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Isolation of *Paenibacillus* sp. and Assessment of its Potential for Enhancing Mineral Weathering

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One mineral-solubilizing bacterial strain designated KT was isolated from a soil in Henan Province, China. The full-length 16S rRNA gene sequence showed 95.2 to 96.7% similarity to the bacteria of the genus *Paenibacillus*, indicating that the strain KT belonged to the genus *Paenibacillus*. The potential of this strain to release potassium from silicate minerals was investigated using a potassium-bearing rock as the sole source of potassium to support its growth. After inoculation for 7 days, the concentrations of water-soluble Al, Ca and Fe released from the potassium-bearing rock in active bacterial culture were higher than those from the control with autoclaved inoculum, but the concentration of water-soluble K in active bacterial culture was similar to that in the control. The concentrations of HNO_3-extractable Al, Ca, Fe and K from the bacterial culture were also higher than those in the control. These results showed strain KT was able to release potassium from potassium-bearing rock. These results have important implications for extraction of potassium from rocks to support plant growth.

Keywords bioweathering, potassium-bearing rock, *Paenibacillus*, potassium-solubilizing bacteria

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INTRODUCTION

Bioweathering as a common geochemical process is the erosion, decay and decomposition of rocks and minerals mediated by living organisms (Burford et al. 2003; Gadd 2007). The bioweathering process plays a fundamental role in the release of nutrients from rocks, and is associated with global climate and environmental changes (Li et al. 2006; Burford et al. 2003). Microorganisms are the main bioweathering agents and contribute to rock degradation and soil formation. Microbes also provide nutrients, such as P, K and Si, to support plant growth by changing environmental pH and oxidation reduction potential to solubilize minerals (Abdulla 2009; Buss et al. 2007; Hameeda et al. 2008; Lian et al. 2008).

K-solubilizing bacteria are able to release potassium from insoluble minerals (Sugumaran and Janarthanam 2007; Basak and Biswas 2009). In addition, researchers have discovered that K-solubilizing bacteria can exert beneficial effects on plant growth through suppressing pathogens and improving soil nutrients and structure. For example, certain bacteria can weather silicate minerals to release potassium, silicon and aluminum, and secrete bio-active materials to enhance plant growth. These bacteria are widely used in biological K-fertilizers and biological leaching (Lian et al. 2001; Bosecker 1997).

Bacteria have wide applications in mining, metallurgy, microbial fertilizer, and feed (Zhao et al. 2008; Sheng 2005; Lian et al. 2002; Li et al. 2006; Li, 2003). A wide range of bacteria, such as members of the genera *Pseudomonas* and *Burkholderia*, *Acidithiobacillus ferrooxidans*, *Bacillus mucilaginosus*, *B. edaphicus* and *B. circulans* can release potassium from potassium-bearing minerals, but only a few bacteria, such as *B. mucilaginosus* and *B. edaphicus,* have high activity in mobilizing potassium from minerals (Zhao et al. 2008; Sheng 2005; Lian et al. 2002; Li et al. 2006; Li 2003). Therefore, it is imperative to isolate more species of mineral-solubilizing bacteria

to enrich the pool of microbial species and genes as microbial fertilizers, which will be of great benefit to the development of ecological agriculture.

The genus *Paenibacillus*, within which *P. polymyxa* is the type species (Ash et al., 1993), can fix nitrogen, suppress plant pathogens, increase soil porosity, and release phosphorus from soil minerals, and is thus considered as an important member to promote plant growth (Rivas et al. 2005; Timmusk et al. 2009). The bacteria within this genus can also promote rock dissolution (Zhou et al. 2008; Zhou et al. 2007). In this paper, we report the isolation of one strain of *Paenibacillus* from soil, using a medium in which potassium-bearing rock (PBR) powders were the sole potassium source. Certain physiological characteristics, the phylogenetic position, and the weathering activity of the strain were described.

MATERIALS AND METHODS

Materials

Soil and PBR. A homogeneous fluvo-aquic soil sample with pH of 7 was collected from a maize field of Xinxiang, Henan Province, China, in October 2009 by mixing three subsamples from an area (0–10 cm depth and 4 cm diameter).

A piece of PBR was collected from Luoyang, Henan Province, China. The rock was crushed and sieved to pass through 0.074 mm mesh size. The mineral composition and the elemental composition of the rock were determined using the K-value method of X-ray diffraction (Rigaku, D/MAx-2200) (Li et al. 2003) and X-ray Fluorescence (Axios, PW4400), respectively, in the Institute of Geochemistry, Chinese Academy of Sciences (Table 1 and Table 2).

