Single-cell exploration of active microbiota in solubilizing fixed phosphorus in soils

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Abstract

Phosphate solubilizing bacteria (PSB) play a crucial role in mobilizing soil fixed phosphorus (P) and mitigating P crisis. However, it is a grand challenge to reveal their in situ P-solubilizing activity and the link between phenotypes and genotypes. Here, single-cell Raman-D$_2$O was employed to discern and quantify soil active PSB. Their abundance and in situ activity differed significantly between soil types and fertilization treatments. Inorganic nutrient input was determined as the key driver for active PSB distributions. Further targeted single-cell sorting and metagenome sequencing of highly active soil PSB revealed novel unculturable genera with a low abundance in bulk soil microbiota. The underlying functional genes and metabolic pathway, especially the previously unrecognized interplay between P and C cycling involved in high P solubilization activity, were elucidated. This study provides a new single-cell approach to exploring PSB from native environments, enabling development of microbial solution for sustainable P utilization in agriculture.

Introduction

Phosphorus (P) is an essential element for all living organisms. Its limited global reserves underscore the importance of efficient P management in agriculture systems $^{1,2}$. When P fertilizers are applied to soils, a significant portion becomes bound to soil particles due to interactions with iron and aluminum hydroxides in acidic soils, and calcium in alkaline soils $^{3}$. This creates a pool of residual P that far exceeds the requirements for crop growth, but is not readily bioavailable for plant uptake. Phosphate solubilizing bacteria (PSB) are crucial for enhancing P bioavailability and regulating P transformation processes through the secretion of organic acid and phosphatase enzymes $^{4}$. Their metabolic activity in the native soil habitat significantly impacts the solubilization efficiency of soil fixed P. Understanding the in situ activity of PSB and their responses to different soil and fertilization types is fundamental for guiding efficient fertilizer application. Additionally, integrating this activity with specific taxa and genetic determinants provides a more comprehensive understanding of the mechanisms underlying the P solubilization process and offers insights for sustainable P management.

Bulk techniques, such as metagenomic sequencing have been widely employed to identify PSB and their functional genes $^{5}$, providing valuable insights into the diversity and genetic capabilities of PSB $^{6,7}$. However, they have limitations in resolving the specific cellular activities of individual microorganism. In addition, a significant portion of microbial diversity (over 40%) may arise from dead or dominant cells, as well as extracellular DNA, and not all functional genes can be actively expressed. These factors constrain the effectiveness of genetic exploration in fully understanding the P solubilization capabilities of soil microorganisms $^{8}$.

Single-cell phenotypic approach is promising in directly elucidating microbial functions in their native habitats in a culture-independent manner. Single-cell Raman spectroscopy combined with heavy water (Raman-D$_2$O) has demonstrated its efficiency in probing the phenotypic traits of PSB and their P-releasing activities. This approach is based on the finding that PSB were significantly more active in
assimilating $\text{D}_2\text{O}$ than non-PSB in the presence of fixed P. The resulting C-D Raman band has been established as a biomarker for distinguishing PSB, and the intensity ratios can be utilized to quantify the P solubilizing activities of both inorganic and organic PSB. This method provides a strategic advantage in discriminating PSB and evaluating their metabolic activity across diverse soil matrices, enabling a deeper exploration of the factors influencing P solubilization processes. The further integration with Raman-activated single-cell sorting and targeted metagenomic sequencing facilitates the investigation of both taxa and genomic attributes of active PSB\(^9,10\), thereby advancing our understanding of the key players and underlying mechanisms involved in P cycling.

The microbial phosphate solubilization process in soil matrix is highly intricate and influenced by many factors, including soil types and fertilization inputs. These factors can impact the stable C: N:P stoichiometry required by PSB to maintain their activity and growth. Fertilizers have been found to exert a profound ecological impact on microbial-driven P cycling in various terrestrial ecosystems\(^6,7,11,12\). For instance, Blanes et al. found that using inorganic fertilizers led to an increase in the abundance of genes associated with inorganic P-solubilization\(^13\). Additionally, the fertilization-induced decrease in soil pH hindered the genetic capacity of microorganisms to solubilize fixed inorganic P by altering the composition of the microbial community\(^14\). Despite these findings from genotypic surveys at the population level, it remains unclear how fertilizer application influences the abundances and in situ activity of PSB in different soils, which specific taxa drive the P solubilization function and the underlying metabolic processes. In addition, microbial-driven P solubilization is not solely related to inorganic and organic P releasing but also to carbon (C) cycling associated with $\text{H}^+$ and organic acids secretion\(^15\). However, our understanding of the interplay between the metabolic processes responsible for P solubilization and C cycling in the active PSB remains limited.

