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D_2O-Isotope-Labeling Approach to Probing Phosphate-Solubilizing Bacteria in Complex Soil Communities by Single-Cell Raman Spectroscopy

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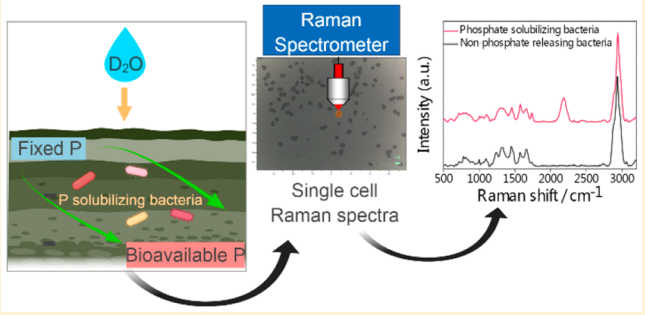
Supporting Information

ABSTRACT: Increasing the bioavailability of immobilized phosphorus (P) in soil by phosphate-solubilizing bacteria (PSB) is an effective strategy for sustainable agronomic use of P and for mitigating the P crisis. Here, D_2O isotope labeling combined with single-cell Raman spectroscopy (Raman−D_2O) was developed as an efficient activity-based approach to characterizing the presence and activity of PSB in a culture-independent way. On the basis of the finding that PSB were significantly more active than non-PSB in the presence of insoluble P, a C−D Raman band from active assimilation of D_2O-derived D was established as a biomarker for both inorganic-phosphate-solubilizing bacteria and organic-phosphate-solubilizing bacteria. C−D ratios (intensities of C−D bands as percentages of the intensities of both the C−D and C−H bands) were further established as semiquantitative indicators of P-releasing activities because of the consistency between the C−D ratio and the concentration of solubilized phosphate or acid phosphatase activity as measured by conventional bulk assays. By applying Raman imaging, single-cell Raman−D_2O clearly discerned PSB in a mixed-soil bacterial culture and even in complex soil communities. Remarkable heterogeneity of microbial activity, ranging from 2 to 30% (close to that in medium without P and that in medium with sufficient soluble P, respectively), was revealed at the single-cell level and clearly illustrated the subpopulation of soil bacteria active in solubilizing P. This work not only enables probing PSB and their P-releasing activities but also opens a window to explore more diverse microbial resources when obtaining related isotope-labeled substrates is prohibitive.



Phosphorus (P) fertilizer is indispensable for crop growth. However, P is a "disappearing nutrient" because of the nonrenewable nature of the phosphate reserves, which are estimated to be exhausted within the next 50−100 years.[1−3] Meanwhile, to promote crop growth and yield, large amounts of P fertilizers exceeding crops needs are applied and accumulate in arable soils. It has been reported that only about 17% of the applied P is utilized by the crops, while the remaining is bound by soil and becomes unavailable to the crops because of the strong adsorption and rapid precipitation of P by Ca^{2+}, Fe^{3+}, and Al^{3+} in soils.[1,4] Long-term P fertilization has built up an abundant soil legacy of P that is largely unavailable to plants. Mobilization of fixed soil P provides a potentially sustainable way to globally reduce the use of P fertilizer and mitigate the aggravating problem of P shortage.[5]

Currently, liming is an established practice used to increase P bioavailability in acidic soils by increasing soil pH to mobilize P fixed by metal cations and mineralize soil organic P.[6] However, long-term liming can result in soil hardening,[7] thus a more sustainable way to release soil P is highly demanded. Microorganisms are the engine driving biogeochemical cycling of essential elements.[8,9] Many naturally occurring soil bacteria and mycorrhizal fungi can mobilize recalcitrant soil P by producing organic acids and phosphatase enzymes.[10−13] The symbiotic cooperation between phosphate-solubilizing bacteria (PSB), mycorrhizal fungi and plant roots can further enhance the P-mobilizing process and increase the uptake of phosphate by plants.[10] Some PSB and fungi have been introduced as biofertilizers in agricultural communities to increase the bioavailability of soil fixed P and improve plant yields, thereby reducing the application of chemical fertilizer.[14,15] Currently, effective PSB strains are normally screened

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and isolated from soil by plating on Pikovskaya's or yolk culture media.[16−19] However, PSB that can grow in an artificial culture medium may not thrive in soil environments. Moreover, because more than 99% of soil bacteria remains uncultivable, it is a great hurdle to discover and characterize functional PSB in soil environments.[20] Developments of novel culture-independent approaches that enable exploration of PSB in their native soil environment are therefore highly important.