Culture media. The medium for isolating mineral-solubilizing bacteria (called isolation medium) was the Aleksanderov's medium(sucrose 5.0 g, $MgSO_4$ 0.5 g, $CaCO_3$ 0.1 g, $Na_2HPO_4{\cdot}12H_2O$ 2.0 g, $FeCl_3{\cdot}6H_2O$ 0.005 g, potassium-bearing rock powders 1.0 g, agar 18.0 g, H_2O 1.0 L, pH 7.0–7.2) (Li et al. 2008), a common medium used to isolate silicate-weathering bacteria. After isolation, the isolate was routinely grown using a similar medium but containing nitrogen sources (sucrose 10.0 g, yeast extract 0.2 g, $(NH_4)SO_4$ 0.5 g, $CaCO_3$ 1.0 g, $MgSO_4$ 0.5122 g, KCl 0.1 g, $Na_2HPO_4{\cdot}12H_2O$ 2.507 g, H_2O 1.0 L, pH 7.0–7.2) (Zhou et al. 2010) to ensure high growth yield (called growth medium). The medium used for rock weathering, called experimental medium, was minimal K-limited salt medium (sucrose 10.0 g, $(NH_4)SO_4$ 0.5 g, $CaCO_3$ 1.0 g, $MgSO_4$ 0.5122g, NaCl 0.1 g, $Na_2HPO_4{\cdot}12H_2O$ 2.507 g, H_2O 1.0 L 1.0 L, pH 7.0–7.2) (Sheng and Huang 2002; Sheng et al. 2008).

Methods

Isolation of K-solubilizing bacteria

Five grams of the homogeneous soil sample were placed into a conical flask, immersed with sterile distilled water, and shaken at 200 rpm for 10 min at 28°C to detach bacteria from soil particles. The liquid mixture was allowed to stand for 20 minutes to obtain the microbe-containing supernatant. The supernatant was serially diluted, and the aliquots of each dilution were spread on plates of Aleksanderov's medium and incubated at 28°C for 2–3 d. Colonies were selected and further purified on these plates. The colonies were stored at 4°C until further use.

Physiological and biochemical characteristics

Physiological and biochemical tests, including gram staining, nitrate reduction, amylohydrolysis, Voges–Proskauer reactions, and catalase activities, were performed according to the method of Wang and Han (Wang et al. 2005). The ability of the isolate to reduce nitrate was tested by incubating the isolate in a nitrate saline peptone water medium for 24 h at 37°C. The amylohydrolysis test was performed using a simple medium with starch to examine the ability of the isolate to produce certain exoenzymes that can hydrolyze starch.

Iodine was added to the incubation plate, and clearing around colonies indicates that the organism is able to hydrolyze starch. The Voges-Proskauer reaction was determined by adding 20 drops of 40% KOH and an equal volume of α-naphthol to a culture grown in MR-VP broth and then incubating for 24 h at 37° C. The change of the solution's color indicates presence or absence of the reaction. Catalase activity was assayed by mixing a pellet of fresh culture from agar slant medium with a drop of 5% hydrogen peroxide.

DNA extraction and PCR amplification of 16S rRNA gene. After the isolate was grown in the inoculum medium at 28°C for 20 h, the culture was transferred to a centrifuge tube (2 ml), and centrifuged at 10,000 *g* for 10 min to concentrate cells into a pellet for extraction of bacterial genomic DNA. DNA was extracted using a Bacterial Genomic DNA Extraction Kit (Omega, US) according to the manufacturer's instructions.

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using bacterial universal primers 16S-fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S-rD1 (5′-ACGGTTACCTTGTTACGACTT-3′) (Weisburg et al. 1991). Each PCR mixture contained 0.4 mM of deoxynucleoside

TABLE 1
Mineralogy of the PBR used in this study

Minerals	K-Feldspar	Quartz	Hematite	Montmorillonite	Illite	Hornblende
Percent	70.99	23.45	3.62	1.66	0.15	0.13

TABLE 2
Element composition of the PBR

Composition	SiO_2	Al_2O_3	Fe_2O_3	MgO	CaO	Na_2O	K_2O	LOI	Others
Percent	70.36	11.52	0.53	0.06	0.38	2.53	6.36	8.02	0.24

triphosphates, 0.4 μM of each primer, 3 μL of 10× PCR buffer, 2 mM magnesium chloride, 1 U of Taq DNA polymerase, and 1 μL (about 5–15 ng) of template DNA in a final volume of 30 μL. Amplification was made using a touchdown protocol. PCR was performed as follows: the annealing temperature was set at 65°C and was decreased by 1°C every cycle until reaching a "touchdown" at 55°C. The amplification program consisted of 5 min at 94°C, and 10 touchdown cycles of denaturation at 94°C for 1 min, annealing at 65°C (with the temperature decreasing 1°C each cycle) for 1 min, and extension at 72°C for 2 min, followed by 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. During the last cycle, the length of the extension step was increased to 10 min. After amplification, PCR product was analyzed by electrophoresis in 1.5% (w/v) agarose gels.

Purification of PCR product and sequencing of 16S rRNA gene. PCR product was excised from 2% low melting point agarose (Sigma, St. Louis, MO) and the DNA was purified using a Gel Isolation Kit following the manufacturer's instructions (Promega, Madison, WI, USA). Purified PCR product was sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

Phylogenetic analysis of 16S rRNA gene sequence. The obtained 16S rRNA gene sequence was compared to those in the National Center for Biotechnology Information database using the BLAST procedure (Altschul et al. 1997). The reference sequences with the highest similarities to the query were retrieved from GenBank.

