Whole-Genome Sequencing and Potassium-Solubilizing Mechanism of Bacillus aryabhattai SK1-7

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To analyze the whole genome of Bacillus aryabhattai strain SK1-7 and explore its potassium solubilization characteristics and mechanism, thus providing a theoretical basis for analyzing the utilization and improvement of insoluble potassium resources in soil. Genome information for Bacillus aryabhattai SK1-7 was obtained by using Illumina NovaSeq second-generation sequencing and GridION Nanopore ONT third-generation sequencing technology. The contents of organic acids and polysaccharides in fermentation broth of Bacillus aryabhattai SK1-7 were determined by high-performance liquid chromatography and the anthrone sulfuric acid method, and the expression levels of the potassium solubilization-related genes ackA, epsB, gltA, mdh and ppc were compared by real-time fluorescence quantitative PCR under different potassium source culture conditions. The whole genome of the strain consisted of a complete chromosome sequence and four plasmid sequences. The sequence sizes of the chromosomes and plasmids P1, P2, P3 and P4 were 5,188,391 bp, 136,204 bp, 124,862 bp, 67,200 bp and 12,374 bp, respectively. The GC contents were 38.2, 34.4, 33.6, 32.8, and 33.7%. Strain SK1-7 mainly secreted malic, formic, acetic and citric acids under culture with an insoluble potassium source. The polysaccharide content produced with an insoluble potassium source was higher than that with a soluble potassium source. The expression levels of five potassium solubilization-related genes with the insoluble potassium source were higher than those with the soluble potassium source. The expression levels of five potassium solubilization-related genes with the insoluble potassium source were higher than those with the soluble potassium source.

Keywords: Bacillus aryabhattai, whole genome, mechanism of potassium solubilization, real-time fluorescence quantitative PCR, potassium-solubilizing bacteria (KSB)

INTRODUCTION

Potassium (K) is an essential element for plant nutrition and plays an important role in the growth and metabolism of plants. Additionally, potassium can improve cold, drought, and stress resistance and promote photosynthesis in plants (Leigh and Wyn Jones, 1984; Marschner, 1995; Schachtman and Shin, 2007; Wang M. et al., 2013). The potassium content (K₂O) in crops is 0.3–5% (dry weight), which is equivalent to that of nitrogen and higher than that of phosphorus. Most of the potassium in soil exists in the form of insoluble potassium, such as potassium feldspar,
Existing studies have confirmed that B. aryabhattai on this kind of bacteria has mainly focused on two areas: potassium solubilization-related genes were mined, the content of by combining second- and third-generation sequencing methods, related genes, the whole genome of this strain was sequenced in this study, to accurately locate potassium solubilization- potassium solubilization of this strain is not clear. Therefore, reached 10.8 µM and the percentage of potassium released by the tested strains was inoculated into LB culture medium at an OD600 value of 0.6. The culture medium was centrifuged until the supernatant was clear, and the samples were stored in a refrigerator at −80°C.

In our previous research, we selected the KSB B. aryabhattai SK1-7 from the rhizosphere of poplar trees (Xi and Ye, 2020). The results showed that the strain could dissolve insoluble potassium and release soluble potassium ions, and it could promote the growth of poplar after being applied to the rhizosphere soil, and the concentration of potassium dissolved reached 10.8 µg/mL and the percentage of potassium released was 32.6% (Chen et al., 2020). However, the mechanism of potassium solubilization of this strain is not clear. Therefore, in this study, to accurately locate potassium solubilization-related genes, the whole genome of this strain was sequenced by combining second- and third-generation sequencing methods, potassium solubilization-related genes were mined, the content of organic acids and polysaccharides produced by this strain under culture with an insoluble potassium source was determined, and the expression patterns of potassium solubilization-related genes under culture with different potassium sources were analyzed, thus revealing the potassium solubilization mechanism of the SK1-7 strain at the molecular level. These studies will undoubtedly contribute to a more comprehensive understanding of the potassium solubilization characteristics and growth-promoting ability of strain SK1-7 and provide a theoretical basis for further exploring the development and application of strain SK1-7 as a microbial fertilizer.