Single-cell Raman spectroscopy is an effective way to study uncultivable microorganisms.[21−23] Raman spectra can provide rich chemical information on bacteria at the single-cell level, including information on nucleic acids, proteins, lipids, pigments (e.g., cytochrome c and carotenoids), and polymers (e.g., poly P).[21−27] More importantly, when combined with stable-isotope probing (SIP), such as with ^{13}C, ^{15}N, or ^{2}D, intracellular assimilation of isotope-labeled substrates generate characteristic Raman shifts as a result of the substitution of light atoms with heavier stable isotopes in the chemical bonds of newly synthesized biomolecules.[21−23] These Raman shifts thus act as simple and quantitative biomarkers of functional or active bacteria. Single-cell Raman spectroscopy combined with ^{15}N and ^{13}C SIP has been applied to reveal N_2-fixing, naphthalene-degrading, and carbon-fixing functional bacteria in complex soil and water communities.[21,28,22,29,30] However, in contrast to C and N with multiple stable isotopes, ^{31}P is the only stable form of P, whereas all other forms varying in mass from ^{24}P to ^{46}P are radioactive.[31] Without a proper stable isotope other than ^{31}P, it is not possible to study PSB in the same way as has been done for C and N. Because P exists primarily as PO_4^{3-} on Earth, a stable isotope of oxygen in phosphate (^{18}O-PO_4) has been utilized to trace microbe-driven P cycling and enzyme activities.[32,33] However, P is a micronutrient, and the amount required by microbes for biosynthesis is much less than those of ^{13}C and ^{15}N. In our early attempts, no Raman shifts were observed using ^{18}O-PO_4 as the sole P source for microbial growth, and up to now, there has been no report using Raman-SIP to investigate PSB.

To apply Raman-SIP for studying PSB without any proper stable-isotope-labeled PO_4^{3-}, alternative SIP has to be sought. Recently, single-cell Raman spectroscopy combined with heavy water (D_2O, Raman−D_2O) provides a sensitive way to detect metabolically active bacteria.[23,34−36] Assimilation of D_2O-derived D into microbial biomass can be explicitly detected via C−D Raman bands.[23,35−38] Moreover, D_2O amendment does not change the natural substrate pool and thus enables a more realistic study of active cells in microbial communities. Raman−D_2O has been used to reveal metabolically active bacteria in mouse cecum in response to different sugars[23] and antibiotic-resistant bacteria in response to antibiotics.[35,38] P is an essential element for synthesizing key microbial components such as DNA, phospholipids, adenosine triphosphate (ATP), and others, and we thus hypothesize that a lack of bioavailable P will decrease or even terminate microbial metabolism, whereas PSB that can release fixed P will exhibit high metabolic activity and therefore a high C−D intensity.

To test this hypothesis and to establish single-cell Raman−D_2O as a new, effective, culture-independent method for revealing PSB in a complex soil community, we studied the effect of P supply on microbial activities and established a C−D Raman band as a proper biomarker to indicate the presence and activity of both inorganic and organic PSB. Raman−D_2O coupled with Raman imaging was further applied to discern PSB in a mixed bacterial culture and even in complex soil communities.

MATERIALS AND METHODS

Bacterial Species, Media, and Growth Conditions. Bacteria used in this study included three inorganic-phosphate-solubilizing bacteria (iPSB) isolated in our laboratory from soil (i.e., *Pseudarthrobacter oxydans* OY2, *Massilia phosphatilytica* OD1, and *Burkholderia cepacia* Y14-15),[18,39] one organic-phosphorus-mineralizing bacteria (oPMB) purchased from the Japan Collection of Microorganisms (i.e., *Bacillus megaterium* JCM 21718^T), and one non-PSB isolated in our laboratory (i.e., *Chryseobacterium gleum* 19). Among them, *B. cepacia* Y14-15 was both an iPSB and an oPMB. Minimal media (MM) supplemented with different forms of P were used in this study. MM without P (MM-NoP) was prepared by adding 2 g of glucose, 0.1 g of $(NH_4)_2SO_4$, 0.06 g of KCl, 0.06 g of NaCl, 0.006 g $MnSO_4{\cdot}H_2O$, 0.006 g of $FeSO_4{\cdot}7H_2O$, and 0.006 g of $MgSO_4{\cdot}7H_2O$ into 200 mL of ultrapure water. MM with dissolvable phosphate (MM-P) was prepared by dissolving 0.1 g of KH_2PO_4 and 0.3 g of Na_2HPO_4 into 200 mL of MM-NoP. MM with tricalcium phosphate (MM-Ca_3P) representing inorganic fixed phosphate was prepared by adding 2.5 g/L $Ca_3(PO_4)_2$ as the only P source into MM-NoP. MM with lecithin representing organic fixed P (MM-orgP) was prepared by amending 0.5 g/L lecithin as the only P source into MM-NoP. For deuterium-isotope labeling, 50% (v/v) D_2O (99.8 atom % D, Aldrich) was used in all the above media. Bacteria were incubated in a 24-well culture plate at 37 °C for different time periods prior to measuring Raman spectra and the concentrations of released phosphate and acid phosphatase. Unless otherwise stated, all chemicals were purchased from Sinopharm Chemical Reagent Company.