The target 16S rRNA gene sequence and the retrieved reference sequences were compared by the ClustalX program (Larkin et al. 2007) with parameters set to default values. Alignments were improved by comparison to the secondary structures and any regions of uncertain alignment were omitted from the subsequent analyses. The best-fit model of nucleotide substitution for the data sets was selected using the program PAUP 4.0b10 (Swofford 2002) and Modeltest 3.06 (Posada and Crandall 1998).

Base composition and sequence variability were examined using the software package MEGA4.0 (Tamura et al., 2007). A minimum evolution (ME) phylogenetic tree with 1000 bootstrap was then constructed using the Tamura-Nei model with pairwise deletion of gaps by MEGA 4.0 package. The outgroup for the Bayesian inference was determined by the phylogenetic result of ME and the Bayesian inference was conducted using MrBayes3_0b4 (Huelsenbeck and Ronquist 2001).

Trees saved below the burn-in generations were discarded, and a majority-rule consensus tree of the remains were calculated in Mrbayes3_0b4, providing posterior probabilities for each clade. The MrBayes3_0b4 was run with the following specifications: The analysis was performed using the GTR model including estimation site's invariants with a gamma distribution (invgamma). The Markov's chains were started from a random tree for 400,000 generations, sampling the Markov chains at intervals of 100 generations. Four chains were run simultaneously, 3 hot and 1 cold, with the initial 200 cycles discarded as burn-in. The 16S rRNA gene sequence of strain KT was submitted to GenBank (HQ451895).

Rock weathering by the bacterial isolate

Preparation of inoculum. Triplicate 250 mL Erlenmeyer flasks containing 100 mL of the growth medium were autoclaved at 121°C for 30 min. After cooling, the flasks were inoculated with isolate KT and incubated at 30°C on a rotary shaker at 200 r/min for 2 days.

Weathering of the rock by the bacterium Triplicate 250 mL Erlenmeyer flasks containing 100 mL of the experimental medium and 20 g of the rock powder were autoclaved at 121°C for 30 min. After cooling, the flasks were inoculated with 5 mL of the inoculum (final concentration 3.5×10^8 cells·mL^{-1}). A sample inoculated with autoclaved inoculum (autoclaved at 121°C for 40 min) was prepared as a control. The flasks were incubated at 30°C on a rotary shaker at 130 rpm for 7 d.

Determination of water-soluble and HNO_3-extractable K, Al, Fe and Ca concentrations

To test the ability of the isolate to solubilize the rock, concentrations of water-soluble and HNO_3-extractable K, Al, Fe and Ca were determined for the samples incubated with the bacterium for 7 days with inductively coupled plasma atomic emission spectrometry (ICP-AES, Optima 2100 DV, Perkin Elmer, USA). The concentrations of these elements were also determined for the controls at time 0 and 7 days.

Water-soluble K, Al, Fe and Ca concentrations were determined using the methods described previously (Zhang et al. 2000; Hosseinifard et al. 2010) with the following modifications: the harvested culture—rock suspension was sterilized (121°C for 45 min), and after cooling filled with five millilitres of 6% H_2O_2. The H_2O_2 treated suspension was incubated at 80° C for 30 min, transferred into a 250 mL volumetric flask, and made up to 250 mL with distilled water. After the suspension was centrifuged and filtered, the supernatant was analyzed for K, Al, Fe and Ca concentrations with ICP-AES.

Nitric acid-extractable K Al, Fe and Ca concentrations were determined using the methods described previously (Zhang et al. 2000; Hosseinifard et al. 2010) with the following modifications: the harvested cell-rock suspension was dried in a flask at 80°C

in an oven to remove water followed by addition of 100 mL of 1 N HNO_3 and boiling for 10 min. The digested mixture was transferred to a 250 mL volumetric flask and diluted with water to 250 mL. Subsequently, the extract was collected following centrifugation to determine HNO_3-extractable K, Al, Fe and Ca concentrations by ICP-AES.

RESULTS

Isolation of Rock-solubilizing Bacteria

Thirty-two strains representing circular, convex, cream-colored and semi-opaque or transparent colonies were selected. One strain (named as KT) grew fast and its colonies were larger than others, so the strain was selected for the subsequent rock-weathering experiments. The colonies appeared to be sticky and elastic. Strain KT was Gram-negative and rod-shaped and its diameter was 1–2 μm. The strain was capable of nitrate reduction, but the tests of amylohydrolysis, Voges–Proskauer reactions, catalase activities were all negative.

16S rRNA Gene and Phylogenetic Relationship

The proportion of A, T, G and C of the full-length 16S rRNA gene of strain KT were 25.17%(364 bp), 19.64% (284 bp), 31.88%(461 bp) and 23.31%(337 bp), respectively. The C+G ratio of strain KT is similar to those of *Paenibacillus* in GenBank.