Here, we aimed to address these knowledge gaps by examining the impact of fertilization on active PSB communities across diverse soil types and elucidating the mechanisms underpinning P solubilization and C cycling. To achieve this, single-cell Raman-$\text{D}_2\text{O}$ was employed to quantify active soil PSB and assess their phenotypic responses, including abundance and in situ activity, to soil types and long-term fertilization practices. This phenotypic information was then leveraged to identify the key factor driving microbial P solubilization in soil. By further integration with targeted single-cell sorting and metagenomic sequencing, we elucidated the taxonomic affiliation of highly active PSB and their genomic determinants. Particularly, some previously unrecognized genera exhibiting a high P solubilization capability were identified, and the underlying P and C cycling genes and interplay mechanisms were clarified. This work contributes to elucidating the metabolically active PSB within their natural habitat and the associated taxa and mechanisms, providing an important guide for the efficient agronomic use of P and mitigating the P crisis.

Results
**Quantifying in situ P solubilization ability of soil microorganisms across different soil types and fertilization treatments**

Understanding the *in situ* P solubilization ability of soil native microbes is important to utilize the abundant soil legacy P and mitigate P crisis. Here, three types of typical Chinese soils across North and South arable land receiving no fertilizer (CK), inorganic fertilizer (IF), and a combination of inorganic and organic fertilizer (CF) were studied. The soil samples from Dezhou (DZ) and Donghu (DH) were alkaline (pH > 8), and those from Qiyang (QY) were acidic (pH < 6, Fig. 1a). Application of fertilizer was not found to affect soil pH. The original Olsen-P concentrations in DZ and DH soils were higher than in QY soils. The application of IF significantly increased Olsen-P concentration in QY soils but had no effect on DZ and DH soils. In contrast, the application of CF significantly increased Olsen-P concentration in DZ and QY soils (p < 0.001), but not in DH soils (P > 0.05, Fig. 1b), indicating varying P fixation and mobilization levels in different soils. In addition, application of CF significantly increased dissolved organic carbon concentration in DH and DZ soils (P < 0.05), but not in QY soils (P > 0.05, Fig. 1c).

Single-cell Raman-D$_2$O was used here to discern active indigenous PSB in these soils and quantify their activities to solubilize fixed P in a culture-independent way$^{16}$ (Fig. 2a). The collected soils were washed with NaHCO$_3$ solution to remove Olsen-P until its concentrations were below 2.0 mg kg$^{-1}$ and leave mostly fixed P in soils (Table S1). Such nearly Olsen-P-free soils were then incubated with 200 µL g$^{-1}$ D$_2$O at 27°C for 16 h, wherein only active PSB capable of solubilizing soil fixed P can use D$_2$O for their metabolism, and display a C-D Raman band (2040–2300 cm$^{-1}$) due to replacement of H by D in CHx of proteins and lipids. C-D ratios of C-D/(C-D + C-H) were calculated to represent the D uptake level$^{16}$. Figure S1a shows the C-D ratios of bacteria in the original and washed (Olsen P-free) soils. The activity (Figure S1a) and abundance (Figure S1b) of active cells (C-D ratio > 8%) in the original soils were significantly higher than the washed soils, indicating that only a fraction of soil cells can solubilize fixed P. It was also noticed that even in the original soils, the C-D ratios of soil microbes varied significantly with soil types and fertilization treatments (Figure S1a, p < 0.05), indicating that the microbial uptake level of D was affected by the soil substrate pool with varying nutrient levels. To eliminate such an effect and allow comparison of PSB across different soils, normalized C-D ratios by dividing the C-D ratios from the washed soils with the average from the original soils were calculated (Fig. 3a). Active PSB was then discerned by setting a threshold at 0.5, which was determined by calculating the mean + 3×standard deviation of normalized C-D ratios of soil bacteria without incubation with D$_2$O (Fig. 3a).

