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Classification of Potassium-releasing Bacteria Isolated from Four Agricultural Soil Samples

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Bacteria capable of potassium-releasing are spread among phylogenetically diverse groups. The purpose of this work was to study the microbial diversity and potassium-releasing capacity of potassium-releasing strains isolated from four rhizosphere soil samples of tobacco from Luzhou, Sichuan Province, China. The density of potassium-releasing bacteria in the S soil sample was significantly higher than in others. Potassium-releasing bacteria isolated from four soils were grouped into 18 strains according to ARDRA. One colony was picked to culture and sequencing from each of the 18 strains to construct the phylogenetic tree, and the tree obtained from the sequences revealed four major branches exhibiting highest identity to the following genera: (i) Bacillus, (ii) Enterobacter, (iii) Pseudomonas and (iv) Providence. Majority of the strains with high potassium-releasing efficiency belong to *Bacillus*, and JK01 (*Bacillus mojavensis*) possesses the highest capability of potassium-releasing (95.01% higher than the control), it has greater potential to serve as plant growth promoting rhizobacteria.

Key words: Soil, Potassium, Isolation, Classification, Capability.

Potassium is an element essential for the growth and development of crop[1]. The deficiency of plant-available potassium is generally considered as a major influencing factor to food production in many agricultural soils[2]. Along with the rapid development of world agriculture, crop removal, leaching, runoff and erosion caused the rapid reduction of the available potassium level in soils[3]. To solve this problem, huge amount of potassium fertilizers are commonly applied to replace the removed minerals and to optimize yield which may cause soil hardening.

Although plant-available potassium is deficient, the total soil potassium reserves are not low in most soils with a content of 1.0~2.5% (based on K_2O) in general, and up to 4.0% in some places[4]. Rock potassium materials are cheaper sources of potassium, and direct application of rock potassium materials may be agronomically more useful and environmentally more feasible than potassium fertilizers[5-6]. However, most of them are not available to a plant because the minerals are released slowly[7]. Besides, many soil microorganisms can markedly modify potassium solution and translocation, such as *Bacillus mucilaginosus*, *Bacillus edaphicus* and *Bacillus subtilis*[8-10]. Therefore, as an alternative approach, it is potential to increase the level of avaliable potassium by fertilizing bacteria which can release insoluble potassium in soil.

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However, few studies have been performed to determine the taxonomic diversity associate with the potassium-releasing capability of the potassium-releasing bacteria isolate from rhizosphere soils of plants[11-14]. In these studies, in addition , most reports have just focused on taxonomic diversity or potassium-releasing capability alone, and have not investigated the relevance.

In this study, bacteria with capability of potassium-releasing were isolated from four soil samples, and all the potassium-releasing colonies we obtained were distinguished by amplified ribosomal DNA restriction analysis (ARDRA). One colony of each ARDRA type was selected to sequence the 16S rDNA for classification. Moreover, in order to evaluate the applicability of potassium-releasing bacteria we isolated, a test of different strains was conducted. We expected to find one strain which is suitable for agricultural application with higher potassium-releasing activity by phylogenetic analysis and potassium-releasing capacity evaluation.

MATERIALS AND METHODS

Soil samples collection

The rhizosphere soil (soil adhering strongly to the root) samples of tobacco were collected from farmland of four reigns: Jinxing (J), Shibao (S), Dazhai (D) and Shuikou (K) in Luzhou, Sichuan Province, China. And all the farmlands had been cropped tobacco plants for at least ten years. Rhizosphere soil samples were collected by uprooting the root system and placed in ice for transport[15-16].

Bacteria enumeration, isolation and culture

Bacteria in soils were enumerated by the spread plate method. Each dilution had three replicate and incubations were performed at 30 °C for 5 days on the plate[16]. Potassium-releasing bacteria (PB) were enumerated on plate with selective medium as follows: 10 g Sucrose, 0.5 g Yeast extract, 1 g $(NH_4)_2SO_4$, 2 g Na_2HPO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 1 g $CaCO_3$, 1g Potash feldspar (9.60% K_2O), 15 g Agar and 1 L sterile water. And total soil bacteria (TB) was enumerated on plate with LB agar medium with following composition: yYeast extract 0.5 g, Tryptone 1.0 g, NaCl 1.0 g, Agar 1.5 g and 100 mL sterile water. Bacteria colonies with transparent zone around after 5 days' culture were selected and purified on the same selective medium and these colonies were considered as potassium-releasing strains.

