the 5% level for multiple comparisons using SPSS 15.0 for Windows (SPSS, Inc., Armonk, NY). Digitized DGGE images were analyzed with Quantity One 4.5 software (Bio-Rad, USA).

RESULTS AND DISCUSSION

Richness and diversity of ACC deaminase-producing bacteria (ADPB) communities in native plant rhizospheres

In winter, there are a total of 20.5, 13.75, and 11.75 ADPB on every enriched medium plate corresponding to SS, IK, and PA rhizosphere soils, each of which contained three OTUs, *Sphingobacterium* (1.5), *Klebsiella* (17.5) and *Acinetobacter* (1.5) in SS, *Chryseobacterium*

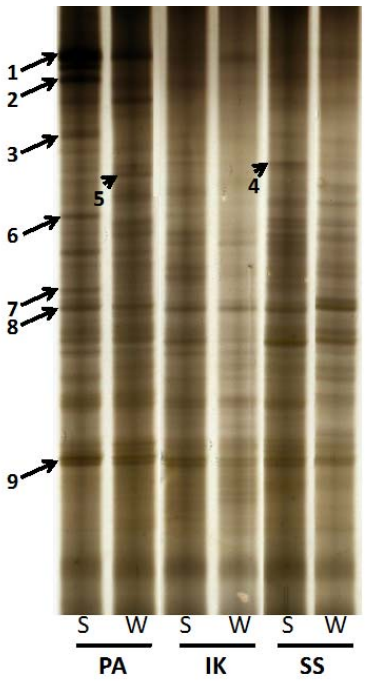

Fig. 1. A comparison of bacterial communities in three types of plant rhizospheres in summer (S) and winter (W) seasons revealed by PCR-DGGE technology analysis. SS=*Saudeasalva*, IK=*Imperata koenigii*, and PA=*Phragmites australis*. SS, IK and PA are three specific species. Bands numbered in the pattern were sequenced and expressed in Table 3.

(2.25), *Klebsiella* (10.25), and *Acinetobacter* (1.25) in IK, *Klebsiella* (8.50), *Enterobacter* (2.00), and *Acinetobacter* (1.25) in PA (Table 1). *Klebsiella* is the dominant genus in each sample with culture

Table 1. Richness and diversity of ACC deaminase producing bacteria (ADPB) communities among native plant rhizospheres revealed by traditional culture method

	OTUs	SS	IK	PA
Winter (February, 2014)	*Sphingobacterium* sp.	1.50	NS	NS
	Chryseobacterium sp.	NS	2.25	NS
	Klebsiella sp.	17.50	10.25	8.50
	Enterobacter sp.	NS	NS	2.00
	Acinetobacter sp.	1.50	1.25	1.25
	Total strains per plate	20.50	13.75	11.75
	H[a]	0.52	0.73	0.77
	E[b]	0.47	0.67	0.7
	ADPB population in rhizosphere without enrichment culture (CFU g^{-1})[c]	1.02×10^5	6.34×10^5	2.44×10^6
Summer (September, 2013)	*Klebsiella* sp.	14.00	17.75	13.25
	Acinetobacter sp.	5.00	NS	1.00
	Agrobacterium sp.	NS	1.75	NS
	*Pseudomonas*sp.	NS	NS	2.00
	*Stenotrophomonas*sp.	1.75	1.50	NS
	Enterobacter sp.	1.00	1.75	1.25
	Chryseobacterium sp.	NS	NS	4.00
	Sphingobium sp.	1.50	1.00	NS
	Total strains per plate	23.25	23.75	21.50
	H[a]	1.14	0.91	1.14
	E[b]	0.71	0.57	0.71
	ADPB populatgion in rhizosphere without enrichment culture (CFU g^{-1})[c]	2.05×10^5	5.31×10^6	7.16×10^6

SS=*Saudeasalva*, IK=*Imperata koenigii*, and PA=*Phragmites australis*. SS, IK and PA are three native plants. [a]*H* is the Shannon index of culturable ADPB community after enrichement inrhizosphere of different plants, which partially reflects the diversity of ADPB in rhizosphere soil. [b] E is evenness of a community; There are three and five OTUs of each kind of plant in winter and summer, which represents the ADPB species richness. [c]Values represent means of bacterial numbersof four plant rhizospheres by dilution plate counting method. NS stands for not screened.

The 16S rRNA gene sequences of represented bacterial strains selected from one OUTwere deposited in Genbank, the accession numbers are listed as follows, *Sphingobacterium*sp. (KM192325), *Chryseobacterium* sp. (KM192326), *Klebsiella* sp. (KM192327),*Enterobacter*sp. (KM192328), *Acinetobacter*sp. (KM192329), *Agrobacterium* sp. (KM192330), *Pseudomonas* sp. (KM192331), *Stenotrophomonas* sp. (KM192332), *Sphingobium*sp. (KM192333).