Using the above defined threshold, the abundance of active PSB in different soils was calculated. Considering the balance between the statistical accuracy and time consumption, the number of soil bacteria for single-cell Raman detection and abundance analysis was optimized. Over 600 single-cell Raman spectra were detected from each soil sample (a total of ~ 5400 spectra for all samples), from them, a total of 200 single-cell spectra were randomly selected from each treatment to calculate the percentage of PSB (Figure S2). With the increase of sample size, the variation of PSB percentage decreased until reaching a plateau at the sample size of 100 (red lines), indicating 100 single cells were...
appropriate for calculating the abundance of active PSB in a complex soil microbial community. The abundance of active PSB in 9 soil samples was calculated and found to be both soil type and fertilization regime dependent (Fig. 3b). DZ soils without fertilization treatment were found to contain the most abundant PSB, followed by QY and DH. In addition, fertilizer application, especially IF, further led to an increase in the active PSB abundance in DH and DZ soils ($p < 0.001$). While in QY soils, a slight decrease in active PSB was observed under IF and CF treatments ($p < 0.05$, Fig. 3b). Considering that the ability of soil microorganisms to mobilize fixed P is associated with both the abundance of PSB and their metabolic activities. These two parameters were multiplied to quantify the phenotypic microbial P solubilization efficiency (Fig. 3c). A similar trend of P mobilization efficiency to that of PSB abundance was observed but with a larger variation, indicating the highly varying activity of individual PSB cells in these soils. In addition, effect of fertilizations on microbial community in three soil types was also investigated. IF treatment was found to significantly increase the abundance of some potential PSB and microbial interactions in comparison with CK and CF treatment (Fig. 3), partly consistent with the Raman detection. These results indicate that tailored fertilization to soils can promote microbial mobilization of soil fixed P.

To further investigate the potential factors affecting the microbial P solubilization function in different soils, the correlation of the phenotypic PSB indexes with Olsen-P and dissolved organic carbon in soils was analyzed, respectively (Figure S3 and S4). The percentage of PSB was found to positively associate with low concentrations of soil Olsen-P ($10–35 \text{ mg kg}^{-1}$, CK, and IF treatments) in DZ and DH soils (Figure S3a) but negatively correlated with high Olsen P concentrations ($35–70 \text{ mg kg}^{-1}$, CF treatment) in these soils (Figure S3b). While in QY soils, a negative correlation between Olsen P and PSB abundance was observed in both low and high concentrations (Figure S3c). Furthermore, P solubilization efficiency was associated with Olsen-P in DZ and DH soils but not in QY soils (Figure S3g-i). The different results between alkaline (DH and DZ) and acid (QY) soils indicated that pH was an important factor affecting microbial P solubilization, and the influence of Olsen-P on phenotypic P solubilization function was dependent on both soil type and fertilization regime. Previous studies through metagenomic sequencing have also proved that soil Olsen-P is a main factor in determining microbial P solubilization $^{17}$. However, the PSB activity in three soils was not influenced by Olsen-P concentrations (Figure S3d-f).

Soil dissolved organic carbon is previously regarded as another main factor determining microbial P solubilization according to the genotype of microorganisms $^{18}$. In this study, no significant correlation was observed between PSB abundance and dissolved organic carbon levels in CK and IF treatments (Figure S4a). However, a negative correlation was identified between PSB abundance and elevated concentrations of dissolved organic carbon, particularly in organic fertilized DZ, DH soils, and QY soils (Fig. 6b and S4c). These results indicated that high carbon input in soils could decrease the abundance of active PSB and further reduce microbial P solubilization function.
Targeted sorting, sequencing, and identifying highly active indigenous PSB in soils

Nondestructive Raman detection enables single-cell sorting and targeted metagenomic sequencing of cells of interest, providing a way to link the P solubilization function to the specific taxa and the underlying mechanisms (Fig. 2b). Single-cell laser ejection method was used to sort out highly active PSB (H-PSB) with normalized C-D ratios > 1.2 (top 7%). A total of 60 single H-PSB (20 single cells from each soil type) were sorted and sequenced. The dominant phyla in the sorted cells belonged to Firmicutes, Proteobacteria, and Acidobacteria (Fig. 4a). All the sorted PSB phyla (post sort) can be found in the bulk soil communities through 16S rRNA gene sequencing (pre sort), demonstrating their prevalence in the original bulk soil (Fig. 4a). Calculation of the relative abundance revealed that the sorted phylum of Firmicutes was present at a relatively low abundance in the initial DH (0.58%) and DZ (0.38%) soils, while they were remarkably enriched by 186 and 115-fold in the sorted fractions. The other two dominant phyla in the sorted fraction also got enriched (Fig. 4a), indicating that these phyla played a more important role in solubilizing fixed P in native soils. A further taxonomic identification revealed the sorted active PSB at the species level, including Bacillus marmarensis and Bacillus pseudofirmus in DH soils, Moraxella osloensis in DZ soil, and Stenotrophomonas maltophilia and Cutibacterium acnes in QY soil (Fig. 4b). These species have been previously reported as typical PSB or can solubilize fixed P, supporting the result of Raman identification of phenotypic PSB. Of note, Bacillus and Propionibacterium sorted from DZ and QY soils, respectively, belonged to the unculturable genus, suggesting the existence of novel efficient PSB worth further exploration.

