Phosphate Solubilization and Gene Expression of Phosphate-Solubilizing Bacterium *Burkholderia multivorans* WS-FJ9 under Different Levels of Soluble Phosphate

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**Introduction**

Phosphate is one of the major essential macronutrients for plant growth and development. However, most phosphates in soil exist as insoluble compounds and phosphate deficiency in soil is a worldwide problem [1]. Phosphate-solubilizing bacteria (PSB) can liberate soluble phosphate from insoluble phosphate and promote plant growth [2]. Subsequently, application of PSB is regarded as an alternative and sustainable method to solve the worldwide soil phosphate deficiency [3]. Nevertheless, the physiological activities of PSB are affected by exogenous soluble phosphate.

Previous studies have reported that the growth rate of microorganisms is related to the level of soluble phosphate [4]. The growth rate of PSB strain *Pseudomonas aeruginosa* under phosphate sufficiency was 25-fold greater than that under phosphate deficiency [5]. The distribution and diversity of PSB in soil can also be influenced by phosphate availability [6]. To date, there is still lack of study on the mechanism of the soluble phosphate affecting the growth of PSB.

As a core phenotype, the phosphate-solubilizing activity of PSB was induced by low levels of exogenous soluble phosphate. Soluble phosphate may induce the release of phosphorus from insoluble phosphate, and this process is called phosphate solubilization. Phosphate-solubilizing bacteria (PSB) have the ability to dissolve insoluble phosphate and enhance soil fertility. However, the growth and mineral phosphate solubilization of PSB could be affected by exogenous soluble phosphate and the mechanism has not been fully understood. In the present study, the growth and mineral phosphate-solubilizing characteristics of PSB strain *Burkholderia multivorans* WS-FJ9 were investigated at six levels of exogenous soluble phosphate (0, 0.5, 1, 5, 10, and 20 mM). The WS-FJ9 strain showed better growth at high levels of soluble phosphate. The phosphate-solubilizing activity of WS-FJ9 was reduced as the soluble phosphate concentration increased, as well as the production of pyruvic acid. Transcriptome profiling of WS-FJ9 at three levels of exogenous soluble phosphate (0, 5, and 20 mM) identified 446 differentially expressed genes, among which 44 genes were continuously up-regulated when soluble phosphate concentration was increased and 81 genes were continuously down-regulated. Some genes related to cell growth were continuously up-regulated, which would account for the better growth of WS-FJ9 at high levels of soluble phosphate. Genes involved in glucose metabolism, including glycerate kinase, 2-oxoglutarate dehydrogenase, and sugar ABC-type transporter, were continuously down-regulated, which indicates that metabolic channeling of glucose towards the phosphorylative pathway was negatively regulated by soluble phosphate. These findings represent an important first step in understanding the molecular mechanisms of soluble phosphate effects on the growth and mineral phosphate solubilization of PSB.

**Keywords:** Transcriptome analysis, mineral phosphate solubilization, *Burkholderia multivorans*, soluble phosphate, glucose phosphorylative pathway
phosphate and inhibited by high levels of exogenous soluble phosphate [7, 8]. Mineral phosphate solubilization by PSB is attributed to the secretion of low-molecular-weight organic acids [9, 10]. The organic acids are generated from carbon source metabolism [11, 12], and the switch of carbon source metabolism pathways is closely related to the concentration of soluble phosphate [4]. It was reported that the production of gluconic and pyruvic acids, etc. by PSB were significantly affected by soluble phosphate [5, 13]. Furthermore, the repression of glucose dehydrogenase activity by high concentration of soluble phosphate was responsible for the decrease of gluconic acid secretion by PSB at a high concentration of soluble phosphate [13, 14]. The ratio of organic acid yield from glucose by PSB at phosphate deficiency was higher than that at phosphate sufficiency, and the regulation of soluble phosphate on organic acid production was speculated to underlie the regulation of soluble phosphate on mineral phosphate solubilization by PSB [5].

Some genes, such as pst (Pi-specific transporter), phoA (alkaline phosphatase), glpQ (glycerophosphoryldiester phosphodiesterase), phyC (phytase), and ushA (nucleotidase), involved in phosphate uptake and organic phosphate solubilization were stimulated by phosphate starvation [15, 16]. Nevertheless, the molecular mechanism of soluble phosphate regulation on mineral phosphate solubilization by PSB is still unclear.