DNA extraction and PCR amplification

Total genomic DNA was extracted as template DNA for PCR (Polymerase Chain Reaction) from each potassium-releasing bacteria with Genomic DNA Isolation Kit (Sangon Biotechnology, China). And 16S rDNA was amplified with the F (Forward) primer of 5'-GCAGGCCTAACACATGCAA-3' and R (Reverse) primer of 5'-GTTACGACTTCACCCCAGTCAT-3'. The reaction mixtures was as follows: 1 μL template DNA, 5 μL 10×PCR buffer, 0.5 μL of each primer, 5 μL dNTP, 1μL Taq DNA polymerase(Takara, Japan), and 37 μL ddH_2O. Thermal cycling was carried out under the following conditions: 95 °C for 2 min, 35 cycles of 95 °C for 10 s, 58 °C for 30 s, 68 °C for 1 min, and a final extension at 68 °C for 10 min[17].

ARDRA

Isolation of chromosomal DNA and PCR amplification of 16S rDNA were performed as described before. The PCR products (10 uL) were digested with the restriction endonucleases AluI and RsaI (Appligene, France) for 16 h at 37°C and BstUI (Ozyme, France) for 4 h at 60°C and the obtained fragments were then separated on a 2-3.5% (w/v) Metaphor agarose gels, depending on the fragment sizes to be distinguished. Restriction profiles were classified according to the presence or absence of fragments for each enzyme.

Sequencing and data analysis

The amplified 16S rDNA products were purified with gel DNA purification kit (Bio Basic, Canada) from agarose gel after subjected to electrophoresis, cloned into pGEM-T Easy Vectors (Promega, USA) and transformed into Escherichia coli DH-5α competent cells (Tiangen, Beijing). The positive transformants were screened out through blue-white selection on agar media containing X-gal and IPTG. And positive clones were sent to sequencing (Sangon Biotech Co., Ltd.).

One colony from each type was picked, purified and cultured for sequencing. And a total of 18 amplified 16S rDNA PCR products were sequenced. And these 16S rDNA sequences were compared to the GeneBank daTablease with BLAST (Basic Local Alignments Search Tool) of NCBI (National Center for Biotechnology Information)

to obtain preliminary phylogenetic affiliation of the strains. Species closely related to our isolate were integrated for phylogenetic tree construction. The 16S rDNA gene sequences were aligned using ClustalX 2.0 program, and the phylogenetic tree was constructed using neighbor-joining (NJ) algorithm of MEGA 4.1[18-19].

Potassium-releasing capability determination

Three biological replicates was carried out to measure their potassium releasing capacity. Details was described as follows. Fifty milligram potash feldspar was mixed with 50 mL nutrient medium and pH value was adjusted to 7.0 before steam sterilization. The culture media were inoculated with 100 μL bacterial suspension (activated for two times before inoculated) after sterilized at 115 °C for 30 minutes, and culture media without inoculation was considered as control. After culture with shaking for 5 days at 35 °C, bacteria suspension was treated with 6% H_2O_2 in water bath(95 °C), and then filtered to separate mineral powder residues from the cells and media. The filtrate solution were digested with 30% H_2O_2 in water bath(95 °C), collected and diluted to a volume of 50ml with distilled water. The potassium concentrations in the solution were measured by flame spectrophotometry.

RESULTS

Chemical characteristics of forest soil sample (Table.1)

Some properties of the soils in four regions used for isolation of potassium-releasing bacteria are given in Table.1. The pH value in J ,S ,D and K samples are 6.42 , 6.00 , 6.71 and 7.08 , respectively. We observed that the S soil sample with a lower pH value.

Enumeration of total bacteria and potassium-releasing bacteria

Table.2 shows the densities of total bacteria and potassium-releasing bacteria in the four soil samples. Among them, the soil sampling from S shows the highest potassium-releasing bacteria density with 1.767×10^7 bacteria$\cdot g^{-1}$ in dry soils. In addition, J, D and K soil samples show similar densities of both total bacteria group and potassium-releasing bacteria group, representing nearly $2\sim4\times10^8$ and $6\sim9\times10^6$ bacteria$\cdot g^{-1}$, respectively (Table 2). One hundred and twenty-one colonies with transparent zone were screened out from 3984 bacteria colonies. The percentages of potassium-releasing bacteria in the total bacteria are 2.37%, 6.24%, 2.34% and 1.80% in J, S, D and K soil samples, respectively. And we found that the PB/TB ratio is higher than in the soils with lower pH value (Table 1, Table 2).