Genetic basis, metabolic pathways and P/C cycling interplay of highly active PSB

The sorted fractions were further investigated to understand the genetic basis underlying P solubilization function and the associated metabolism pathways. In DZ-sorted metagenome, one typical gene encoding inorganic P solubilization (ppa, Figure S5) and five genes encoding organic P acquisition including glucose 1-dehydrogenase, lysophospholipase, phospholipase A2, phospholipase C, and glycerophosphodiester phosphodiesterase were found (Figure S6), suggesting that H-PSB in DZ soils possessed the genetic capabilities to recycle P from soil organic matter, especially from phospholipid and glycerophospholipid. Considering the incomplete bacterial genome from the sorted PSB with limited number of P cycling related genes, we searched seven genomes from the NCBI database that are phylogenetically congruent with our sorted bacterial samples, which facilitated the prediction of genes involved in P cycling. These genomes were annotated against the KEGG database, and a total of 24 genes associated with P metabolism were identified, including genes related to P solubilization, mineralization, regulatory functions, and transporters (Fig. 5a). This finding suggests that these bacteria possess complete genetic pathways involved in the release of fixed P. Interestingly, our analysis revealed that C. acnes and M. osloensis had the potential to dissolve fixed inorganic P, while Enhydrobacter
aerosaccus, *S. maltophilia*, *Bacillus okhensis*, *B. pseudofirmus*, and *B. marmarensis* possessed the capacity to release both fixed organic and inorganic P.

In addition to the P solubilization genes, carbon catabolism was also reported to participate in the inorganic P solubilization process. For instance, the anaerobic metabolism of glucose is followed by the synthesis of small molecule organic acids, which can be involved in the solubilization of fixed inorganic P. Here, a total of 22 functional genes involved in catabolism were identified in the sorted active PSB cells (Figure S5). Among them, the sorted cells contained genes related to pyruvate and purine metabolism, such as *pyk*, *adk*, *guaA*, *pps*, *purK*, *purE*, and *guaA*. Meanwhile, the DZ sorted cells contained genes participating in pentose phosphate (*prsA, gnl*), oxidative phosphorylation (*ppa*), purine (*adk, guaB, purH, ndk, spoT, purA, purD, purT, nrdD*), pyruvate (*pyk, pps*), and pyrimidine (*thyA, ndk, dcg, pyrF*) catabolic pathway. All the sorted H-PSB communities contained genes encoding enzymes involved in the degradation of carbon substrates (Fig. 5b). For example, genes encoding hemicellulose and pectin degradation were detected in all sorted H-PSB cells, genes encoding cellulolytic and ligninolytic enzymes were found in DH and DZ sorted cells, and genes encoding starch-degrading enzymes were found in DZ sorted H-PSB community.

To gain a deeper understanding of the relationship between P solubilization and carbohydrate metabolism, it is important to recognize the core metabolism that contributes to microbial P solubilization. By annotating metagenomes of the sorted PSB against the KEGG database, the core metabolic pathways involved in carbon metabolism and the tricarboxylic acid cycle in the sorted H-PSB cells from the DZ, DH, and QY soils are summarized in Fig. 5c. The carbohydrates serving as energy sources can be directly oxidized to glucose and ketones, which can then be further oxidized to produce ATP and proton, thereby contributing to dissolution of inorganic fixed P. In addition, enzymes such as NADH dehydrogenase, cytochrome c oxidase, cytochrome bcl complex, and ATPase, also play a critical role in generating and transferring H⁺ out of cells, accelerating inorganic P solubilization.

**Discussion**

**Microbial P solubilization efficiency and their responses to soil and fertilization types**

Solubilization of the large amount of soil fixed legacy P by PSB is an effective strategy for sustainable use of P, however, identifying metabolically active PSB inhabiting in soils and quantifying their in situ P solubilization efficiency are still a big challenge. Here, by standardizing a discrimination threshold based on the normalized activity of bacteria in Olsen P-free soils against that in original soils, single-cell Raman-D₂O was advanced to identify and quantify active indigenous PSB populations in soils. Based on the quantitative information, a new index of phenotypic fixed-P solubilization efficiency of soil organisms by multiplying the abundance and in situ activity of PSB was proposed. The PSB abundance and P solubilization efficiency were found to vary with soil and fertilization by comparing three soils of different
pH with two long-term fertilization treatments. Compared with previous genome-centric sequencing that can only assess the potential function for P solubilization by quantifying the abundance of P functional genes (e.g., *gcd*, *phod*, *pstA*)\(^6, 7, 26, 27, 28\), this work provides a direct *in situ* phenotypic evaluation of microbial P solubilization function. It thus can serve as an important approach to guide tailored management to stimulate native PSB to mobilize soil legacy P, reducing the reliance on P fertilizer and promoting sustainable agriculture. Moreover, the proposed P solubilization efficiency of soil organisms can serve as a new phenotypic index to evaluate soil health.