Materials and Methods

Strains
Bacillus aryabhattai SK1-7 was isolated from the rhizosphere of Populus alba L. and preserved at the Laboratory of Forest Pathology, Nanjing Forestry University.

Culture Media for the SK1-7 Strain
Luria–Bertani (LB) medium was composed of 10.0 g of tryptone, 5.0 g of yeast extract, 10.0 g of NaCl, and 1,000 mL of deionized water (pH 7.2).

Two groups of fermentation media with different potassium sources were set up. Fermentation medium A had an insoluble potassium source (potassium feldspar) and was composed of the following: sucrose 10.0 g, Na2HPO4 1 g, MgSO4·7H2O 1 g, FeCl3 0.0005 g, (NH4)2SO4 0.5 g, yeast 0.2 g, potassium feldspar powder 12 g, deionized water 1,000 mL, pH value 7.2. Fermentation medium group B was supplemented with a soluble potassium source (K2HPO4): the potassium feldspar powder in group A was replaced by 0.1% K2HPO4, and the other components remained unchanged.

Potassium feldspar was purchased from Rongshide Co., Ltd. (Hefei, China), ground and sieved, soaked in a hydrochloric acid solution for 24 h, washed with deionized water, and dried for later use.

Genome Sequencing and Analysis of Strain SK1-7
Sample Preparation for Genome Sequencing of Strain SK1-7
The tested strains were inoculated into LB culture medium at 30°C and 200 r/min and cultured to the logarithmic growth stage with an OD600 value of 0.6. The culture medium was centrifuged at 10,000 r/min at 4°C for 10 min, and the supernatant was removed. Then, 1 × PBS buffer was applied as a wash 3–4 times until the supernatant was clear, and the samples were stored in a refrigerator at −80°C.

Genome Sequencing, Assembly, and Annotation
The samples were sent to a sequencing company (Personal, Shanghai), and the extracted and tested qualified total DNA samples of B. aryabhattai SK1-7 were sequenced. A5-miseq v20150522 and SPAdes v3.9.0 were used to assemble the sequencing data without linker sequences from scratch, the assembly effects were compared, and the results of SPAdes
Different Potassium Sources

Contents of Strain SK1-7 Cultured With Different Potassium Sources and Determination of the Polysaccharide

The tested strains were inoculated into LB medium at 30°C and 200 r/min and cultured to the logarithmic growth stage as seed solution. The seed solution was inoculated with 5% inoculum into two groups, fermentation media A and B with different potassium sources, and each group was set up with three replicates. The groups were cultured with shaking at 30°C and 200 r/min. Samples were taken at 24, 48, 72, 96, 120, 144, and 168 h, the fermentation broth was centrifuged at 8,000 r/min for 10 min, and 5 mL supernatant was collected. Then, 15 mL absolute ethyl alcohol was added and precipitated at 4°C for 2 h, the mixture was centrifuged at 8,000 r/min for 10 min and the supernatant was removed. Then, 1.0 mL absolute ethyl alcohol was added to precipitate polysaccharides, and the mixture was centrifuged at 8,000 r/min for 10 min and the supernatant was removed. Next, 4.0 mL deionized water was added for precipitation, and the polysaccharide precipitate was dissolved. Then 1.0 mL Sevag solution was added to remove protein under continuous mixing for 30 min followed by standing until layering was observed. The sample was centrifuged for 10 min at 8,000 r/min, and the volume of the supernatant was adjusted to 10 mL, followed by the addition of 1 mL deionized water to 4.0 mL. Then, 6 mL sulfuric acid-anthrone solution was added, the mixture was placed in boiling water for 10 min and cooled with running water after treatment. A standard curve was established as follows. First, 200 mg of anhydrous glucose standard was mixed with deionized water to a constant volume of 1,000 mL and shaken well. Then, 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of glucose standard solution were removed by suction, and deionized water was added to keep the volume constant at 4.0 mL. Then, 6.0 mL of sulfuric acid-anthrone solution was added, and the solution was shaken in boiling water for 10 min. After treatment, the solution was cooled with cold water, and its absorbance was measured at 625 nm wavelength (Ma, 2011).