*Burkholderia multivorans* is regarded as one of the components of the *B. cepacia* complex and belonged to genomovar II [17]. Moreover, *B. multivorans* is recognized as a versatile gram-negative microbe. It is an important opportunistic pathogen to individuals with cystic fibrosis [18]. It can also be found in soil [19], water [20], and plants [21]. PSB strain *B. multivorans* WS-FJ9 was isolated from the rhizosphere of slash pine (*Pinus elliotii* Engelm.) [22]. Previous research showed that *B. multivorans* WS-FJ9 is a nonpathogenic strain, showing remarkable phosphate-solubilizing and plant growth-promoting activities, and could also colonize internal plant tissues [23]. In the current study, the growth and phosphate-solubilizing activity of *B. multivorans* WS-FJ9 under different exogenous soluble phosphate levels (0, 0.5, 1, 5, 10, and 20 mM) were investigated, and the global transcriptomes of WS-FJ9 at three exogenous soluble phosphate levels (0, 5.0, and 20.0 mM) were analyzed via high-throughput sequencing technique to elucidate the molecular mechanisms of soluble phosphate on PSB growth and mineral phosphate solubilization.

### Gene Expression of WS-FJ9 under Soluble Phosphate

#### Strain and Culture Conditions

Phosphate-solubilizing bacterium *B. multivorans* WS-FJ9 was isolated from rhizospheric soil of a 28-year-old slash pine (*P. elliotii*) in Guangzhuang Forestry Centre, Fujian, China, and had been deposited to the China Center for Type Culture Collection (Accession No. CCTCCM2011435) [22]. Strain WS-FJ9 was routinely grown and maintained in LB medium. The media of six levels of exogenous soluble phosphate (0, 0.5, 1, 5, 10, and 20 mM) were prepared by using simple phosphate (SP) medium and 100 mM KH2PO4 solution as described previously [13]. A bacterial suspension of *B. multivorans* WS-FJ9 (106 CFU/ml) was prepared for inoculation by centrifuging and washing three times with sterile saline from culture of LB broth medium.

#### Treatments under Different Levels of Soluble Phosphate

Ten microliters of the bacterial suspensions was pipetted to the center of SP agar plates with each soluble phosphate level, in triplicates. Growth and phosphate solubilization of the bacterium were observed after 5 days of incubation at 30°C. The phosphate-solubilizing activity was determined by the ratio between the clear zone diameter and colony diameter.

One milliliter of the bacterial suspension was inoculated into each soluble phosphate level of SP broth medium, in triplicates. Medium without bacterium inoculation served as the control. The growth was measured by optical density (OD600nm) using NanoDrop 2000c (Thermo Fisher Scientific, USA) at regular intervals. The measurements were terminated once the growth reached the late exponential phase (OD600nm value stopped showing significant increase (p < 0.05)). The growth rate was calculated by following the formula: (OD

\[
\text{OD}_{t} - \text{OD}_{0} = \frac{\ln N - \ln N_0}{t_N - t_0}
\]

where OD600nm0 was the OD600nm value measured at the beginning of cultivation. The bacterial cells were harvested by centrifugation (10,000 xg, 4°C, 5 min) and immediately frozen in liquid nitrogen for the next RNA extraction. The supernatant was collected to test other physiological parameters. For RNA library preparation, the bacterial cells from three replicates were pooled together as one sample of each soluble phosphate level treatment (0, 5, and 20 mM). For quantitative real-time RT-PCR (qPCR), the bacterial cells were collected from the three replicates individually of each soluble phosphate level treatment.

#### Phosphate Solubilization Measurement

The supernatants of each soluble phosphate level treatment and control were filtered through 0.22-µm-pore-sized medical millex-GP filters (Millipore, USA). The concentrations of soluble phosphate in the filtrates were measured using the ascorbate method [24]. The phosphate-solubilizing activity of *B. multivorans* WS-FJ9 is reflected by the released soluble phosphate, which is calculated by subtracting the concentration of soluble phosphate of relevant control from the concentration of soluble phosphate of
treatment. The pH was measured by using a basic pH meter (Sartorius, Germany). The filtrates were analyzed for the presence of gluconic, pyruvic, malic, maleic, 2-ketogluconic, lactic, malonic, acetic, methanoic, tartaric, oxalic, crylic, and citric acids by high-performance liquid chromatography (HPLC) (1200 liquid chromatograph; Agilent, USA).