To identify strain KT's phylogenetic position, the 16S rRNA gene sequences of 14 bacterial species, including 7 species of the genus *Paenibacillus*, 3 species of *Bacillus*, 2 species of *Geobacillus*, *Streptococcus agalactiae*, and *Lactococcus piscium*, were downloaded from GenBank. Figure 1 showed that there were small sequence divergence values (3.3–4.8%) between strain KT and the bacteria from the genus *Paenibacillus.* Sequence divergence values of 6.8–12.8% were observed between KT and the bacteria from the genus *Bacillus.* The sequence divergences between KT and other bacteria including the members from *Geobacillus*, *Streptococcus* and *Lactococcus* were more than 13.8%.

After multiple alignments using Clustal X, the 16S rRNA gene sequences were 1456 bp (including gap) and the average divergence values among these sequences was 10.7% (p-distance). In these sequences, the average contents of A, T, C, G, A+T and C+G were 25.2%, 19.9%, 23.5%, 31.4%, 44. 1% and 54.9%, respectively. The transition-transversion ratio was 1.229 and the transition number was higher than transversion number, showing a strong transition-transversion bias. Based on

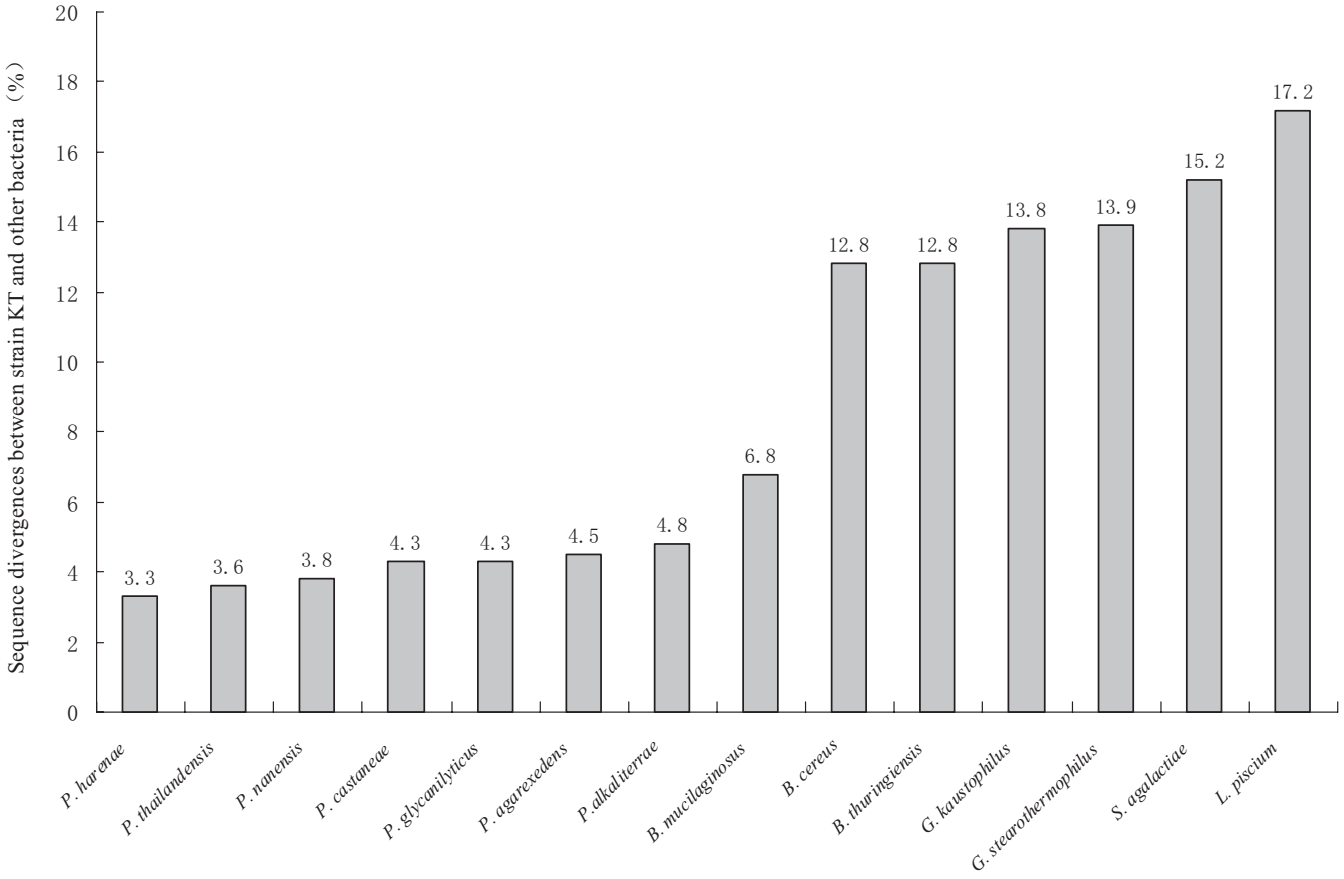

FIG. 1. The sequence divergence comparisons of the 16S rRNA genes between strain KT and other bacteria.

the results from PAUP 4.0b10 and Modeltest 3.06, the best-fit model of nucleotide substitution for the data sets was TrN+I+G (restriction of 6ST GTR model).