Table 2. The diversity of the bacterial communities in rhizosphere soils of different kind of plants revealed by PCR-DGGE

Samples	PA		IK		SS	
	Summer	Winter	Summer	Winter	Summer	Winter
H	3.11	2.76	3.00	2.82	2.81	2.84

SS=*Saudeasalva*, IK=*Imperata koenigii*, and PA=*Phragmites australis*. SS, IK and PA are three specific species. [a]*H* is the Shannon index of rhizosphere bacterial diversity as calculated basing on the PCR-DGGEfingerprinting (Fig. 1).

method. The diversity of ADPB of each sample is 0.52, 0.73, and 0.77 as indicated by *H* value. The number of OTUs of samples collected in summer increased by 60% as compared to those of winter. *Klebsiella*, similarly, is the dominant genus in each sample, and accounts for 60.21%, 74.74% and 61.63% respectively. The diversity of ADPB of each sample (*H* value) is 1.14, 0.91, and 1.14. Both richness and diversity in summer season were higher than that in winter, which probably due to the distinct growth vigour in different seasons. Soil microbial community can adapt to changing environmental conditions on very short time scales[29], because changes between summer and winter may be a certain control on annual patterns of nitrogen and salt uptake[30,31].

Surprisingly, bacteria on the selective medium were all gram-negative, though ADPB have been found in a wide range of Gram-negative[13,32,33] and Gram-positive bacteria[27], as well as in fungi[34]. However, from this work, the isolates were all Gram-negative. Besides, most bacteria obtained by searching *acdS* gene (encoding ACC deaminase) in Genbank are Gram-positive, which implies that ADPB, at least the culturable ADPB, in nature belong to Gram-negative group.

The culturable population of ADPB in PA rhizosphere was more abundant than that in SS and IK (Table 1). In both sampling seasons, SS has the smallest population of ADPB. In addition, there were no obvious differences in diversity of bacterial communities among three plant rhizospheres with PCR-DGGE technology (Table 2). These indicated that although SS has the best ability among the three types of plants to resist salt stress, and thus largely exist in the intertidal zone of Yellow Sea[35], which, however, was not due to the assumed capability of attracting more ADPB, because the population of ADPB was fewest compared to other plants. Some studies have suggested that changes in rhizosphere bacterial community composition occurred along with shift of plant communities[36-39], and vegetation type contributed more of the variation in bacterial community composition than pH[39]. A study pointed out that diversity and abundance of bacterial community in rhizosphere of *Spartina alterniflora* were important factors for their rapid and salt-tolerant growth[40]. Another

Table 3. The most closely related bacteria or clones deposited in GenBank to the sequences derived from DGGE bands using BLAST analysis

Band	Accession number	Closest GenBank match (accession number)	Identity %
1	KM192303	*Pseudomonas koreensis* HR150 (LK020945)	100
2	KM192304	*Pseudomonas fluorescens* 2Re2-6 (AJ581955)	100
	KM192305	Uncultured bacterium clone BaMNPK2RC07 (FR751105)	98
	KM192306	Uncultured alpha proteobacterium clone G231 (HG917267)	100
3	KM192307	*Pseudomonas putida* strain 3Re4-21 (AJ581987)	100
	KM192308	*Paenisporosarcina* sp. G03 48-4 (KF974301)	99
	KM192309	Uncultured *Chryseobacterium* sp. clone WCD 121 (KJ123819)	99
4	KM192310	Uncultured bacterium clone BT1123 (JF800781)	99
	KM192311	Uncultured bacterium clone GB7N87003FP2ZP (HM646802)	99
	KM192312	Uncultured bacterium clone ty80 (JF523591)	98
5	KM192313	Uncultured *Flavobacterium* sp. clone RLBp4219 (KC451768)	99
	KM192314	Uncultured alpha proteobacterium clone D12L-2 (HE614761)	100
6	KM192315	Uncultured soil bacterium clone 127 (EF667435)	98
	KM192316	Uncultured beta proteobacterium clone NL5BD-01-A08 (FM252788)	99
	KM192317	*Sphingobium* sp. A1b-3 isolate A1b-3 (HG738904)	100
7	KM192318	*Bosea* sp. Cd36e (HG937602)	100
	KM192319	Bacteroidetes bacterium CHC2 (FN554387)	99
8	KM192320	Alpha proteobacterium 1153-i2a (KF768980)	99
	KM192321	*Sphingomonas* sp. enrichment culture clone MRHull-Fe-12D (KC404011)	100
	KM192322	Uncultured Verrucomicrobia bacterium clone LJ-J99 (JF319253)	98
9	KM192323	Hyphomicrobiaceae bacterium LX49 (KC921159)	100
	KM192324	*Pseudomonas fluorescens* strain HR95 (JF700468)	99