RNA Isolation
RNA was isolated with Trizol (Invitrogen, USA). Fifty microliters of cell pellet was ground to a fine dry powder in a mortar and pestle under liquid nitrogen. The powder was transferred into an RNAase-free tube and vortexed at maximum speed for 15 sec after 1.3 ml of Trizol was added. The suspension was placed at room temperature for 5 min, and the subsequent steps followed the Trizol extraction method as described by Kang et al. [25]. The concentration, RIN (RNA integrity number), 28S/18S, and size of total RNA sample were detected by using the Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit). The purity of the sample was tested by a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA). One microgram of total RNA sample was treated with Ribo-Zero Magnetic Gold Kit (epicenter) (Illumina, USA) to deplete rRNA.

Whole-Transcriptome Library Preparation
Fragmentation, cDNA synthesis, end-repair, A-tailing addition, adapter ligation, UNG digestion, and PCR amplification were carried out using TruSeq RNA Sample Prep Kit v2 (Illumina). Briefly, the RNA was fragmented into small pieces with Elute, Prime, and Fragment Mix and used as templates in reverse transcription. The first-strand cDNA was generated by using the First Strand Master Mix and Super Script II Reverse Transcriptase Kit (Invitrogen) (reaction condition: 25°C for 10 min; 42°C for 50 min; 70°C for 15 min). The product of the transcription was purified with Agencourt RNA Clean XP Beads (Beckman Coulter) and used as the template to synthesize the second-strand cDNA by adding with Second Strand Master Mix and dATP, dGTP, dCTP, dUTP mix (reaction condition: 16°C for 1 h). The end of the purified double-stranded cDNA was repaired by incubating with End Repair Mix at 30°C for 30 min. After recovery with Agencourt Ampure XP Beads (Beckman Coulter), the 3'-end of the repaired cDNA was added to dA-tailing by using A-Tailing Mix and reacted at 37°C for 30 min. The RNA index adapter was combined to the adenylate 3'-end cDNA by using Ligation Mix at 30°C for 30 min and the product was purified with Agencourt Ampure XP Beads. Afterwards, the second-strand cDNA was digested by uracil-N-glycosylase enzyme. For this, the uracil-N-glycosylase enzyme was added into the adapter-ligated cDNA and incubated at 37°C for 10 min. The subsequent PCR amplification was run for 11 rounds with PCR Primer Cocktail and PCR Master Mix to enrich the cDNA fragments. The PCR product was purified with Agencourt Ampure XP Beads and the whole-transcriptome library was completed.

The quality and quantity of the library were assessed by two methods. The distribution of the fragment sizes was detected by using an Agilent 2100 bioanalyzer (Agilent DNA 1000 reagents), and quantification of the library via qPCR (TaqMan Probe). The qualified library was used to amplify on cBot to generate the cluster on the flowcell (TruSeq PE Cluster Kit V3-cBot-HS; Illumina). The amplified flowcell was sequenced pair end on the HiSeq 2000 system (TruSeq SBS KIT-HS V3; Illumina).