Expression Patterns of Potassium Solubilization-Related Genes in Bacillus aryabhattai SK1-7 Cultured With Insoluble Potassium Sources

Total RNA Extraction and cDNA Synthesis of Strain SK1-7

The tested strains were inoculated into LB medium at 30°C and 200 r/min and cultured to the logarithmic growth stage as seed solution. The seed solution was inoculated with 5% inoculum into two groups, fermentation media A and B with different potassium sources, and each group was set up with three replicates, which were cultured with shaking at 30°C and 200 r/min. When the culture time was 4, 6, 8, 10, and 12 h, the fermentation broth was centrifuged at 4°C for 10 min at 10,000 r/min, the supernatant was removed, 200 µL lysozyme was added, and the mixture was placed in a 37°C metal bath for 10 min. Then a total RNA Extraction Kit (Beijing Tianmo Technology Development Co., Ltd., Beijing) was used to extract the strain SK1-7 RNA; all equipment used in the extraction process such as centrifuge tubes, pipette tips, etc. were treated with 0.1% diethyl carbonate (DEPC) and sterilized. The total RNA mass of the extracted bacteria

software analyses were selected for construction. GeneMarkerS (version 4.32 April 192015) software was used to predict the whole gene sequence. The TRNA gene was predicted by tRNAscan-SE (version 1.3.1), and the rRNA gene was predicted by Barrnap (0.9-dev). DRs (forward repeats) and spacers (spacers) in the whole genome were predicted by CRISPR finder.1 Sequence alignment of protein-coding genes was completed by blastall software.

Determination of Organic Acids Produced by Strain SK1-7

The tested strains were inoculated into LB medium at 30°C and 200 r/min and cultured to the logarithmic growth stage as seed solution. The seed solution was inoculated into 20 mL fermentation medium with the insoluble potassium source (potassium feldspar) with a 5% inoculation amount and 3 replicates in each group. In addition, LB medium with the same volume was inoculated as a blank control and cultured at 30°C and 200 r/min for 168 h. Samples were taken at 24, 96, and 168 h, the fermentation broth was centrifuged at 8,000 r/min for 10 min, and 5 mL supernatant was collected. The types and contents of organic acids in the potassium-decomposing fermentation broth inoculated with strain SK1-7 were determined by high-performance liquid chromatography with three replicates. Chromatographic conditions were as follows: Agilent InfinityLab Poroshell120 SB-C8, 4.6 mm × 100 mm, 2.7 µm, mobile phase 0.02 mol/L NH4H2PO4-H3PO4 (pH value 2.9), flow rate 0.4 mL/min, column temperature 30°C, detection wavelength 210 nm. Organic acid standards were as follows: oxalic acid, citric acid, succinic acid, fumaric acid, tartaric acid, formic acid, acetic acid, gluconic acid and malic acid.

Determination of the Polysaccharide Contents of Strain SK1-7 Cultured With Different Potassium Sources

The tested strains were inoculated into LB medium at 30°C and 200 r/min and cultured to the logarithmic growth stage as seed solution. The seed solution was inoculated with 5% inoculum into two groups, fermentation media A and B with different potassium sources, and each group was set up with three replicates. The groups were cultured with shaking at 30°C and 200 r/min. Samples were taken at 24, 48, 72, 96, 120, 144, and 168 h, the fermentation broth was centrifuged at 8,000 r/min for 10 min, and 5 mL supernatant was collected. Then, 15 mL absolute ethyl alcohol was added and precipitated at 4°C for 2 h, the mixture was centrifuged at 8,000 r/min for 10 min and the supernatant was removed. Then, 1.0 mL absolute ethyl alcohol was added to precipitate polysaccharides, and the mixture was centrifuged at 8,000 r/min for 10 min and the supernatant was removed. Next, 4.0 mL deionized water was added for precipitation, and the polysaccharide precipitate was dissolved. Then 1.0 mL Sevag solution was added to remove protein under continuous mixing for 30 min followed by standing until layering was observed. The sample was centrifuged for 10 min at 8,000 r/min, and the volume of the supernatant was adjusted to 10 mL, followed by the addition of 1 mL deionized water to 4.0 mL. Then, 6 mL sulfuric acid-anthrone solution was added, the mixture was placed in boiling water for 10 min and cooled with running water after treatment. A standard curve was established as follows. First, 200 mg of anhydrous glucose standard was mixed with deionized water to a constant volume of 1,000 mL and shaken well. Then, 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of glucose standard solution were removed by suction, and deionized water was added to keep the volume constant at 4.0 mL. Then, 6.0 mL of sulfuric acid-anthrone solution was added, and the solution was shaken in boiling water for 10 min. After treatment, the solution was cooled with cold water, and its absorbance was measured at 625 nm wavelength (Ma, 2011).