Determination of P-Releasing Ability of Bacteria. Bacteria from a culture grown overnight in LB media were washed three times with ultrapure water to eliminate residual phosphate. One milliliter of the as-prepared bacteria was inoculated in 100 mL of MM-Ca_3P or MM-orgP in triplicate. After 5 days of incubation in MM-Ca_3P at room temperature, bacterial suspensions were centrifuged at 5000 rpm, and the supernatants were filtered through a 0.22 μm filter to remove bacteria and Ca_3P. The concentrations of released water-soluble phosphate from Ca_3P in the culture filtrate were determined using Mo−Sb−ascorbic acid colorimetry.[40] Briefly, 10 mL of culture filtrate was mixed with 1 mL of molybdate (prepared by dissolving 25 g of ammonium molybdate in 1 L of 4.6 M sulfuric acid) and 0.15 g of ascorbic acid. After incubation for 15 min at room temperature, the concentration of released soluble phosphate was determined by measuring the absorbance at 660 nm via a multiplate spectrophotometer (Thermo Scientific) and comparing with a standard curve (R^2 = 0.9993). A KH_2PO_4 solution of 1 mg/L was used to calculate the recovery rate (instrument reading/true value) after 10 measurements. The recovery rate of KH_2PO_4 was 96−103%. In addition, the acid phosphatase (ACP) activities of bacteria after 24 h of incubation in MM-orgP were measured via an ACP test kit (Nanjing Jiancheng Bioengineering Institute).[41] Briefly, the bacterial culture was incubated with disodium phenyl phosphate for 15 min to allow enzyme−substrate reaction. The absorbance at 520 nm was then measured using a microplate spectrophotometer to determine ACP activities by comparison with a standard curve (R^2 = 0.9996). A certified phenol solution used as a reference material was provided by

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the test kit, and its recovery rate was calculated as 98−102%. Both Mo−Sb−ascorbic acid colorimetry and ACP tests were performed in biological triplicate for each bacterial sample.

Soil-Sample Collection. Surface soil samples at a depth of less than 10 cm were collected from a grassland at JingXian Park, Xiamen, China (24° 34′ 40.8″ N, 118° 06′ 00.0″ E). The soil samples were homogenized and sieved through a 0.6 mm sieve to remove stones and then stored at 4 °C until use.

Extracting Bacteria from Soil Microcosms by Nycodenz Density-Gradient Separation (NDGS). A modified NDGS protocol from previous reports was used to extract bacteria from soil.[21,42] Briefly, soils (1 g) were homogenized in 5 mL of phosphate-buffered saline (PBS, 8 g·L^{-1} NaCl, 0.2 g·L^{-1} KCl, 1.44 g·L^{-1} Na_2HPO_4, 0.24 g·L^{-1} KH_2PO_4) amended with 25 μL of Tween 20. After being vigorously vortexed for 30 min, the soil-associated bacteria were detached. To separate bacteria from soil particles, the as-prepared slurry was gently added to 5 mL of Nycodenz (≥98%, Aladdin) solution (1.42 g/mL), prepared by dissolving 4 g of Nycodenz in 5 mL of sterile water, and then centrifuged at 14 000 g for 90 min at 4 °C. After centrifugation, the middle layer containing bacteria was collected into a new tube containing PBS. The microorganisms inside were collected by centrifugation at 5000 rpm for 10 min at room temperature and washed twice with ultrapure water to remove residual PBS and other traces of reagents.

Preparation of Soil Bacteria in Artificial Media. The soil bacteria extracted via NDGS were then inoculated in MM-NoP, MM-P, and MM-Ca_3P amended with 50% D_2O and incubated at 37 °C for 24 h prior to Raman analysis.