Table 4. Plant growth-promoting (PGP) traits of selected isolates

Isolates [a]	ACC deaminase activity (¼M ±-KB mg^{-1} h^{-1}) [b]	Protease activity (U mL^{-1}) [bc]	IAA production (mg L^{-1}) [b]	Inorganic-phosphate-solubilizing activity (mg P L^{-1}) [b]	Organic-phosphorus-solubilizing activity (mg P L^{-1}) [b]	Potassium-solubilizing activity (mg K L^{-1}) [b]	Nitrogen-fixing ability (mg TN mL^{-1}[bd]
Sphingobacterium sp. K-1	2.95±0.74	ND [e]	ND	26.83±1.62	ND	ND	41.15±4.03
Sphingobacterium sp. SY-1	3.33±0.82	ND	8.52±0.77	ND	63.69±2.21	ND	56.92±3.13
Chryseobacterium sp. K-2	1.94±0.45	45.75±3.63	10.56±0.63	22.56±1.73	ND	3.42±0.73	62.15±2.01
Chryseobacterium sp. SY-2	2.66±0.76	56.22±2.56	78.34±3.12	16.04±1.13	ND	2.38±0.61	70.72±2.75
Klebsiella sp. K-3	4.96±0.86	42.84±1.34	52.42±1.05	46.48±3.68	26.56±3.04	2.13±0.22	86.13±5.73
Klebsiella sp. SY-3	5.02±1.01	30.53±3.49	77.24±2.63	6.56±0.91	42.62±2.33	ND	79.21±2.92
Enterobacter sp. K-4	3.24±0.67	ND	34.56±2.52	3.53±0.82	41.62±3.34	2.52±0.21	63.29±3.11
Enterobacter sp. SY-4	3.22±0.54	21.42±1.67	75.77±4.24	ND	ND	1.69±0.15	37.58±2.81
Acinetobacter sp. K-5	4.24±0.73	84.55±4.83	14.84±0.98	1.75±0.63	ND	ND	22.40±1.24
Acinetobacter sp. SY-5	1.84±0.42	64.23±3.57	12.62±1.60	0.95±0.35	ND	ND	ND
Agrobacterium sp. K-6	3.56±0.88	ND	80.54±2.32	ND	6.42±0.46	2.62±0.43	48.26±2.60
Agrobacterium sp. SY-6	2.67±0.23	ND	26.27±1.64	ND	ND	ND	35.31±2.71
Pseudomonas sp. K-7	3.82±0.55	60.64±7.36	22.51±0.62	103.42±6.42	56.62±2.62	ND	28.17±1.86
Pseudomonas sp. SY-7	2.95±0.42	14.33±2.61	ND	37.54±2.26	39.82±1.93	2.71±0.72	ND
Stenotrophomonas sp. K-8	1.46±0.54	44.35±2.74	ND	ND	ND	ND	50.91±3.23
Stenotrophomonas sp. SY-8	2.35±0.53	16.56±0.88	43.27±4.23	52.23±2.62	ND	ND	ND
Sphingobium sp. K-9	4.23±0.84	35.54±1.62	6.32±0.56	53.74±2.05	ND	1.27±0.24	ND
Sphingobium sp. SY-9	0.76±0.11	20.76±1.95	ND	ND	ND	ND	ND

[a] In each OTU, two strains were selected randomly to test their PGP traits.[b] Values are expressed as means±SD, four repeats for each isolate.[c] One unit (U) of protease is defined as the amount of enzyme that hydrolyses casein to produce equivalent absorbance to 1 µg of tyrosine per min.[d] TN indicates total-N in culture solution, including both bacterial biomass-N and soluble-N.[e] ND means no detection.

nonparametric estimation further revealed that Operational Taxonomic Unit (OUT) richness decreased in response to the shift from early to late successional communities in the marshes[36]. So, it is logical that microbial factor may be important to plant succession. From this work, we negate the possibility of the positive role of ADPB around rhizosphere in plant succession. Nevertheless, we suggest another possibility that endophytic bacteria especially those dormant bacteria in plant seeds may have more diverse and abundant ADPB, which may play some roles in plant succession. In plant succession area, a study showed that OUT richness decreased in response to the shift from early to late successional communities in the marshes[36]. But in our study, we found the opposite results (Table 1), which may be due to the elimination of impact of different soil characterstics on ADPB communities, for soil samples were all collected within the reserve rather than from different successional areas.