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1http://crispr.i2bc.paris-saclay.fr/Server/
was detected by agarose gel electrophoresis at a concentration of 1%, and the total RNA concentration and purity were determined by a Nanodrop series ultramicro spectrophotometer (Thermo Fisher Scientific, Waltham, United States). Using an RNA reference reverse transcription kit (Accurate Biology Co., Ltd., Changsha), 1 µg of total RNA was taken to prepare the reaction solution and reacted in a PCR instrument (Eppendorf no. 5345/015458, Germany). The reverse transcribed cDNA concentration was diluted to approximately 100 ng/µL and stored at 4°C until use.

**Real-Time Fluorescent Quantitative PCR of Potassium Solubilization-Related Genes Under Insoluble Potassium Source Culture Conditions**

To understand the potassium solubilizing mechanism of strain SK1-7 from multiple perspectives, the relative expression levels of SK1-7 genes under different potassium sources were determined by real-time quantitative PCR. The control group was group B supplemented with a soluble potassium source (K₂HPO₄), and the experimental group was group A supplemented with an insoluble potassium source (potassium feldspar). Different potassium solubilizing genes were selected: genes ackA, epsB, gltA, mdh, and ppc. Primer Premier 5.0 software was used to design specific primers for real-time fluorescence quantitative PCR. The specific primer design is shown in Table 1. A SYBR green Pro Tap Hs premixed qPCR (Low ROX Premixed) kit
Livak and Schmittgen, 2001). The culture conditions were calculated by the 2−ΔΔCT method (Livak and Schmittgen, 2001).

The original reading of the SK1-7 strain obtained by sequencing was used for quality control, quality evaluation and assembly. The whole genome of the strain consisted of a circular chromosome and four circular plasmid (Figure 1). The length of the chromosome was 5,188,391 bp, and the GC content was 38.2%. The genome encoded 5,307 genes, accounting for 81% of the genome. The total length of the coding genes was 4,207,257 bp, the average length of the coding genes was 792.78 bp, and the size of N50 was 18,721 bp, and the size of N90 was 2,442 bp, a total of 5,307 ORFs were predicted in the chromosome, and the length of the ORFs was 4,207,257 (Table 2). The genome of strain SK1-7 predicted 120 tRNA structures, 323 ncRNA structures, 14 SS rRNA structures, 13 16S rRNA structures, 13 23S rRNA structures and 4 CRISPR structures (Table 2). Strain SK1-7 was found to contain plasmids, P1, P2, P3 and P4, with sequence sizes of 136,204 bp, 124,862 bp, 67,200 bp and 12,374 bp, and 4 CRISPR structures (Table 2). Genome sequencing data of B. aryabhattai SK1-7 were submitted to NCBI with the GenBank BioProject number: PRJNA716807.

Functional Annotation of the Protein-Coding Genes of Strain SK1-7
The main purpose of functional annotation of protein-coding genes is to analyze the function of all protein-coding genes to deeply examine a species at the molecular level. According to the functional annotation results of the protein-coding genes of the strain SK1-7 genome (Table 4), 5,159 protein-coding genes were compared in the NR database, and 2,513 protein-coding genes were compared in the KEGG database. The differences were mainly related to the capacity and focus of the databases.