The Tamura-Nei's substitution model, which considered not only Ts/Tv but also the base composition, was used to perform minimum evolution (ME) analysis according to the characteristics of the sequence data set and the selection result of the substitution model using PAUP 4.0b10 and Modeltest 3.06. Strain KT and all the bacteria of genus *Paenibacillus* were clustered into a group (Figure 2), showing that KT should be a member of genus *Paenibacillus.*

The minimum evolution (ME) tree showed *S. agalactiae* and *L. piscium* were the sister group of the remaining bacteria, so they were used as the outgroups to construct a Bayesian tree. The Bayesian tree (Fig. 3) was similar to the ME tree, indicating with a high posterior probability value that strain KT was a member of the genus *Paenibacillus*, but was different from the seven bacteria of the genus *Paenibacillus.*

The Effect of Strain KT on Weathering of PBR

The effect of strain KT on weathering of the PBR was observed through changes in the aqueous concentrations of major elements (Figures 4 and 5). The concentrations of water-soluble Al, Ca, Fe and K in the experiments incubated with autoclaved inoculum (i.e., control) for 7 d were higher than those in the fresh medium. The concentrations of Al, Ca, and Fe in those experiments incubated with live inoculum for 7 d were higher than those in the abiotic control, but the concentration of water-soluble K was lower than that in the abiotic control. Likewise, the concentrations of HNO_3-extractable Al, Ca, Fe and K in those experiments incubated with live inoculum were higher than those in the two controls (Fig. 5).

DISCUSSION

Phylogeny and Identification of Strain KT

Bergey's classification of prokaryotes, well-recognized and widely used for bacterial identification, is based on the phylogeny of prokaryotes from the 16S rRNA gene. If the sequence of 16S rRNA gene of an unknown organism is >95% similar to those in the GenBank (Clarridge 2004), it is generally considered as the same genus. If the sequence of the 16S rRNA gene is >97% similar to those sequences of any cultures in the GenBack, it should be considered as the same species but may be a different strain (Embley and Stackebrandt 1994). Strain KT had a 3.3–4.8% sequence divergence from the seven related bacteria of the genus *Paenibacillus*, thus strain KT should be a member of the genus *Paenibacillus* but is a new species.

In addition, the ME molecular phylogenetic tree also showed that KT should be a member of genus *Paenibacillus.* To more

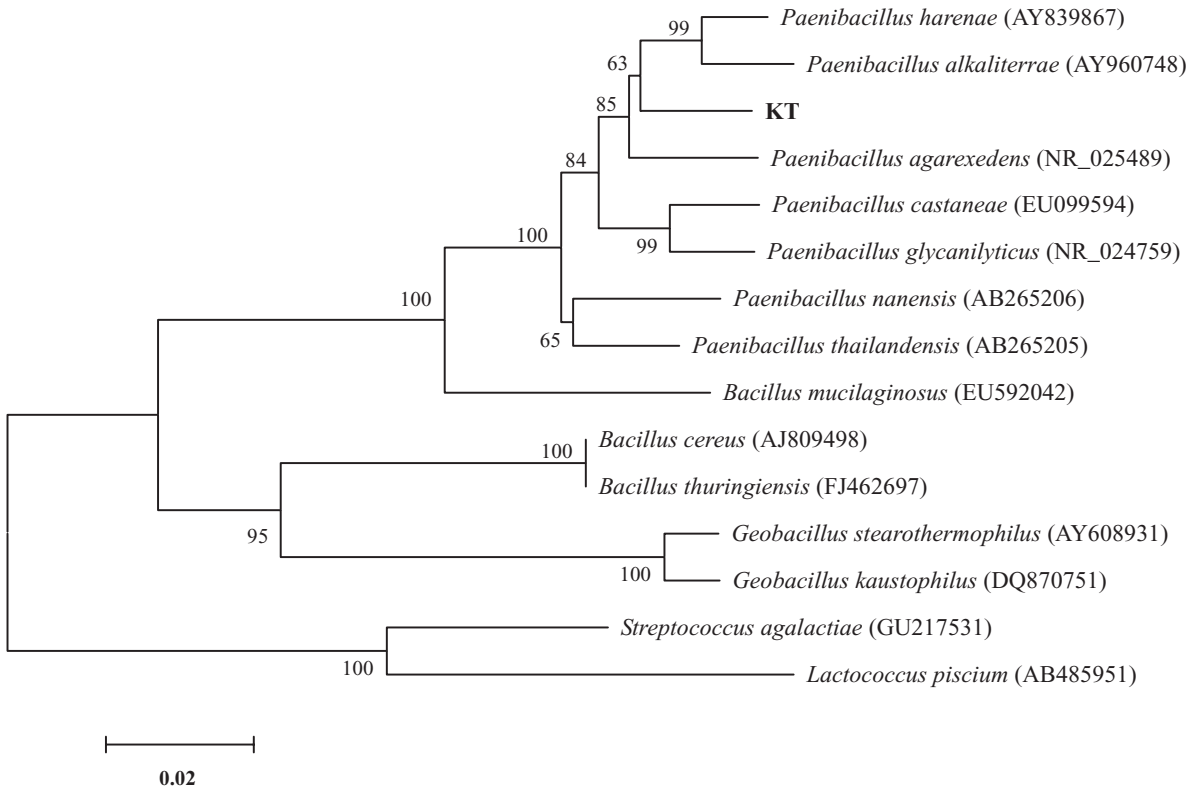

FIG. 2. The minimum evolution tree resulting from the analyses of the 16S rRNA gene sequences of strain KT and other bacteria (the numbers on the nodes correspond to percentage bootstrap values for 1000 replicates).