Preparation of Soil Bacteria in Bioavailable Phosphate-Free Soil. To prepare soil free of bioavailable P, the soil was washed successively with water and a 0.5 mol/L $NaHCO_3$ solution, which is widely used to extract the soil dissolvable-phosphate pool, in a modified Hedley method.[43] Briefly, 1 g of soil was added to 30 mL of ultrapure water, incubated at 25 °C and 180 rpm for 16 h, and then centrifuged to remove supernatant. The same procedure was applied to the $NaHCO_3$ solution. The obtained soil pellets were dried at 108 °C for 12 h. The soils were weighed before and after drying in order to calculate soil moisture. A certain volume of D_2O was added to reach a D_2O content of around 50% and a soil moisture of 20% (analogous to that of natural soil). The soil bacteria extracted via NDGS were added to the as-prepared soil without soluble phosphate and incubated at 37 °C for 24 h prior to Raman analysis.

Single-Cell Raman Measurements and Raman-Mapping Acquisition. All bacterial samples were washed with ultrapure water three times in order to remove residual media. An aliquot of 2 μL of the as-prepared bacterial solution was loaded on Al foil and air-dried at room temperature prior to Raman measurements.[44] Raman spectra and Raman mapping were acquired from a LabRAM Aramis confocal Raman microscope (HORIBA Jobin-Yvon) equipped with a 532 nm Nd:YAG laser (Laser Quantum), 300 g/mm grating and a 100× objective (Olympus, NA = 0.09). The Raman band of a silicon wafer at 520.6 cm^{-1} was used to calibrate the Raman spectrometer before each measurement. Twenty individual cells from each sample were randomly selected for single-cell Raman measurements. A total of three independent experiments were performed. The spectra were acquired in the range of 500−3200 cm^{-1} at an acquisition time of 15 s for each point. Raman imaging was acquired at a step size of 1 μm, and a pseudocolored Raman image was generated on the basis of the C−H and C−D bands. All spectra were preprocessed by baseline correction and normalization to the C−H bands via LabSpec5 software (HORIBA Jobin-Yvon). Peak intensity was quantified by calculating the peak area. To indicate the degree of D substitution in C−H bonds, the intensities of the C−D peak (2040−2300 cm^{-1}) and the C−H peak (2800−3100 cm^{-1}) were used to calculate a C−D ratio of CD/(CD + CH). The relative percent differences of C−D ratios in the three independent experiments ranged from 1.13 to 7.37% for the five bacterial species incubated in MM-NoP, MM-Ca_3P, MM-P, and MM-orgP for 24 h (Table S2). OriginPro 8.5 was used to plot each graph. Correlation and variance analyses were performed using GraphPad Prism 5.

RESULTS AND DISCUSSION

Effect of P Availability on Microbial Activity Revealed by Raman−D_2O Labeling. To understand the effect of P availability on microbial activity and ascertain the possibility of using C−D as a biomarker for PSB, we incubated the four bacterial species, *B. cepacia* Y14-15, *M. phosphatilytica* OD1, *P. oxydans* OY2, and *C. gleum* 19, in MM-P and MM-NoP containing 50% D_2O for 24 h. Single-cell Raman spectra were then acquired, and the obtained results are shown in Figure 1.

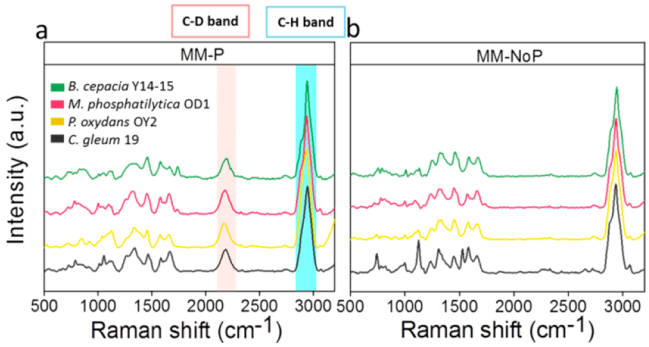

Figure 1. Single-cell Raman spectra of *B. cepacia*, *M. phosphatilytica*, *P. oxydans*, and *C. gleum* incubated in minimal media containing 50% D_2O (a) with and (b) without dissolvable P (MM-P and MM-NoP, respectively) for 24 h.