Transcriptome De Novo Assembly and Annotation
The filtered reads were obtained from raw data of three levels of exogenous soluble phosphate-treated WS-FJ9 by filtering out adaptors, N-proportion (>10%), and low-quality (Q ≤ 20) reads. The RNA sequence reads are deposited in the NCBI Sequence Read Archive under Accession No. SRR75875. The de novo assembly for each treatment was performed to generate transcript assembly by using the Trinity method [26] with optimized k-mer length of 25. Contigs were firstly assembled from clean reads by SOAPdenovo, and then further extended and assembled to long sequence denominated “unigenes.” The transcripts from three levels of exogenous soluble phosphate treatments (0, 5, and 20 mM) were denominated as WS-FJ9-0, WS-FJ9-5, and WS-FJ9-20, respectively. A nonredundant unigenes database was finally generated by merging the three transcripts together as a transcript reference and used for the rest of the analyses. The resulting unigenes were used for BLAST searches (E-value b ≤ E-5) against databases like NCBI Nr (http://www.ncbi.nlm.nih.gov/), Swissprot (http://www.expasy.ch/sprot/), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), and Clusters of Orthologous Groups (COG) (http://www.ncbi.nlm.nih.gov/cog/). To classify the unigenes, Gene Ontology (GO) annotation was carried out by the Blast2GO program [27]. The resulting BLAST hits were processed by Blast2GO to retrieve associated GO terms. All unigenes were mapped to the KEGG metabolic pathway database to predict perform pathway assignments [28].

Gene Expression Analysis
Gene expression in transcript was calculated by the reads per kilobase of transcript per million reads (RPKM) method [29]. The RPKM value of each gene was used in the consequent differential gene expression analysis. Differentially expressed genes were identified between two pairs of transcripts (WS-FJ9-0 versus WS-FJ9-5, WS-FJ9-5 versus WS-FJ9-20) by Cuffdiff, and rigorously detected based on the description by Audic and Claverie (FDR ≤ 0.001 and |log2Ratio| ≥ 1) [30]. The expression data for all genes were clustered by using Multi Experiment Viewer (ver. 4.9; Dana-Farber Cancer Institute, USA) with the hierarchical clustering method. We focused on those genes whose expression showed a consistent variation trend with the increase of soluble phosphate concentration.

Quantitative Real-Time RT-PCR
Quantitative real-time RT-PCR was used to validate the digital gene expression (DGE) by using SYBR Premix Ex Taq (Tli RnaseH
Phosphate Solubilization at Different Levels of Soluble Phosphate

The phosphate solubilization on SP plate showed that as the soluble phosphate concentration increased, the diameters of WS-FJ9 colonies also increased but the diameters of the clear zones decreased (Fig. 1, Table 1). The ratio of clear zone/colony diameter generally showed a declining trend as soluble phosphate concentration increased. Quantitative detection of the phosphate-solubilizing activity of WS-FJ9 in SP broth displayed that the soluble phosphate released by the strain remained above 6.2 mM when the concentration of soluble phosphate was 0.3 mM (10 mM), and 21.4 ± 0.1 mM (20 mM), respectively. The pH values in all the treatments dropped as compared with the initial pH value (6.9 ± 0.1), and the pH values in the media were closely related to the activity of phosphate solubilization and generally increased with an increase of soluble phosphate. However, the detection of OD$_{600
\text{nm}}$ showed that the growth of WS-FJ9 gradually improved as the concentration of soluble phosphate increased. In the organic acids analysis, only gluconic and pyruvic acids were detected, but the concentrations of pyruvic acid and gluconic acid decreased with the increase of soluble phosphate.

Table 1. Physiological parameters of B. multivorans WS-FJ9 at different levels of soluble phosphate.

<table>
<thead>
<tr>
<th>Soluble phosphate level (mM)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of colony (mm)</td>
<td>128.3 ± 3.6$^a$</td>
<td>134.0 ± 4.0$^b$</td>
<td>147.2 ± 4.4$^c$</td>
<td>164.2 ± 9.9$^d$</td>
<td>187.3 ± 12.2$^e$</td>
<td>205.6 ± 11.3$^f$</td>
</tr>
<tr>
<td>Diameter of clear zone (mm)</td>
<td>291.1 ± 4.6$^a$</td>
<td>288.0 ± 12.6$^b$</td>
<td>285.0 ± 12.1$^c$</td>
<td>259.2 ± 4.8$^d$</td>
<td>210.0 ± 8.1$^e$</td>
<td>0.0 ± 0.0$^f$</td>
</tr>
<tr>
<td>Clear zone/colony</td>
<td>2.27 ± 0.09$^a$</td>
<td>2.15 ± 0.12$^b$</td>
<td>1.94 ± 0.09$^c$</td>
<td>1.58 ± 0.10$^d$</td>
<td>1.12 ± 0.04$^e$</td>
<td>0.00 ± 0.00$^f$</td>
</tr>
</tbody>
</table>
| Growth rate (OD$_{600
\text{nm}}$/h) | 0.0046 ± 0.0004$^a$ | 0.0056 ± 0.0003$^b$ | 0.0061 ± 0.0001$^c$ | 0.0078 ± 0.0003$^d$ | 0.0081 ± 0.0004$^e$ | 0.0091 ± 0.0004$^f$ |
| Gluconic acid secretion (µg/ml) | 124.9 ± 0.58$^a$ | -      | -     | 123.4 ± 0.52$^c$ | -     | 123.2 ± 0.41$^d$ |
| Pyruvic acid secretion (µg/ml) | 1,369.0 ± 27.53$^a$ | -      | -     | 759.6 ± 5.18$^c$ | -     | 83.3 ± 6.88$^f$ |