Raman phenotypic characterization revealed the distributions of active PSB in different soil types. Similar trends in PSB abundance and P solubilization efficiency were observed in DZ and DH soils, with a significant increase with IF when compared to CK treatments. While a decreasing trend was found in QY soils under fertilization application. Factors such as pH, Olsen-P, and DOC were revealed to influence the PSB abundance in different soils. For example, PSB abundances were increased in DZ and DH soils (pH > 8) under IF treatments but decreased in acidic QY soils (pH < 6). The decreased abundance of PSB in IF-QY treatment could be due to the exacerbated soil acidification after long-term chemical fertilizer addition. This is consistent with previous reports that found a significant reduction in the relative abundance of P solubilization-related genes with pH decreasing\(^7\). pH has been previously reported as the key role affecting soil microbial community composition\(^29, 30\), and low pH in acidic sites could be harmful to bacterial activity\(^31\). In QY soils, low pH has been assumed to be the most common limiting factor for bacterial growth. Additionally, the extra nutrient input by fertilization could activate the originally dormant microorganisms in QY soils (Figure S1), resulting in a decrease in the relative abundance of PSB (Fig. 3b). These results indicate that neutralizing acidic soil could be an important strategy for promoting soil microbial P solubilization and improving P application efficiency.

Olsen P was revealed to have different effects on PSB abundance depending on fertilization regimes. It was positively correlated with the abundance of PSB in DZ and DH soils with CK and IF treatments, reinforcing the previous view that P bioavailability regulates the biogeochemical cycle of element\(^32\). Based on trait-based microbial strategies under no organic matter input conditions, microbes are driven to capture more resources for their own catabolism. For example, more microorganisms are selected to produce extracellular enzymes to break down complex resources with increasing organic acid secretion\(^33, 34\), which enhances P solubilization. In contrast, negative correlations between PSB abundance and soil Olsen P were found in DZ and DH soils after organic fertilization. The absence of resource limitation favors a high-yield strategy in soil microbes\(^35\). This strategy allows microbes to maximize resource uptake by allocating the added Olsen P to invest in associated assimilatory pathways, such as the synthesis of amino acid, nucleotide, and fatty acid, rather than resource acquisition in which microbes are selected for catabolism processes\(^34\). Therefore, the microbial high-yield strategy would explain the decrease in PSB abundance under organic fertilization. In addition, we found that soil bacterial community associations were more tightly linked in IF treatment, indicating cooperative and synergistic interactions among community members. These complementary activities within the community can improve overall P solubilization efficiency (49). The concentrations of DOC were observed to negatively
impact the abundance of PSB across three types of soils. The reason could be that elevated soil DOC stimulated the growth of some microorganisms that engaged in resource competition with PSB.

**Active PSB in native soils and P mobilization strategy**

The non-destructive Raman approach enables downstream single-cell sorting and targeted metagenomic sequencing of H-PSB in soils. The identified H-PSB taxa included *Bacillus marmarensis, Bacillus pseudofirmus, Moraxella osloensis, Stenotrophomonas maltophilia*, and *Cutibacterium acnes* spanning in three phyla of *Firmicutes, Acidobacteria* and *Proteobacteria* (Fig. 4a, b). All these taxa can be found in soil bulk microbial communities but represent a small fraction of the overall soil microbial diversity, indicating that these taxa play a key role in solubilizing soil fixed P. In addition, although the majority of the sorted bacteria have been previously reported as inorganic PSB, this is the first time that their *in situ* P solubilization activity in soils was demonstrated here. This study also identified three novel H-PSB affiliated with uncultured bacteria, including *g_Bacillus, g_Propionibacterium*, and *K_bacteria*. These novel PSB represent new important beneficial microbial resources that worth further exploration. Considering the high *in situ* activity and the long-term adaption of H-PSB in original soils, these H-PSB are superior in terms of *in situ* function and colonization ability than those obtained via the traditional pure culture-based method. Therefore, the presented function-driven single-cell Raman sorting also enhances the capability to mine indigenous soil beneficial bacteria for a sustainable agriculture.