TABLE 5 | The organic acid contents in fermentation broth after B. aryabhattai SK1-7 inoculation.

| Organic acid (ng/μ L) | CK | SK1-7 |
|-----------------------|----|-------|
| Oxalic acid           | –  | 16.74 ± 1.01 |
| Citric acid           | –  | 42.29 ± 3.24 |
| Butanedioic acid      | 127.7 ± 1.4 | 37 ± 2.36 |
| Fumaric acid          | –  | 0.4 ± 0.01 |
| Tartaric acid         | 0.4 ± 0.03 | 0.11 ± 0.01 |
| Formic acid           | 8.38 ± 0.6 | 0.24 ± 0.02 |
| Acetic acid           | 6.62 ± 0.12 | 84.2 ± 2.96 |
| Gluconic acid         | –  | 40.79 ± 1.32 |
| Malic acid            | 0.054 ± 0.01 | 44.56 ± 2.1 |

TABLE 6 | Changes in the polysaccharide contents of B. aryabhattai SK1-7 under different potassium sources.

| Incubation time (h) | Soluble potassium source (K2HPO4) | Insoluble potassium source (potassium feldspar) |
|---------------------|----------------------------------|-----------------------------------------------|
| 24                  | 1.31 mg/mL                        | 2.12 mg/mL                              |
| 48                  | 3.91 mg/mL                        | 3.65 mg/mL                              |
| 72                  | 3.41 mg/mL                        | 4.98 mg/mL                              |
| 96                  | 3.94 mg/mL                        | 5.44 mg/mL                              |
| 120                 | 4.55 mg/mL                        | 6.34 mg/mL                              |
| 144                 | 5.16 mg/mL                        | 6.82 mg/mL                              |
| 168                 | 4.43 mg/mL                        | 5.1 mg/mL                                |

RESULTS

Genome Assembly and Annotation of Strain SK1-7

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Determination of Organic Acid Production by Strain SK1-7

As shown in Table 5, the organic acid production of strain SK1-7 was the highest under the insoluble potassium source culture, with 183.14 ng/µL at 168 h, followed by formic acid, citric acid, acetic acid and gluconate, with yields at 168 h of 81.68, 40.27, 38.63 and 36.39 ng/µL; small amount of fumaric acid and oxalic acid were also detected. Thus, it can be suggested that under the condition of an insoluble potassium source, the strain could secrete many organic acids, which were presumed to play a role in the process of potassium solubilization.

Determination of Polysaccharide Yields of Strain SK1-7 Cultured With Different Potassium Sources

As shown in Table 6, the yield of polysaccharides was higher than that of soluble potassium under the condition of an insoluble potassium source. The polysaccharide yield of strain SK1-7 increased gradually after 24–144 h of culture with an insoluble potassium source and then decreased to 5.1 mg/mL after reaching the highest value of 6.82 mg/mL at 144 h. Strain SK1-7 can produce a large amount of polysaccharides under culture with an insoluble potassium source. This may be because during the process of potassium solubility, bacteria secrete polysaccharides and feldspar to form a bacteria-mineral complex and then secrete acidic substances for acid dissolution.

Expression Analysis of Potassium Solubilizing Genes in Strain SK1-7

To understand the role of potassium solubilization-related genes of strain SK1-7 from multiple perspectives, potassium solubilization-related genes were selected and tested by qRT-PCR under different potassium sources. As shown in Figure 2, the expression levels of the five genes in KSB in the fermentation medium supplemented with a soluble potassium source (K$_2$HPO$_4$). In the first 8 h, the expression of the ackA gene increased with increasing culture time; the expression was 4.26 times at 8 h and decreased after 10 h, while it was 3.48 times at 12 h. The expression of epsB increased from 4 to 10 h, and was 9.55 times and 8.99 times at 10 h and 12 h, respectively. The expression of gltA increased from 1.67 times to 3.66 times at 4–8 h and decreased to 1.06 times and 1.02 times at 10–12 h. The expression of mdh increased gradually from 1.23 times to 6.88 times at 4–12 h. The ppc gene also increased with increasing culture time, and the highest expression level was 4.99 times at 12 h.