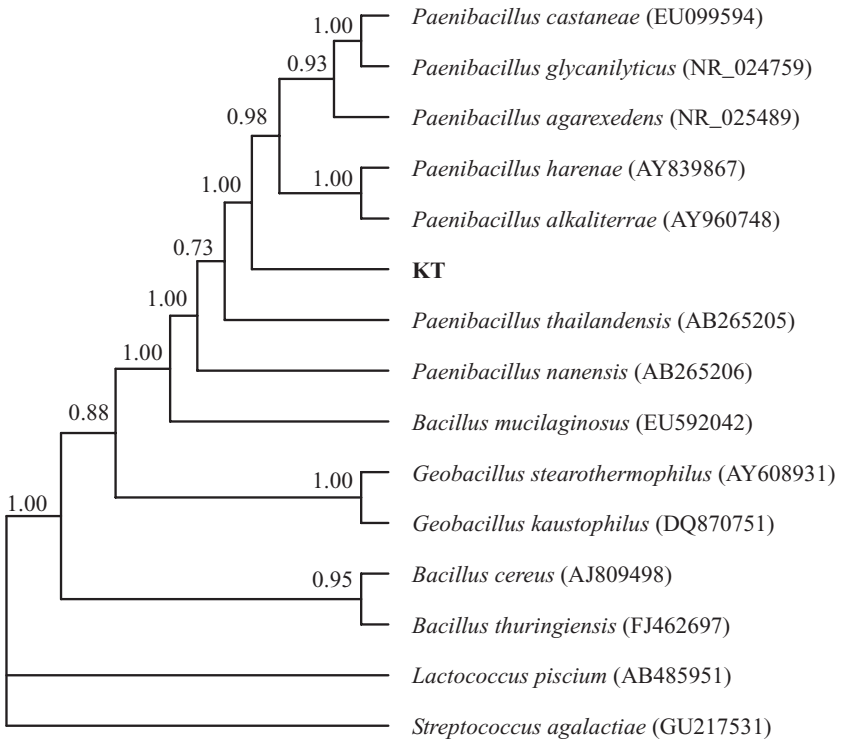

FIG. 3. The Bayesian tree resulting from the analyses of the 16S rRNA gene sequences of strain KT and other bacteria (the numbers on the nodes correspond to posterior probability values).