The identified H-PSB were found to be at a low-abundance level in bulk soil communities. Low-abundance species are increasingly recognized as important drivers of biogeochemical cycling in terrestrial ecosystems. Currently, bulk methods such as metagenomic sequencing and DNA-SIP techniques are still challenging to resolve bacteria at low abundances. By comparison, single-cell Raman sorting presented here and single-cell fluorescence sorting that was previously reported to study cellulose-degrading bacteria from the rare biosphere demonstrated their ability to meet this challenge.

These findings illustrate the importance of applying function-driven single-cell approaches to identify key functional microorganisms in the soils that maybe overlooked by bulk genome-based approaches.

The metagenome of the sorted PSB also sheds light on the genetic basis and functional traits in relation to P and C metabolism. PSB mainly employs two strategies to obtain P nutrients: producing organic acids and phosphatase to release fixed P and constructing high-efficiency P transport pathways (Fig. 5a). Typical genes encoding inorganic P solubilization and organic P mobilization enzyme were detected (Figure S5, 7), supporting their role in mobilizing both inorganic and organic fixed P. In addition, the carbon cycle is inextricably linked to the phosphorus cycle, but the role of C cycling processes in phosphorus solubilization has rarely been analyzed in previous studies. Here, in addition to P cycling-related genes, genes encoding C cycling including carbon metabolism and TCA cycles (Fig. 5) were also detected in the sorted H-PSB. Our results indicated that the sorted PSB can produce protons for solubilizing recalcitrant forms of P through hydrocarbon degradation, such as organic carbon compound oxidation. The protons produced as the by-products of hydrocarbon metabolism can be transferred to the extracellular space through electron transfer mediated by cytochrome bc1 and
cytochrome c oxidase\textsuperscript{40,41}. Among the three metagenomes analyzed, the genome of sorted PSB from the DZ soil contained genes encoding the catabolism of a broad spectrum of carbon sources (Fig. 5b and Figure S5), suggesting their greatest potential to exploit nutrient sources. Microbes with a broader catabolic capacity may, therefore, become strong candidates for long-term survival and P solubilization activity in the soil. This is attributed to the fact that microbial communities exhibiting a strong capacity for resource acquisition tend to be selected, leading to enhanced colonization ability even in challenging soil conditions, as proposed by the optimal foraging theory\textsuperscript{42}. This finding suggests that DZ-PSB holds more potential for the development of advanced bio-fertilizers.

However, we could not obtain a metagenome-assembled genome from the sorted PSB, probably due to the amplification bias of multiple displacement amplification. Moreover, the current single-cell Raman and sorting methods yield a limited number of cells that are not enough to completely depict the composition of active soil PSB microbiota. Future efforts should focus on improving the throughput of single-cell sorting techniques to obtain more comprehensive metagenomic insights into highly efficient PSB.

In conclusion, we employed a function-driven single-cell Raman-D\textsubscript{2}O approach to identify and quantify active PSB in native soils. Our findings revealed a distinctive response of PSB abundance and \textit{in situ} P solubilization activity to different soil types and fertilization regimes. Interestingly, the addition of organic fertilizer input was not effective in enhancing microbial P solubilization ability in nutrient-rich soils, whereas inorganic fertilizer notably increased the abundance of active PSB. These findings shed new light on tailored agricultural management for inorganic and organic fertilization in different soils by evaluating the efficacy of indigenous active PSB. Moreover, through Raman-activated single-cell sorting and metagenomic sequencing, we successfully identified highly active PSB, including previously uncultured strains mainly from the low-abundance soil microorganism pool. By analyzing the genomes of H-PSB, C cycle processes involved in substrate degradation provide protons, organic acids and energy for P solubilization. Overall, this work advances our understanding of soil active functional PSB pool, providing a promising microbial approach to mobilize soil legacy P and improve sustainable agricultural practices and soil health.

**Methods**

**Soil samples collection**

In this study, nine different soil samples were collected from three long-term field experiments in Hunan (QY), Zhejiang (DH), and Shandong (DZ) provinces in China. These experiments utilized three different fertilization treatments: without fertilization, inorganic fertilizer, and a combination of organic (pig manure) and inorganic fertilizers. Additional information about the long-term fertilization of the soils can be found in Table S2. Soil samples were collected from the top layer of soil, which was less than 15 cm deep, and three replicates were collected for each soil type. The soils were then air-dried in the dark and sieved using a 0.6 mm sieve to remove large particles.
Analysis of soil properties

Soil pH and bioavailable nutrients (i.e., dissolved organic carbon, Olsen P) were detected in order to understand the comprehensive effects of different fertilization on soil systems. The pH of the soils was determined by measuring the pH of soil suspensions at a soil/water ratio of 1:1.25 (w/v). The study used a TOC analyzer (TOC-LCPH, Shimadzu, Japan) to detect soil dissolved organic carbon (DOC) at a soil/water ratio of 1:1.5 (w/v). Soil Olsen-P was measured using the molybdenum-blue colorimetric method, which involved extracting the nutrient using a NaHCO₃ solution.