Diameters of clear zone and colony were measured on SP plates. Growth rates were determined using late-log phase cultures in SP liquid medium. The data are the means of three replicates ± standard deviation. Lowercase letters within each row indicate significant differences at the $p < 0.05$ level. Uppercase letters within each row indicate significant differences at the $p < 0.01$ level.
detected during phosphate solubilization of *B. multivorans* WS-FJ9 (Table 1). The production of gluconic acid by *B. multivorans* WS-FJ9 was unaffected by the increase in concentration of soluble phosphate. At 0 mM, pyruvic acid was secreted at a rate 11 times higher than gluconic acid, but significantly declined when the soluble phosphate concentration was increased.

**Assembling and Annotation of *B. multivorans* WS-FJ9 Transcriptome**

After barcode removal and quality control, 1 Gb of paired-end sequencing data with 90 bp read length was obtained for each sample (WS-FJ9-0, WS-FJ9-5, WS-FJ9-20). After de novo assembly, 2,009, 1,725, and 1,743 unigenes with length ranging from 150 to 71,553 bp were obtained from WS-FJ9-0, WS-FJ9-5, and WS-FJ9-20, individually. Finally, a transcript of 2,047 unigenes was generated after merging all three unigene datasets. All the integrated unigenes were mapped against the NCBI Non-redundant (Nr) and Swiss-Prot protein databases using the BLAST program with a threshold E-value of 1E-5 and it was observed that 1,889 (92.3%) unigenes were annotated successfully.

Among the 2,047 unigenes, 446 differentially expressed genes (FDR ≤ 0.001 and fold change |log₂ Ratio| ≥ 1) were found (Fig. 3A). As the exogenous soluble phosphate concentration increased from 0 to 5 mM, 33 unigenes were up-regulated and 337 unigenes were down-regulated. Among these regulated unigenes, 5 unigenes were up-regulated >2-fold, and 107 unigenes were down-regulated >2-fold. As the exogenous soluble phosphate concentration increased from 5 to 20 mM, 42 unigenes were up-regulated, whereas 39 unigenes were down-regulated. Among these regulated unigenes, 7 unigenes were up-regulated >2-fold, and 6 unigenes were down-regulated >2-fold.

Clustering analysis of the differentially expressed genes in the three transcripts showed that 44 differentially expressed unigenes were continuously up-regulated with the increase of soluble phosphate concentration, whereas 81 differentially expressed unigenes were continuously down-regulated (Figs. 3B and 3C).

**Functional Analysis**

Functional analysis was performed to assign the differentially expressed unigenes of *B. multivorans* WS-FJ9 into three main GO terms; namely, “biological process,” “cellular component,” and “molecular function.” The mass of categories was differentially enriched between WS-FJ9-0 and WS-FJ9-5 (Fig. 4A). Most of the unigenes up-regulated at 0 mM of exogenous soluble phosphate in “biological process” were related to metabolic process and cellular process, in “cellular component” were related to cell and cell parts, and in “molecular function” were related to catalytic activity and binding, whereas metabolic process, membrane, and catalytic activity were the prominent categories of up-regulated unigenes at 5 mM of exogenous soluble phosphate. Based on the annotations of differentially expressed unigenes in WS-FJ9-5 versus WS-FJ9-20 (Fig. 4B), most of the unigenes related to metabolic process in “biological process,” cell in “cellular component,” and catalytic activity in “molecular function” were up-regulated at 5 mM of exogenous soluble phosphate. Meanwhile, metabolic process, and membrane and catalytic activities were prominent categories of up-regulated unigenes at 20 mM of exogenous soluble phosphate among “biological process,” “cellular component,” and “molecular function,” respectively. These differences reflected the response of WS-FJ9 to soluble phosphate levels directly or indirectly. Among the differentially expressed genes in WS-FJ9, we focused on those that showed uniform continuous change to the increase of exogenous soluble phosphate concentration.