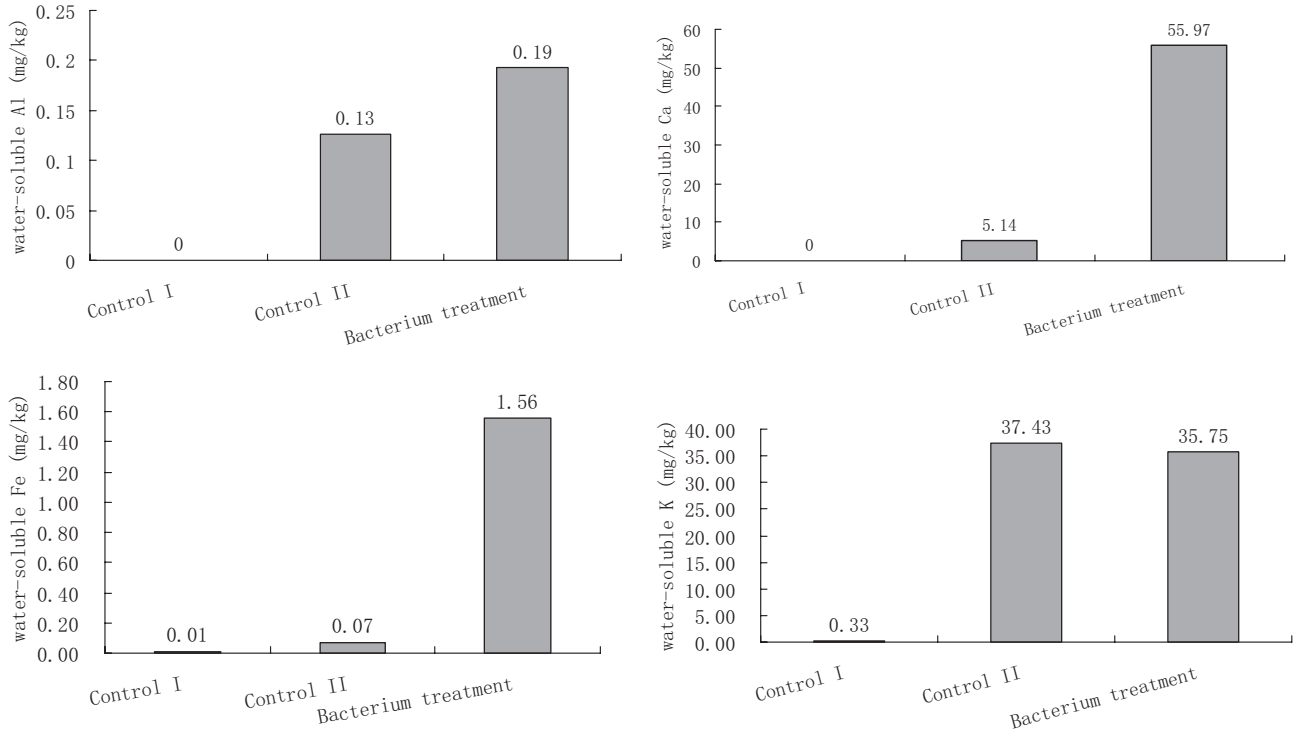

FIG. 4. The concentrations of water-soluble elements in the experiments inoculated with strain KT strain for 7 days. Each value represented the average of two independent measurements. Control I was the fresh medium with autoclaved inoculum (i.e., 0 day). Control II was the medium incubated with autoclaved inoculum for 7 days.

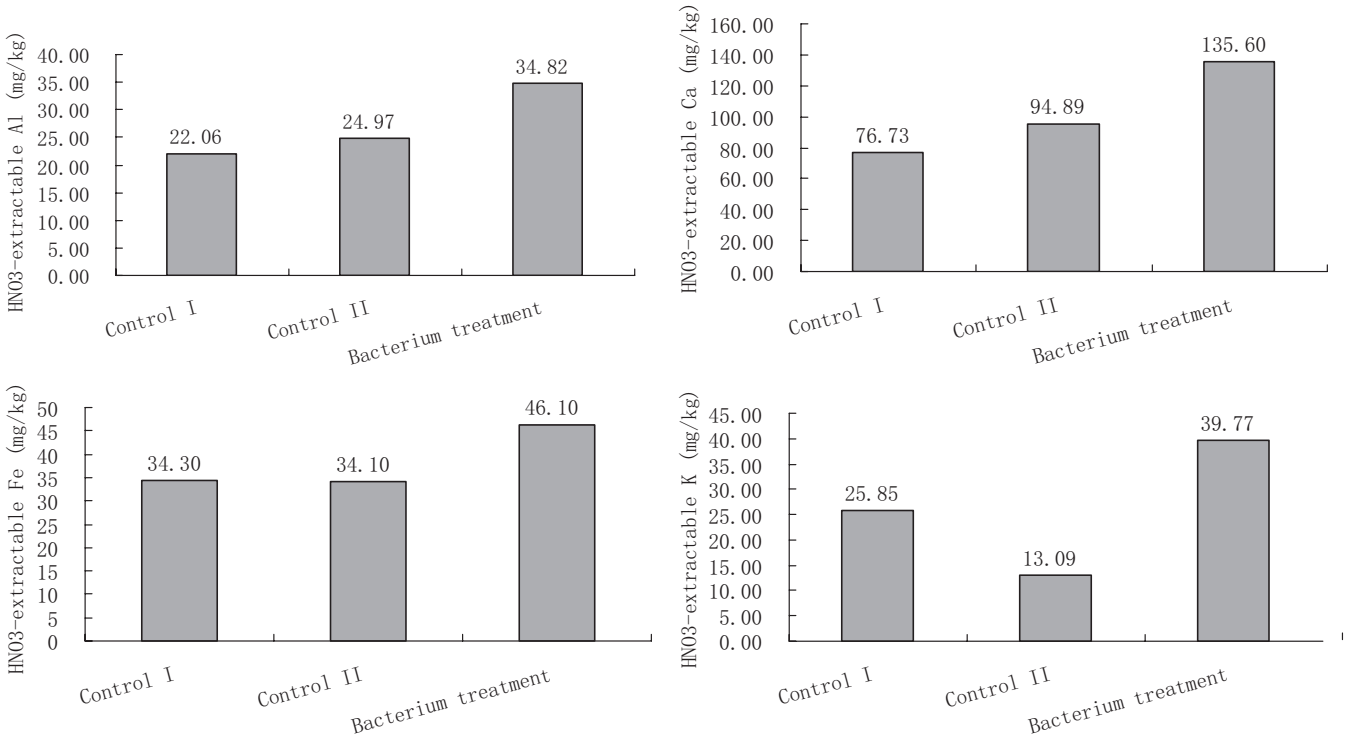

FIG. 5. The concentration of HNO_3-extractable elements in the experiments inoculated with strain KT for 7 days. Each value represents the average of two independent measurements. Control I was the fresh medium with autoclaved inoculum (i.e., 0 days). Control II was the medium incubated with autoclaved inoculum for 7 days.

accurately identify strain KT's taxonomic position, Bayesian molecular phylogenetic tree was constructed using the outgroup inferred from the result of ME analysis. Bayesian inference,based on the likelihood function, not only inherits many of the good statistical properties of the maximum likelihood method, but also has a short computation time due to the usage of a technique called Markov chain Monte Carlo (or MCMC) to approximate the posterior probabilities of trees.