Active PSB extraction

To prepare the soil for analysis without bioavailable P, we added 1 g of soil to 30 mL of 0.5 mol·L⁻¹ NaHCO₃ solution, which was then incubated at 25°C and 180 rpm for 6 h. After incubation, the solution was centrifuged at 6000 g for 3 min to remove the supernatant and reserve the soil microorganisms. This washing process was repeated three to five times until the concentration of Olsen-P was low in the final supernatant. The washed soils were then air-dried at room temperature. The study also weighed the soils before and after drying to calculate soil moisture. To extract soil bacteria, a certain volume of D₂O was added to the soil, and the final D₂O content reached 20% of the soil moisture. To further extract soil bacteria, the study utilized Nycodenz Density-Gradient Separation. Specifically, 1 g of soil sample was homogenized in 5 mL of phosphate-buffered saline solution (PBS, containing 8 g·L⁻¹ NaCl, 0.2 g·L⁻¹ KCl, 1.44 g·L⁻¹ Na₂HPO₄, and 0.24 g·L⁻¹ KH₂PO₄) amended with 25 µL of Tween 20. The slurry was vigorously vortexed for 30 min to detach soil-associated bacteria. The solution was then added to the top of 3 mL of Nycodenz solution (4 g of Nycodenz was dissolved into 5 mL of sterile water) and centrifuged at 14,000 g for 60 minutes. The bacteria-containing layer was carefully collected into a new tube, and the collected bacteria were washed twice with ultrapure water at 5000 rpm for 3 minutes to remove residual PBS and other reagents.

Single-cell Raman measurement and cell sorting

To perform Raman measurements, 2 µL of as-prepared soil microorganisms were loaded onto an Al foil sorting chip and air-dried at room temperature. We used a LabRAM Aramis confocal Raman microscope (HORIBA Jobin-Yvon), equipped with a 300 g/mm grating, a 100× objective (Olympus, NA = 0.09), and a 532 nm Nd:YAG laser (Laser Quantum) to acquire Raman spectra. Each cell was typically exposed for 9 s for Raman spectra acquisition. To evaluate the metabolic activity of the cell, which quantitatively evaluates the ability of microbial P solubilization, we calculated the Raman C-D ratio. To preprocess the obtained Raman spectra, we performed baseline correction using LabSpec5 software (HORIBA Jobin-Yvon). The intensity of the Raman band was calculated by the area of the peak. The Raman C-D ratio was calculated by dividing the intensity of the C-D peak (2040–2300 cm⁻¹) by the sum of the C-D peak and C-H peak (2800–3100 cm⁻¹).
We considered bacteria from free bioavailable P soils containing a significant C-D band as PSB\textsuperscript{45}. To obtain the targeted single PSB, we used Active Cell Laser Ejection (ACLE) with PRECI SCS (HOOKE Instruments Ltd, China). We sorted PSB from three soil samples, and 20 targeted PSB of each soil sample and one control ejection without any bacteria were sorted into a receiver. We then transferred the cells to a 200 µL tube containing cell lysis buffer (Qiagen, Germany), and lysed the sorted cells at 65 °C for 10 min. We amplified the DNA of lysed cells using multiple displacement amplification (MDA) with Phi29 DNA polymerase (Qiagen, Germany). We checked DNA quality by PCR of the 16S rRNA gene (primer: 515 F- GTGYCAGCMGCCGCGGTAA, 907 R- GGACTACNVGGGTWTCTAAT). We sequenced only the sample with a positive PCR result in sorted DNA and a negative result in the control treatment using the Illumina HiSeq X-Ten sequencer (Majorbio, China).