Bacteria incubated in MM-P amended with soluble phosphate displayed obvious C−D Raman bands in the region of 2040−2300 cm^{-1} (Figure 1a), whereas bacteria incubated in MM-NoP without P did not show any detectable C−D band (Figure 1b). Without P supply, bacteria lack the key ingredient to synthesize energy-providing adenosine triphosphate (ATP) and basic microbial-composition molecules, such as phospholipids and nucleic acids, leading to a cessation of metabolic activity and intracellular D incorporation.[45] In contrast, bacteria grown with a large-enough P supply are metabolically active and can assimilate D from heavy water for biosynthesis and therefore display a strong C−D band.[37]

In the case of PSB, when bioavailable P is unavailable, PSB are capable of dissolving insoluble phosphate to maintain their activity and growth. Because tricalcium phosphate and lecithin are the typical inorganic and organic forms of insoluble P, minimal media containing tricalcium phosphate (MM-Ca_3P) and lecithin (MM-orgP) as the only P sources amended with D_2O were used to incubate the bacteria. Bacterial activities and the utility of the C−D band in distinguishing PSB from non-PSB are examined below.

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Utility of C−D Band as a Biomarker for Inorganic-Phosphate-Solubilizing Bacteria (iPSB). Solubilizing inorganic phosphate is generally associated with bacteria-secreted organic acids, which can chelate cation-bound P and convert it into soluble forms.[46] The microbial ability to solubilize inorganic phosphate was examined by measuring released water-soluble phosphate via conventional Mo−Sb−ascorbic acid colorimetry after incubation of the bacteria in MM-Ca_3P for 5 days. Among the four bacteria, the concentrations of phosphate released by *B. cepacia* Y14-15, *M. phosphatilytica* OD1, and *P. oxydans* OY2 were from around 400 to 1400 ppm, whereas almost no phosphate was released by *C. gleum* 19 (Figure 2a), indicating that the first three were capable of solubilizing inorganic phosphate and can be classified as iPSB, whereas the last one is a non-iPSB.

The metabolic activities of the four bacteria incubated in MM-NoP (no P), MM-Ca_3P (inorganic fixed P), and MM-P (soluble P) containing 50% D_2O for different time periods (from 0 to 24 h) were examined by single-cell Raman−D_2O. Figure 2b shows the C−D ratios obtained from approximately 20 individual bacteria at each time point and incubation condition. In MM-NoP, the C−D ratios from all four bacteria after incubation for 0−24 h remained at a similarly low levels compared with those observed in Figure 1b. This finding indicated that the bacterial activity was lost in the absence of dissolvable phosphate and their biosynthesis metabolisms were thus shut down. By comparison, the C−D ratios of *B. cepacia* Y14-15, *M. phosphatilytica* OD1, and *P. oxydans* OY2 in MM-Ca_3P were found to increase over time until reaching a plateau after around 12 h. However, the C−D ratios of *C. gleum* 19 in MM-Ca_3P did not increase over time and remained at a low level, indicating a remarkable difference in activity between *C. gleum* and the other three bacteria. In MM-P which supplied enough soluble P, the C−D ratios of all four bacteria increased over time and were higher than the ratios in MM-Ca_3P, indicating that nutrient-rich MM-P was better at promoting bacterial activity than MM-Ca_3P, which supplied a limited amount of soluble phosphate dependent on the phosphate-solubilizing ability of the bacteria. We also noticed that the C−D ratios of *C. gleum* were lower than those of the other three bacteria in MM-P. The reason should be related to its intrinsic low metabolic activity or growth rate, which has been demonstrated to be species-specific.[23]

To relate microbial activity to P-releasing ability, both absolute and normalized C−D ratios from incubation in MM-Ca_3P for 24 h were plotted in Figure 2c. Normalized C−D ratios were calculated by dividing C−D ratios from MM-Ca_3P with those from MM-P, aiming at excluding the possible effects of intrinsic microbial activity on C−D ratios. It is clear that both the absolute and normalized C−D ratios of *B. cepacia* Y14-15, *M. phosphatilytica* OD1, and *P. oxidans* OY2 were significantly higher than those of *C. gleum* 19 (Figure 2c). This result corresponded well with the concentrations of phosphate released by the bacteria (Figure 2a), demonstrating that C−D ratios can distinguish iPSB from non-iPSB well. We also noticed that *M. phosphatilytica* OD1, which released the highest concentration of phosphate (Figure 2a), showed similar metabolic activity to that of *B. cepacia* Y14-15. After measuring the optical density of a bacterial suspension at 600 nm (OD_{600}), *M. phosphatilytica* OD1 was found to grow to an OD_{600} level almost double that of of *B. cepacia* Y14-15. The larger bacterial number due to the rapid growth rate could explain the observed higher phosphate concentration. In addition, absolute and normalized C−D ratios were found to display highly similar patterns among the four bacteria, indicating that bacteria with intrinsically low metabolic activity were also inferior in releasing fixed P. Therefore, the absolute C−D ratio was enough to discern iPSB. This result is especially important when applying Raman−D_2O to discern iPSB in complex microbial communities that contain highly diverse microbes with distinct intrinsic metabolic activities, where normalized C−D ratios are impossible to calculate.