ARDRA

Stringent PCR conditions allow amplification of a single DNA fragment. All the 18 colonies yielded a band ranging from 1050 to 1400 bp (base pair) after amplification. Potassium-releasing isolated from all soils were grouped into 18 ARDRA types according to the similarities of the restriction patterns obtained (Fig. 1). It is apparent that the fore major branches can be consistently associated with fore major groups: (i) B, F, I; (ii) K, N, O, P, R; (iii) A, C, D, L; (iv) G,J,Q. Every minor group is not associated with any other major groups and the group showes low similarity with the other major groups. The number of individual colonies found in each ARDRA type appears to be highly variable. Of them, A, K and L contain 11, 13 and 15 colonies, respectively, while F, Q and R contain only 1 or 2 colonies (Fig. 1). Table 3 shows that the distribution of each type is variable, too. Colonies isolated from J, S, D and K distributed into 10, 16, 12 and 10 ARDRA types, respectively. Some ARDRA types (A, H and K) are observed at all the four sampling soils, but type G and R present only in J soil, and type F presents only in S soil, type G presents only in J soil (Table 3).

16S rDNA sequence analysis

Potassium-releasing bacteria isolated from all soils were grouped into 18 strains based on their colonial morphology, and we numbered them JK01 to JK18 (A-R). One colony from each strains was selected for 16S rDNA sequencing analysis. And these sequence informations were deposited to GenBank of NCBI with accession numbers of KF135453-KF135466 (JK01-JK14), KF148637-KF148638 (JK15-JK16) and KF181164-KF181165 (JK17-JK18). Phylogenetic trees were generated with 16S rDNA sequence of these strains as well as their close related strains (Fig.2). It shows that these strains could be clustered to four major genera, including JK01, JK03, JK04, JK05, JK07, JK08, JK10, JK12, JK13 and JK17 for group I (*Bacillus*); JK11, JK14, JK15, JK16 and JK18 for

Table 1. Basic properties of the rhizosphere soil used in this study

Soil Sample	Location	pH	Organic carbon(mg·L^{-1})	Inorganic carbon(mg·L^{-1})	Soluble nitrogen(mg·L^{-1})
J	27º54'N, 106º03'E	6.42	2.043	2.720	8.455
S	27º53'N, 106º09'E	6.00	1.928	2.033	1.452
D	28º07'N, 105º38'E	6.71	6.986	0.573	6.914
K	27º50'N, 106º14'E	7.08	2.147	4.089	3.612

Table 2. Enumeration(bacteria numbe·g^{-1} soil) of total bacteria (TB) and potassium-releasing bacteria (PB) from soils J, S, D and K and the relative abundance of the potassium-releasing bacteria of each soil

Soil sample	J	S	D	K
TB(×10^8)	3.24	2.83	3.70	3.51
PB(×10^6)	7.67	17.67	8.67	6.33
PB/TB(%)	2.37	6.24	2.34	1.80

Table 3. Isolate number from the soil samples J ,S ,D and K, classified by Gram typing, sequence analysis and ARDRA typing. A Gram deduced from sequence analysis

Gram[a]	Sequence analysis	ARDRA Group(i)	J	S	D	K	Total
+	Bacillus	A	5	3	1	2	11
		C	0	3	2	2	7
		D	1	4	2	0	7
		E	4	2	0	0	6
		G	3	0	0	0	3
		H	1	3	2	2	8
		J	2	6	2	0	10
		L	0	7	5	3	15
		M	0	4	3	1	8
		Q	0	1	0	1	2
		Group(ii)					
-	Providencia	B	0	1	1	3	5
		Group(iii)					
-	Pseudomonas	K	2	7	2	2	13
		N	0	4	3	1	8
		O	2	3	0	2	7
		P	2	1	2	0	5
		R	1	0	0	0	1
		Group(iv)					
-	Enterobacteria	I	0	2	1	0	3
		Group(v)					
-	Unknown	F	0	2	0	0	2
		total	23	53	26	19	121