**Mini-metagenome sequencing and analyses**

We generated 12 gigabases of sequencing data for each sample. In total, 36 gigabases of data were obtained. To ensure data quality, we eliminated clean reads with a length less than 50 bp and an average quality score lower than 30 using Fastp. The remaining clean reads were assembled using IDBA-UD with default parameters\textsuperscript{46}. We clustered the contigs using CD-HIT with 90% identity and 90% coverage\textsuperscript{47}. The read counts of each predicted ORF were calculated using htseq-count v0.6.1 with the ‘intersection strict’ parameter, and normalized by sequencing depth and ORF length, which were expressed as RPKM values\textsuperscript{48,49}. The contigs were queried against the NCBI nr database using DIAMOND. Metabolic pathways and carbohydrate hydrolases in each mini-metagenomics were annotated by BLASTP against the KEGG and CAZyme databases, respectively\textsuperscript{50,51}. To analyze the P cycling-related functional genes within the sorted H-PSB, we acquired the complete genomes of bacterial strains that fall within the sorted bacterial category from the NCBI database. Subsequently, we constructed a phylogenetic tree using FastTree v2.1.10\textsuperscript{52}. To identify the P cycling-related genes, we performed a sequence alignment against the KEGG database. This alignment was carried out using BLASTP with a stringent E-value threshold of $\leq 10^{-5}$.

**Soil DNA extraction and high-throughput sequencing**

We extracted soil DNA using the DNeasy PowerSoil Kit (QIAGEN, Germany) following the manufacturer instructions. The concentration of soil DNA was determined using a Qubit™ 4 Fluorometer. We sequenced the bacterial 16S rRNA gene in the V4 region using an Illumina MiSeq PE 300 platform (Majorbio, China). To analyze the sequencing data, we used Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1). High-quality sequences were assigned to operational taxonomic units (OTUs) using UCLUST based on a similarity threshold of 97%. We assigned representative OTUs using the PyNAST aligner based on the SILVA database v. 132. The raw reads of nine amplicons and three mini-metagenomics were deposited in the NCBI sequence Read Archive.

**Statistical analyses**

Shapiro-Wilk analysis was applied to test for normality. One-way analysis of variance (ANOVA) and T-tests were employed for all statistical examinations using GraphPad Prism 5, and a $p$ value below 0.05...
was considered significant.

**Declarations**

**Data availability**

The relevant data are available within the paper and its Supplementary Information file. Source data are provided with this paper.

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**Author contributions**

H.Z.L., L.C. and Y.G.Z. conceived the study. H.Z.L. and K.Y. performed the experiments. H.Z.L., J.J.P. and Y.Y.Z. performed the data analysis. L.C. and Y.G.Z. provided financial support. H.Z.L. wrote the manuscript. L.C., Q.L.C. and Y.G.Z. reviewed and edited the manuscript.

**Competing interests**

The authors declare no competing interests.

**References**


**Figures**
Figure 1

**Effect of long-term fertilization on soil physiochemical properties.** Changes of soil pH (a), Olsen-P (b), and dissolved organic carbon (c) under different fertilization in DH, DZ, and QY soils, respectively. Mean ± standard deviation (n = 3). One-way ANOVA was used to test the significance, ***$P < 0.005$; ns represents no significance. CK, IF, and CF represent no fertilization, inorganic fertilization, and a combination of organic and inorganic fertilization treatments, respectively.
Figure 2

The workflow for *in situ* PSB identification through Raman-D$_2$O (a) and targeted single-cell sorting and metagenomic sequencing (b).
**Figure 3**

**Evaluation of phenotypic microbial P solubilization of soils.** The normalized C-D ratios (a), PSB percentage (b), and phenotypic P solubilization efficiency (c) in DZ, DH, and QY soils, respectively. The grey dotted line at 0.5 represents the threshold for distinguishing PSB and non-PSB. It is determined as the mean + 3×SD of the cell without D₂O addition (D free). One-way ANOVA was used to test the significance, *P < 0.05, **P < 0.005; ns represents no significance.
Figure 4

**Bacterial composition of the sorted H-PSB.** (a) Sorting enrichment of H-PSB recovered from bulk soils. Relative abundance of bulk soil bacterial communities (Pre-sort) and Raman-sorted H-PSB (Post-sort) at phylum level. OTUs of bulk soil microorganisms that match to a H-PSB that were enriched during the sorting are marked with a grey alluvium indicating taxonomy (phylum) that links the pre- and post-sort relative abundances. (b) Relative abundance of the Raman-sorted H-PSB at the species level from DH, DZ, and QY soils.
Figure 5

Genetic information of sorted H-PSB. (a) Phylogenetic tree displaying the nearest neighbors of seven sorted H-PSB based on BLAST searches against the NCBI database. The heatmap showing the P cycling relevant functional genes in these PSB based on whole-genomic data. (b) The detected CAZyme groups involved in the degradation of cellulose, hemicellulose, lignin, starch, and pectin. (c) An outline of carbon metabolism and TCA cycle. The color squares represent the presence of functional genes or enzymes.

Supplementary Files

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