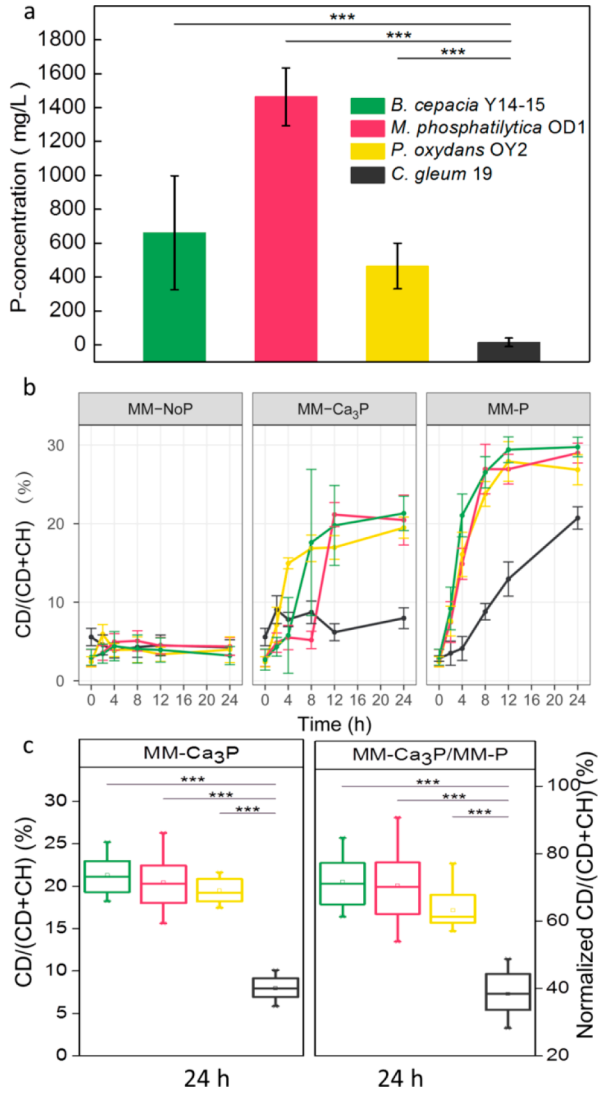

Figure 2. (a) Concentrations of dissolvable phosphate released from $Ca_3(PO_4)_2$ in minimal media by *B. cepacia* Y14-15, *M. phosphatilytica* OD1, *P. oxydans* OY2, and *C. gleum* 19 after incubation for 5 days. Error bars represent the standard deviations of three measurements from biological triplicates. (b) Time-dependent C−D ratios of the four bacterial strains incubated in minimal media containing 50% D_2O without P (MM-NoP), with $Ca_3(PO_4)_2$ (MM-Ca_3P), and with dissolvable P (MM-P). (c) C−D ratios (left) and normalized C−D ratios (right) after incubation in MM-Ca_3P for 24 h. Normalized C−D ratios were calculated by dividing the C−D ratios in MM-Ca_3P by those in MM-P. Twenty individual cells from each bacterial sample were measured by Raman spectroscopy. All comparisons denoted by asterisks (***) and a black bar are significantly different (one-way ANOVA, $P < 0.001$).

Utility of C−D Band as a Biomarker for Organic-P-Mineralizing Bacteria. In addition to inorganic fixed P, a

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considerable portion (30−65%) of soil P is present as organic P, such as that in phytate, nucleic acids, ATP, phospholipids, and phosphoproteins.[10,47,48] oPMB play an equally important role in soil-P cycling as iPSB. Different from the secretion of organic acids by iPSB, oPMB utilize enzymes such as phosphatase and phytase to mineralize organic P into plant-bioavailable inorganic P. In this study, a conventional ACP assay was used to evaluate the organic-P-solubilizing abilities of four bacteria after incubation in MM amended with lecithin as the sole organic-P source (MM-orgP) for 24 h.[41] On the basis of the ACP activity shown in Figure 3a, it was clear that *B. megaterium* JCM exhibited the highest phosphatase activity, followed by *B. cepacia* Y14-15, whereas the other two bacteria (i.e., *M. phosphatilytica* OD1 and *P. oxydans* OY2) exhibited the lowest ACP activity.