Genes annotated as cellular structure biosynthesis, such as flagellar protein (Unigene560_All, CL49.Contig1_All, CL49.Contig3_All, Unigene916_All), were continuously up-regulated with the increase of exogenous soluble phosphate concentration, as well as some enzyme genes involved in physiological activities, such as peptidyl-prolyl *cis-trans* isomerase SurA (Unigene716_All) and mannose-1-
Fig. 3. Heat map representation of 446 differentially expressed genes of *B. multivorans* WS-FJ9 at three levels of soluble phosphate (A), and clustering of the genes continuously up-regulated (B) and down-regulated (C) with the increase of soluble phosphate. WS-FJ9-0, WS-FJ9-5, and WS-FJ9-20 indicate the transcripts from 0, 5, and 20 mM exogenous soluble phosphate treatments, respectively. The expression data for the differentially expressed genes (FDR ≤ 0.001 and fold change $|\log_{2}\text{Ratio}| \geq 1$) were clustered using MultiExperiment Viewer with the hierarchical clustering algorithm. The list of continuously up-/down-regulated genes has been shown next to the heat map. Genes up-regulated are in red, down-regulated in green, and no change in black.
phosphate guanylyltransferase (Unigene183_All). Some genes associated with regulation and signal transportation also showed positive responses to the increase of exogenous soluble phosphate concentration, such as transcriptional regulator LysR (Unigene508_All), sigma-54-dependent transcriptional regulator (Unigene1448_All), tyrosine kinase (Unigene372_All), and histidine kinase (CL55.Contig2_All). Besides these, translocator genes, such as sodium solute symporter (CL210.Contig1_All) and some hypothetical protein coding genes, such as hypothetical protein Bpse38_06297 (CL44.Contig3_All), were up-regulated along with the increase of exogenous soluble phosphate concentration. Of these, the expression of Unigene560_All for flagellar MS-ring protein, Unigene716_All for isomerase SurA, and Unigene372_All for tyrosine kinase were further analyzed by qPCR (Fig. 5).

However, as exogenous soluble phosphate concentration increased, there were many genes that underwent down-regulation rather than up-regulation. Genes of glycerate kinase (CL79.Contig1_All), 2-oxoglutarate dehydrogenase (CL102.Contig5_All), and glucose-methanol-choline oxidoreductase (CL94.Contig5_All) were involved in glucose metabolism. Histidine protein kinase PhoR (CL233.Contig1_All), transcriptional regulator LuxR (Unigene498_All), and secreted protein EspA (CL77.Contig1_All, CL77.Contig4_All) were related to regulation and signal transportation. Some genes were also divided into translocator genes, such as sugar ABC transporter (Unigene794_All), cation ABC transporter (Unigene598_All), and porin (Unigene1417_All). In addition, some hypothetical proteins (Unigene1443_All, Unigene819_All) were also constantly down-regulated. Of these, the expression levels of CL79.Contig1_All for glycerate kinase, CL102.Contig5_All for 2-oxoglutarate dehydrogenase, CL233.Contig1_All for histidine protein kinase PhoR, and Unigene794_All for Sugar ABC transporter were further verified by qPCR (Fig. 5).