DISCUSSION

Potassium bacteria, also known as potassium-solubilizing bacteria and silicate bacteria, can decompose aluminosilicate rocks in soil, and the decomposed nutrients can be used for plant growth (Saha et al., 2016). At present, many researchers have paid attention to the screening of high-efficiency potassium solubilizing strains, but there is still a lack of potassium bacteria strains with stable and high-efficiency potassium solubilizing effects and clear potassium solubilizing mechanisms, which seriously restricts the development of these potassium bacteria. In the research and application of plant rhizosphere growth-promoting bacterial mechanisms, with the help of genome sequencing technology, we can carry out in-depth analyses of gene regulation mechanisms and expression (Liu et al., 2020).

In this study, a complete chromosomal sequence and four plasmid sequences of the whole genome of B. aryabhattai SK1-7 were determined. The length of the chromosome was 5,188,391 bp, the GC content was 38.2%, the N50 was 18,721 bp, and the N90 was 2,442 bp. The chromosome encoded 5,307 genes, accounting for 81% of the genome length. The total length of the coding genes was 4,207,257 bp. Yan et al. (2016) sequenced the whole genome of B. aryabhattai T61, the total length of the genome was 5,325,933 bp, and the GC content was 38%.
A total of 5,534 genes were encoded, accounting for 83.4% of the genome length. Bhattacharyya et al. (2017) sequenced the whole genome of B. aryabhattai AB211, the circular chromosome was 5,403,026 bp, and the GC content was 37.8%, a total of 5,468 ORFs were predicted in the genome of which 5,226 were putative protein-coding DNA sequences (CDS). The sequencing results of these strain were similar to those of the SK1-7 genome in this study.

In a previous study, strain SK1-7 was inoculated into poplar rhizosphere soil. The results showed that the pH value of rhizosphere soil inoculated with SK1-7 was lower than that of the blank control. These results indicate that SK1-7 may secrete acidic substances during the growth process, reduce the pH value of rhizosphere soil, and convert the insoluble potassium in soil into soluble potassium that can be absorbed and utilized by plants, thus increasing the content of available potassium in the rhizosphere soil and promoting the growth of poplar (Chen et al., 2020). In this study, strain SK1-7 mainly produces malic acid, formic acid, citric acid, acetic acid and gluconic acid under culture with an insoluble potassium-source. Most scholars believe that the production of organic acids (acetic acid, malic acid, citric acid, gluconic acid and oxalic acid) is the main mechanism of potassium solubilization (Badr, 2006; Sheng and He, 2006; Saiyad et al., 2015). The results of the species and contents of organic acids produced by strain SK1-7 in this study also support this view. Combined with previous studies, it is possible that organic acids secreted by SK1-7 in this study also support this view. Combined with previous studies, it is possible that organic acids secreted by SK1-7 in this study also support this view. Combined with previous studies, it is possible that organic acids secreted by SK1-7 in this study also support this view. Combined with previous studies, it is possible that organic acids secreted by SK1-7 in this study also support this view. Combined with previous studies, it is possible that organic acids secreted by SK1-7 in this study also support this view.

In previous studies, based on the potassium solubilizing ability of the SK1-7 strain, its potassium solubilizing effect in different mediators was analyzed (Chen et al., 2020). In this study, combined with the previous results, we further explored the potassium solubilizing mechanism of strain SK1-7 and analyzed its potassium solubilizing characteristics, which can provide theoretical support for its development and application as a biological potassium fertilizer. Transcriptome and gene knockout technology of SK1-7 and the compounds of bacteria and fertilizer need further study.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: PRJNA716807).

**AUTHOR CONTRIBUTIONS**

YC performed the majority of the experiments and data analysis and drafted the link content of the manuscript in the manuscript. JY participated in the planning of research work, interpretation of data, and supervision of manuscript writing. HY and ZS involved in the planning and execution of the research, analysis, and interpretation of the data. All authors read and agreed to the published version of the manuscript.

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