Comparison of dominant bacterial communities with- or without enrichment culture

Given that plants in salt stress environment can produce large amount of ACC[33], thus by enrichment with ACC, the changes in dominant bacterial community can partly reflect those may be beneficial to helping plants to resist salt stress. Before enrichment, the dominant bacterial genera of PA, IK and SS were *Pseudomonas* and *Sphingomonas* (Fig. 1, Table 3). Band 4 of SS, an uncultured bacterium clone revealed by sequencing, which in summer was more obvious than that in winter. There were also some specific bands existing in both certain sampling season and plants, for example, band 5 and band 6 were specific in PA-W and PA-S, which indicated that *Flavobacterium* was specific in rhizosphere of PA in winter, whilst *Sphingobium* was specific in rhizosphere of PA in summer. However, there was no similar result from bacteria culture experiment that corresponding to DGGE, because neither *Sphingobium* nor *Flavobacterium* was isolated from PA rhizosphere by enrichment screening.

For both understanding the changes in dominant bacterial community and screening for ADPB, we use traditional culture method to perform the experiment. Although there were many ADPB belong to *Pseudomonas* genus reported[10,32,41], yet it was not dominant in culturable ADPB communities (Table 1). *Sphingomonas*, a seldom reported ADPB, were founded both in DGGE pattern and culturable ADPB communities despite it was not the absolutely dominant genus. To our surprise, *Klebsiella* became the overwhelming dominant genus in culturable ADPB communities of three plants rhizosphere, which was not remarkable in DGGE pattern at all. Although *Klebsiella* is the rare genus in rhizosphere without enrichment as revealed by DGGE, it may represent a reservoir of genetic diversity and be metabolically active, which is capable of responding rapidly to environmental change[42-44], such as enrichment with ACC in this study. When under stressed environment, excessive ACC produced by plant may be a certain inducer to promote these previously rare genera such like *Klebsiella* to be dominant. It is also possibly because many *Klebsiella* bacteria have nitrogen-fixing abilities[45,46], as a result, these bacteria may grow faster and thus generate competition against other microbes, but if so, the selective medium seems not so suitable to isolate diverse ADPB and needs to be improved in future.

Among the isolates, *Sphingobacterium* sp. (KM192325), to our knowledge, was the first reported isolate with the ability of producing ACC deaminase, which were always focused on their ability of degration of and pesticide[47,48]. The ability of producing ACC deaminase is a possible factor for adaption to stressed environments such as petroleum or pesticide contaminated soils, the function of ACC deaminase production in degration of contaminants should be proven by generating knock-out and complementation strains targeting ACC deaminase encoding genes.

It is worth mentioning that the use of amplifed ribosomal DNA restriction analysis (ARDRA) may lead to an overestimate of bacterial population without sequencing, because of the multiple heterogeneous 16S rRNA gene copies[49]. In this work, taking *Klebsiella* as an example, if without sequencing, OTUs would be overestimated by up to ten fold only through ARDRA patterns (data not shown). So, care must be taken when using those methods based on 16S rRNA gene phylogenetic diversity.

ADPB isolates and their plant growth-promoting (PGP) traits

With the enrichment culture method, a total of 116 isolates were screened for both comparison and further determination of PGP abilities, from which, 9 OTUs were obtained by ARDRA analysis followed by sequencing. We randomly selected two strains in each OUT and thus altogether 18 isolates were prepared for further study.

As exhibited in Table 4, all the selected isolates showed different extent of ACC deaminase abilities (ranging from 0.76 to 5.02 μM α-KB mg^{-1} h^{-1}), indicating the feasibility of isolation of ADPB by enrichment and selective culture with ACC as the sole nitrogen source[12]. Both strains of *Klebsiella* spp. exhibited the highest ACC deaminase activity among 18 strains. Compared with other publications[1,2,12], this is the first report showing that *Klebsiella* is the dominant genus with great potential for producing ACC deaminase in Sheyang Reserve, China. In addition, *Klebsiella* strains have relatively comprehensive coverage of PGP traits especially in ACC deaminase, IAA synthesis and nitrogen fixation, which may be an important factor in plants growth under salt stressed and barren soil condition. The high accumulation of IAA can induce the transcription of plant enzyme ACC synthase that catalyzes the formation of ACC, which along with other small molecule components of root exudates are exuded into the rhizosphere[50], and thus stimulate the population of ADPB. It is obvious that every strain has more than one PGP trait, suggesting they may be promising resources for further explorations in soil remediation, plant growth-promoting agents as well as some industrial applications.

CONCLUSION

This work uncovered diversity and abundance of bacteria with ACC deaminase activities in three native plant rhizospheres in the Red-crowned Crane National Nature Reserve, China, revealed by culture-based method and DGGE technology. A total of 116 isolates (classified as 9 OTUs) with ACC deaminase activities were screened for further developments. The stimulation of ACC leads to changes in dominant genus from *Pseudomonas* to *Klebsiella* in rhizospheres. *Sphingobacterium* sp. (accession number: KM192325) is the first reported isolate with the ability of producing ACC deaminase.

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