+:positive -:negative

group II (*Pseudomonas*); JK09 for group III (Enterobacter) and JK02 for group IV (Providencia). In addition, it could be found that group I (*Bacillus*) contains more colonies than other groups both in types and numbers (Fig. 1, Fig. 2). And we didn't found a species which is similar to JK06, and we'll

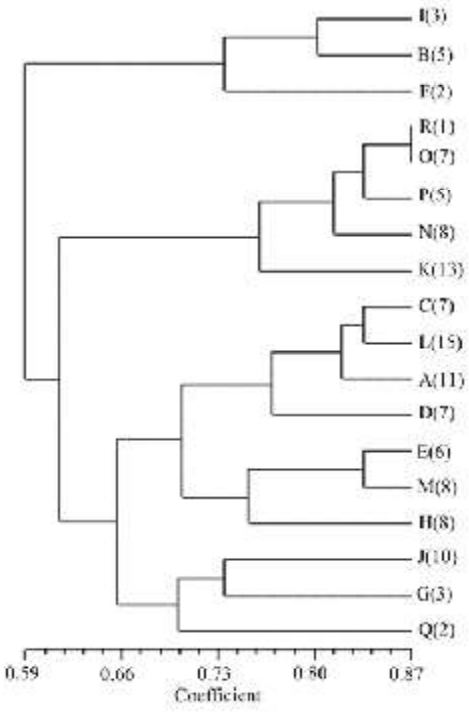

Fig. 1. Dendrogram of the different potassium-releasing isolate types. The dendrogram was based on ARDRA patterns obtained from soils J, S,D and K. The letter indicates the ARDRA type and the numbers in parentheses are the numbers of colonies for each type

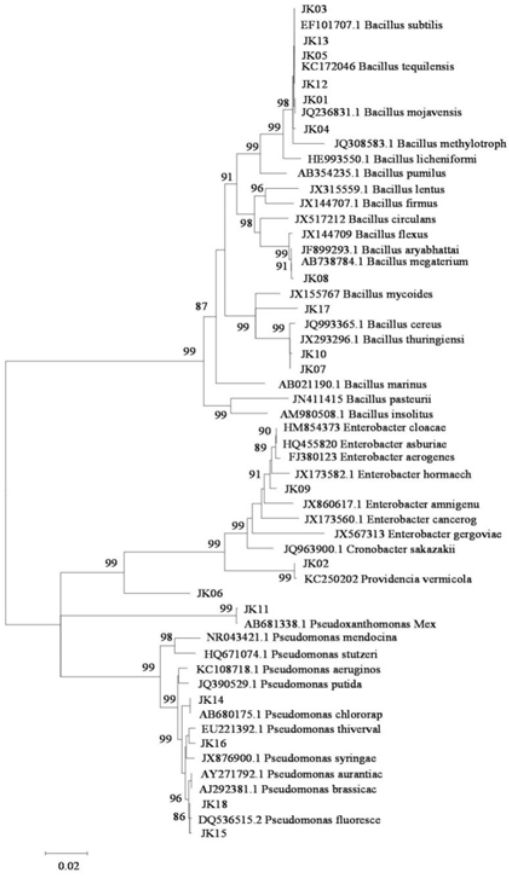

Fig. 2. Phylogenetic tree based on analysis of the 16S rDNA sequences of the potassium-releasing strains (JK01~JK18). Closest related strains, and strains represented with accession number from GeneBank, are integrated. Bootstrap percentage values of 85% or more, obtained from the 1000 re-samplings of data set, are given at the nodes

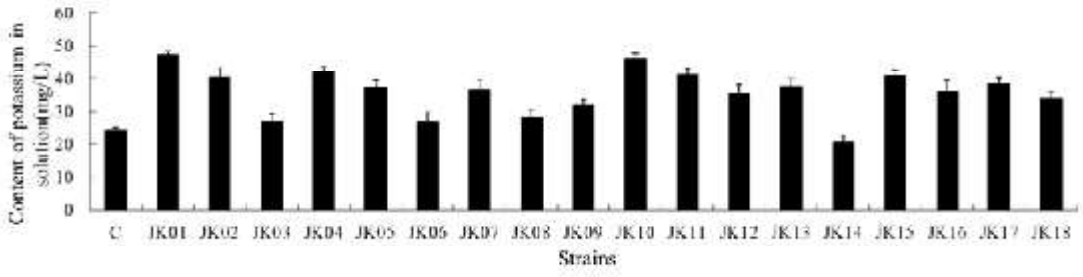

Fig. 3. Results of potassium-releasing capability determination of different strains. Content of potassium in solution after deal with H_2O_2 shows potassium-releasing capacities of different strains. C is control (non-inoculation) and others are inoculated with corresponding bacteria

do further research about it in next study.