To demonstrate the ability of Raman−D_2O in identifying oPMB, the four bacteria were incubated in MM-orgP amended with 50% D_2O for different time periods (from 0 to 24 h) prior to Raman measurement. Time-dependent C−D ratios from about 20 individual cells were plotted in Figure 3b.

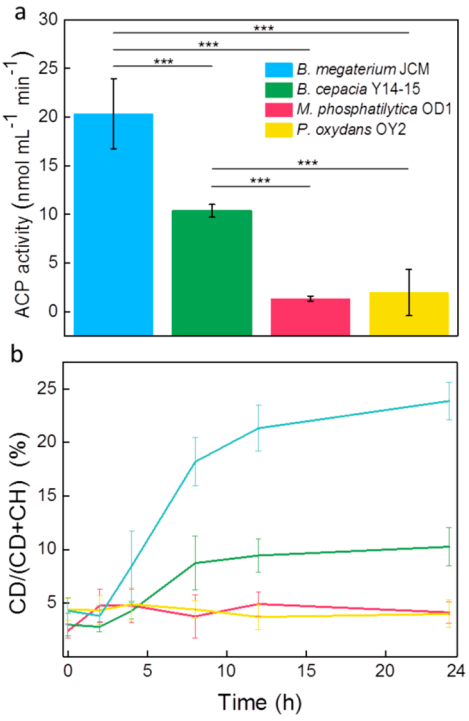

Figure 3. (a) Acid phosphatase (ACP) activity of *B. megaterium*, *B. cepacia*, *M. phosphatilytica*, and *P. oxydans* after incubation in minimal media containing organic P (MM-orgP) for 24 h. Error bars represent the standard deviations of three measurements from biological triplicates. (b) Time-dependent C−D ratios of the four bacterial strains incubated in MM-orgP containing 50% D_2O. Twenty individual cells from each bacterial sample were measured by Raman spectroscopy. All comparisons denoted by asterisks (***) and a black bar are significantly different (one-way ANOVA, $P < 0.001$).

Interestingly, C−D ratios from the two bacteria exhibiting higher ACP activities (i.e., *B. megaterium* JCM and *B. cepacia* Y14-15) also increased with time until reaching a plateau after 12 h. Furthermore, *B. megaterium* JCM, which had the highest ACP activity also exhibited the highest C−D ratios (Figure 3b), whereas the C−D ratios from the other two bacteria without ACP activity (*M. phosphatilytica* OD1 and *P. oxydans* OY2) remained at low levels during all incubations (Figure 3b). These results clearly indicate that the metabolic activities of the bacteria, measured as C−D ratios, are consistent with the ACP activities of the respective bacteria. Therefore, Raman−D_2O provides a good means to distinguish oPMB from non-oPMB and can even quantitatively evaluate the organic-P-releasing ability of bacteria. Similar to with the iPSB, the absolute and normalized C−D ratios were found to display identical patterns (Figure S1), enabling the use of absolute C−D ratios as a reliable biomarker to discern both oPMB and iPSB in complex microbial communities. Interestingly, *B. cepacia* Y14-15 were found to exhibit high C−D ratios with both inorganic and organic forms of fixed P, indicating its potency for releasing different forms of fixed P and thus its high potential as a bacterial fertilizer.

Probing and Raman Imaging of Soil-Dwelling PSB in Artificial Media. Raman−D_2O can be performed at the single-cell level, endowing its potential ability to discern PSB, including uncultivable bacteria, in complex communities without the necessity of pure culturing, and it can furthermore reveal the heterogeneous P-releasing ability of microbes at a high-resolution single-cell level.[21,36] Here, by inoculating soil bacteria in MM-NoP, MM-P, and MM-Ca_3P amended with heavy water for 24 h, respectively, single-cell Raman−D_2O was utilized to identify PSB from a mixed culture of soil bacteria. Figure 4a shows C−D ratios of soil bacteria in the three kinds