Therefore, it is widely known that Bayesian phylogenetic inference outperforms some methods of phylogenetic estimation such as the maximum parsimony (MP) method, the neighbor-joining (NJ) and the minimum evolution (ME) method. The constructed Bayesian molecular phylogenetic tree was the same as the ME tree, suggesting the strain KT should be a member of the genus *Paenibacillus.* Similar to the sequence similarity values of the 16 S rRNA gene analysis, the two trees verified that strain KT was indeed a new species.

The Weathering of Potassium-Bearing Rock by *Paenibacillus*

Possible reasons for the higher concentrations of water-soluble Al, Ca, Fe and K in the medium incubated with autoclaved inoculum than those in the fresh medium may be related to hydrolysis. More importantly, the higher concentrations of water-soluble of Al, Ca and Fe in these experiments incubated with live inoculum than those of the control indicated that strain KT significantly enhanced weathering of the potassium-bearing rock.

The elements of Ca and Fe are the main components of hornblende and hematite, respectively, thus we inferred the isolate may have a strong ability to weather these two minerals. The lower concentration of water-soluble K in the experiment incubated with the live culture than that in the abiotic control was unexpected. There may be a couple possibilities. First, potassium (K) is a macronutrient and microbial demand for K is higher than Al, Ca and Fe. The isolate may have utilized some of water-soluble K to support its growth. Second, a great deal of exo-polysaccharides (EPS) excreted by bacterial cells can enwrap mineral particles and form a bacteria-mineral complex (Lian 1998; Lian et al. 2008; Chen et al. 2008), which would prevent the K release.

This explanation was supported by the HNO_3-extractable K concentration data, which showed that the live inoculum that reacted with the rock for 7d released higher amounts of K than those in the control (Figure 5) (Zhang et al. 2001; Basak and Biswas 2009). After incubation for 2 d, the medium with live strain KT was very ropy, which indicated strain KT excreted a great deal of exo-polysaccharides (EPS). After experimental tubes were dried at 80°C in an oven, the dry and dense materials formed, which may have contained EPS and other aqueous components from the media. The dry and dense material still existed after extraction with boiling HNO_3 (1mol/L). Therefore, some

EPS may still have remained even after HNO_3 extraction and may have prevented potassium extraction to some extent. The actual amount of potassium released by strain KT should have been higher than the value determined by the HNO_3 extraction.

The reason for the lower HNO_3-extractable K concentration in the experiment incubated with autoclaved inoculum than that in the fresh (starting) medium can be explained by the formation of dry and dense materials. These materials formed after culture was dried at 80°C in an oven and still remained after HNO_3 extraction. We suspect some amounts of K, including some released from PBR powders and some from the fresh medium, were trapped in these materials and could not be extracted by HNO_3.

Microbial weathering is a common geological process on earth surface. Numerous studies have documented the release of K during the weathering of silicate minerals by bacteria since the role of silicate bacteria in mineral weathering was discovered (Basak and Biswas 2009; Friedrich et al. 1991; Sheng and He 2006; Welch et al. 1999). The recent research on the microbial weathering of potassium-bearing minerals has mainly focused on the roles of *B. mucilaginosus* and *B. edaphicus* (Zhao et al. 2008; Wu et al. 2010). More recently, species of the genus *Paenibacillus* have been shown to possess the ability to solubilize some minerals and rocks such as biotite, bauxite, microperthite and basalt (Zhou et al. 2007; Zhou et al. 2008; Uroz et al. 2009).

For example, *P. polymyxa* can promote dissolution of microperthite by direct and indirect mechanisms and enhance the release of K, Al and Si from the mineral (Zhou et al. 2007). *P. polymyxa* and its metabolites are able to remarkably promote dissolution of basalt (Zhou et al. 2008). Under the bacterial growth condition, olivine is the most bioweathered mineral followed by augite but feldspar is the most stable (Zhou et al. 2008). Furthermore, a large number of studies have documented that *Paenibacillus* can promote the growth of plants by preventing their diseases (Lal and Tabacchioni 2009).

If *Paenibacillus* can promote the dissolution of potassium-bearing rocks, these bacteria can be used not only as a crop disease control agent, but also for the production of K-fertilizer. So these bacteria have a great potential application in agriculture. In study, strain KT has demonstrated its ability to promote the weathering of potassium-bearing rock.

CONCLUSIONS

Isolate KT was identified as a member of the genus *Paenibacillus* on the basis of the sequence similarity of the 16S rRNA gene and molecular phylogeny. The results of weathering of PBR powders by the isolate showed that the bacterium had the ability to solubilize PBR powders and release elements such as K, Ca, Fe and Al from minerals to aqueous solutions. These results would have important implications for enhancing elemental release from rocks for the benefit of agriculture.

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