Validation of Expression Pattern of Unigenes by qPCR

Ten genes were randomly selected from highly expressed genes to validate the DGE results. For this purpose, their expression patterns at three soluble phosphate levels were analyzed by qPCR. Four out of the 10 genes exhibited constant up-regulation and 6 out of the 10 genes showed constant down-regulation with an increase in soluble phosphate concentration. The expression patterns of the 10 candidate genes detected by qPCR were similar to their DGE results (Fig. 6), which demonstrates the reliability of the high-throughput RNA sequencing data.
Fig. 5. Expression of continuously up-/down-regulated genes involved in cellular structure biosynthesis (flagellar MS-ring protein), physiological activities (isomerase SurA), transcriptional regulator (tyrosine kinase and histidine protein kinase PhoR), glucose metabolism (glycerate kinase and 2-oxoglutarate dehydrogenase), and translocator (sugar ABC transporter). The names and tags of genes are shown below the subplots. The digital gene expression data are shown on the left and the qPCR data are on the right in each pair of subplot. Error bars for qPCR indicate the standard deviation of three replicates.
Discussion

The PSB strain *B. multivorans* WS-FJ9 grew better at high concentrations of soluble phosphate in both plate and broth cultures. This finding is in agreement with the study on a PSB strain, *P. aeruginosa* P4, which reported that the growth rate of the strain P4 under sufficient phosphate condition is higher than that under deficient phosphate condition [5]. However, the mechanism through which soluble phosphate affects the growth of PSB is not well understood.

Results of transcriptome analyses showed that there was a global up-regulation of genes involved in WS-FJ9 growth. Among these genes, cellular structures, such as flagellar MS-ring protein (Unigene560_All), flagellar hook protein FlgE (CL49.Contig1_All), and Fe-S protein (Unigene455_All) assembly chaperone HscA were promoted by high concentrations of soluble phosphate. Mutation of the mannose-1-phosphate guanylyltransferase gene (Unigene183_All) revealed that this enzyme is involved in N-glycosylation and construction of the cell wall [32]. D-Amino acids are recognized as common regulatory substances in bacterial growth, and accumulation of D-amino acids enabled the down-regulated synthesis of peptidoglycan, the stress-bearing component of the bacterial cell wall [33]. An important enzyme in D-amino acids metabolism [34], up-regulation of D-amino-acid dehydrogenase (Unigene897_All) would relieve the repression of D-amino acids on growth. In *Escherichia coli*, genetic inactivation of isomerase gene SurA resulted in the decrease of the growth rate [35]. There were also several genes involved in nucleic acid replication and translation, including genes of ATP-dependent DNA helicase RecQ (Unigene973_All), and elongation factor G (CL9.Contig2_All). In this expression investigation, these genes displayed up-regulation in response to the increase in soluble phosphate concentration and showed the same trends with the growth of *B. multivorans* WS-FJ9, which might account for the growth promotion at high concentrations of soluble phosphate.

The mineral phosphate solubilization by *B. multivorans* WS-FJ9 was affected by soluble phosphate. The phosphate-solubilizing activity of the strain showed a significant repression at 5 mM soluble phosphate concentration and was completely inhibited at 20 mM soluble phosphate concentration. The activity of mineral phosphate solubilization by PSB showed a common in trait that it is repressed by soluble phosphate as a feedback mechanism [13]. Sensitivity to soluble phosphate is a serious constraint to the extensive application of PSB. However, the molecular mechanism of soluble phosphate regulation on phosphate-solubilizing activity of PSB is still not clear.

Secretion of various low-molecular-weight organic acids by PSB is considered the main mechanism of mineral phosphate solubilization [9, 10]. The organic acids produced by PSB lead to acidification of surrounding soils and, consequently, the release of soluble phosphate by proton substitution or carboxyl group chelation of metal ions such as Ca$^{2+}$, Fe$^{3+}$, and Al$^{3+}$ from mineral phosphate [3, 36]. During the phosphate solubilization in the SP media, only gluconic and pyruvic acids were detected in the organic acid analysis of *B. multivorans* WS-FJ9.