Potassium -releasing capability of different strains

All the eighteen strains were selected to measure their potassium-releasing capability and 17 of them (94.4%) showed potassium-releasing capacity with potassium content increased ranged from 10.19% (JK06) to 95.01% (JK01). And the content of potassium in solution of JK14 was similar to the control. In addition, JK01, JK04, JK10 and JK11 showed higher potassium-releasing capacities than other strains (Fig.3), and most of them belong to *Bacillus* genera (Fig.2).

DISCUSSION

Spread plate method analysis and ARDRA in the four soils revealed higher densities and more types of potassium-releasing bacteria in the S soil samples than in J, K and D soil samples, though the densities of TB were similar in four samples (Table 2, Table 3). We observed S soil samples have a lower pH value, this may associate with production of carboxylic acids like citric, tartaric and oxalic acids by potassium-releasing bacteria[9]. And some studies found potassium-releasing ability was positively related to the decreasing of pH value during culture process[20]. The composition of potassium-releasing community may also associated with the influence of plant roots, a variety of sites, soil type, and environmental factors[16]. The difference in relative abundance of the potassium-releasing bacteria indicates a big difference among soil samples, and suitable soil is important for isolating potassium-releasing bacteria. In our study, a PB to TB ratio (3.04%) was obtained with 121 potassium-releasing colonies isolated out of 3984 colonies. And we got 17.67×10^6 bacteria in one gram dry soil, it's higher than we got in our previous study[21].

Until recently, there is only a few studies focusing on taxonomic diversity associate with potassium-releasing capability of potassium-releasing bacteria in the rhizosphere soil of tobacco. In this study, 16S rDNA-based analysis reveals that four groups relate to generas of *Bacillus*, Enterobacter, *Pseudomonas* and Providence represent most of the strains. Of them, *Bacillus*, Enterobacter and Pseudomonas have already been found in others study[22-24]. And we most interest in group *Bacillus* because, more than half of the colonies with transparent zone around belong to the *Bacillus* genera. The second genus of interest in this study is Providence genera because, there are no literatures have published to report any isolate had the potassium-releasing ability. This phenomenon indicates that ability of potassium-releasing may distribute more widely in phylogeny than found until now.

All the eighteen strains were selected to measure their potassium-releasing capability, and 17 of them (94.4%) showed potassium-releasing capacity. In addition, JK010JK040JK10 and JK11 showed higher potassium-releasing capacities than other strains (Fig.3). In addition to potassium-releasing function, these bacteria may possess other functions. For example, *Bacillus megaterium* (JK08) is usually regarded as phosphate-solubilizing bacteria[25-26]; *Bacillus thuringiensise* (JK10) is considered as a friendly safe bacterial insecticide which has been widely used[27]; *Bacillus subtilis* (JK03) has wide scope of application such as, controlling plant fungous diseases[28] and serveing as microbial feed additives[29] and Pseudomonas fluoresce (JK15) is verified to have the ability of phosphate-solubilizing[30].

Most of the strains we isolated show capacity of release insoluble potassium from potash feldspar (Fig.3). It is obvious that the potassium-releasing capacities of JK01 (95.01%), JK04 (72.99%), JK10 (90.52%) and JK11 (70.52%) are higher than other strains, and most of them (JK01, JK04 and JK10) belong to *Bacillus* genera. Bacteria belonging to *Bacillus* genera usually has high capacity of stress resistance, and could be stored for a long time[31]. JK01 (*Bacillus mojavensis*) possesses the highest capacity of potassium-releasing in our study. And in our follow-up experiments, we found JK01 can produce large numbers of spores in the later stage of growth. And this makes it to be a potential source of bacterial fertilizer used under the surface soil.

By characterizing and sequencing the potassium-releasing communities, we have identified a variety of bacteria that may be important in releasing rock potassium and the use of potassium-releasing bacteria might influence the bacterial and fungal community structure. Future work which focuses on the physiology of these bacteria as well as the edaphic factors of active

soil communities will improve our understanding of potassium-releasing bacterial that influence this process.

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