The low-molecular-weight organic acids are generated from glucose oxidation by bacteria via the periplasmic direct
oxidative pathway [11] and the intracellular phosphorylative pathway [12, 37]. In PSB strain *P. aeruginosa* P4, the promotion of phosphate-solubilizing activity by phosphate deficiency contributed to the enhancement of gluconic acid secretion, which was caused by the change of predominant distribution of glucose available from the phosphorylative pathway to the direct oxidative pathway [5]. Gluconic acid was demonstrated to be an effective component of organic acid in mineral phosphate solubilization in *S. marcescens* [38] and *E. herbicola* [39]. In *P. frederiksenensis* JW-SD2, the production of gluconic acid was gradually repressed with the increase of soluble phosphate. The reports on *E. herbicola* [7] and *R. leguminosarum* [8] indicated the negative regulation of soluble phosphate on the direct oxidative pathway of glucose metabolism. However, the production of gluconic acid by *B. multivorans* WS-FJ9 was not affected by the changes in soluble phosphate concentrations, which would be due to the constitutive nature of the direct oxidative pathway of glucose metabolism [5, 14]. Nevertheless, the phosphorylative pathway of glucose metabolism in *B. multivorans* WS-FJ9 was affected by soluble phosphate. Expression of the glycerate kinase gene (CL79.Contig1_All) and 2-oxoglutarate dehydrogenase gene (CL102.Contig5_All) was continuously up-regulated under phosphate deficiency with the decrease of soluble phosphate. In other words, these genes were promoted by phosphate deficiency. Glycerate kinase and 2-oxoglutarate dehydrogenase are rate-limiting enzymes of the Entner-Doudoroff pathway and the tricarboxylic acid cycle pathway, respectively [40, 41]. These two pathways of glucose metabolism are serial routes of the phosphorylative pathway [42]. In addition, pyruvic acid generated from the phosphorylative pathway was detected in *B. multivorans* WS-FJ9 and its secretion was inversely proportional to the soluble phosphate concentration, which indicates a repression of the phosphorylative pathway by soluble phosphate.

In *P. putida* CSV86, the phosphorylative pathway regulation was carried out by regulation of glucose metabolizing enzymes and/or the glucose transport process [43]. Indeed, genes involved in sugar ABC-type transporter (Unigene794_All) were overexpressed under phosphate deficiency in *B. multivorans* WS-FJ9, which was regarded as an important transport system for uptake and transport of glucose [44, 45]. Therefore, soluble phosphate develops a negative regulation on the phosphorylative pathway of glucose metabolism in *B. multivorans* WS-FJ9, which might affect the organic acid secretion and subsequently the phosphate-solubilizing activity. This novel mechanism of soluble phosphate regulation on the glucose metabolism pathway of *B. multivorans* WS-FJ9 would help to further elucidate the effects of soluble phosphate on phosphate solubilization by PSB.

Besides this, the histidine protein kinase PhoR gene (CL233.Contig1_All) was up-regulated at a lower soluble phosphate concentration. PhoR is an important component of the two-component regulatory operon PhoB-PhoR (Pho regulon) [46], named PhoP-PhoR in *Bacillus subtilis* [47], which acts as a sensor responding to soluble phosphate in signaling processes [48]. This two-component transcription factor regulates the expression of several Pho regulon genes, such as alkaline phosphatase genes *phaA* and *phaB* [49] and APase-alkaline phosphodiesterase gene *phoD* [50], the enzymes encoded by which take active part in mineralization of organic phosphate. However, how the Pho regulon is involved in the regulation of soluble phosphate on glucose metabolism of *B. multivorans* WS-FJ9 still needs further research.

In addition to the genes involved in phosphate solubilization, the expression levels of many transcriptional regulator genes and genes involved in signal transduction were regulated at different soluble phosphate concentrations. As a ubiquitous transcriptional regulator, the LysR family transcriptional regulator (Unigene508_All) negatively regulates a diverse set of genes, including those involved in virulence, metabolism, quorum sensing, and motility [51]. TonB-dependent regulatory systems (CL3.Contig9_All) sense signals from outside the bacterial cell and transmit them into the cytoplasm, of which TonB-dependent receptor is involved in both transport [52] and trans-envelope signal transduction [53]. In this study, the expression levels of these genes were regulated by soluble phosphate. These genes would directly or indirectly play a role in responding to exogenous soluble phosphate.

The transcriptome sequencing results revealed a highly complex transcriptional response for *B. multivorans* WS-FJ9 to different exogenous soluble phosphate levels. These findings would help us to understand the molecular mechanism of soluble phosphate regulation on PSB physiological activities, especially phosphate solubilization, which provides the basis for a molecular engineering strategy to reduce the sensitivity of PSB strains to soluble phosphate, which could improve the scope of their application.

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