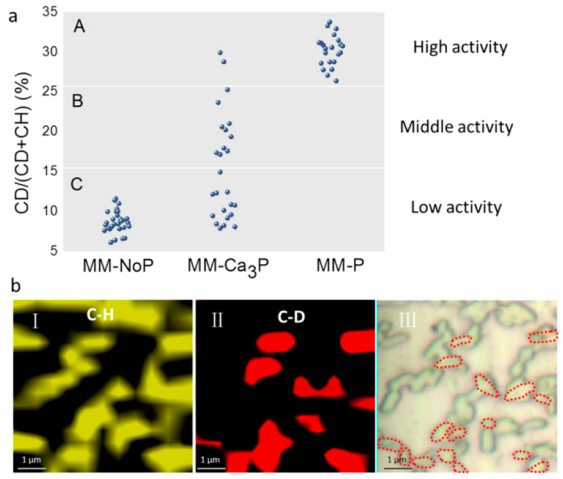

Figure 4. (a) C−D ratios of soil bacteria incubated in the artificial culture media of MM-NoP, MM-Ca_3P, and MM-P with 50% D_2O for 24 h. Low, high, and middle activity were defined by the C−D ratios in MM-NoP, the ratios in MM-P, and those in between. Each point is a measurement of a single cell. (b) Raman-mapping images based on C−H (I, yellow) and C−D (II, red) bands and corresponding photomicrographic images (III) of soil bacteria incubated in MM-Ca_3P with 50% D_2O for 24 h.

of media, where each globule represents a measurement of a single cell. In MM-NoP, all soil bacteria showed low C−D ratios ranging between 5 and 15%, indicating very low activities in the absence of P, whereas in MM-P, which contains adequate bioavailable P, all bacteria displayed high activity, as observed from the significantly elevated C−D ratios ranging between 25 and 35%. Different from the results with MM-NoP and MM-P, the C−D ratios in MM-Ca_3P spanned a broad range from 5 to 30%, covering low, middle, and high activity (Figure 4a), thereby illustrating the very different abilities of soil bacteria in solubilizing P. On the basis of the C−D ratios,

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the subpopulations with middle and high activity in MM-Ca_3P were regarded as active P releasers, and two of them, with C−D ratios as high as those observed in MM-P, were potentially the most efficient PSB. In contrast, the subpopulation with an activity as low as the one observed in MM-NoP was regarded as non-PSB. To confirm the presence of PSB in the artificial media as revealed by Raman−D_2O, the *pqqC* gene, which is required for gluconic acid synthesis and is widely used as a marker gene to identify PSB,[49] was amplified and visualized on an agarose gel (Figure S2). A bright band specific to the *pqqC* gene was observed in the soil bacteria incubated in both MM-P and MM-Ca_3P, confirming the presence of PSB.

To clearly distinguish PSB from diverse soil bacteria after incubation in MM-Ca_3P, Raman imaging was constructed on the basis of both the C−H and C−D bands, representing all bacteria and PSB, respectively (Figure 4b). Because all biotic particles such as bacteria have a C−H bands originating from lipids or proteins, a yellow C−H Raman image illustrating all soil bacteria (I) was created, and it was consistent with the corresponding photomicrographic image (III). By comparison, iPSB, as indicated by red in the C−D Raman image (II), were clearly less abundant than all soil bacteria (I and III). Comparison of photomicrographic and C−D Raman images allowed identification of PSB, which were outlined by red dotted lines in the micrographic image (III). These observations demonstrate that single-cell Raman−D_2O and related Raman imaging provide a good means to reveal PSB from mixed bacterial cultures as well as their heterogeneous P-solubilizing ability.

Probing and Raman Imaging of Soil-Dwelling PSB in Complex Soil Communities. Incubation of soil microbes in artificial culture media demonstrated the applicability of Raman−D_2O in probing active PSB in a mixed culture. However, artificial media are very different from soil conditions. Soil bacteria that grow well in artificial culture media can be enriched, leaving large amounts of uncultivable soil bacteria to be overlooked. In situ incubation of D_2O in soil will overcome this problem and provide more insights into soil PSB.

To probe PSB in soil via Raman−D_2O, a soil environment lacking bioavailable P was created in order to promote PSB to dissolve fixed P. Ultrapure water and a $NaHCO_3$ solution were used to wash out bioavailable P from soil. After washing, the amount of bioavailable P in the soil was largely reduced, from around 410.68 ± 34.37 to 8.17 ± 1.43 mg/L. Soil bacteria extracted from fresh soil via NDGS were added to the washed soil in order to supplement the loss of soil microbes during the washing and drying processes. A certain amount of 50% heavy water was also added to create a water content of around 20%, which was close to that of the original soil. After incubation for 24 h at room temperature, bacteria were separated from soil particles via NDGS to eliminate the interference of soil particles in the single-cell Raman measurement.[21,42] Figure 5a shows eight typical Raman spectra of individual soil bacteria with obviously different spectral fingerprints, indicating the high diversity of soil bacteria. More importantly, soil microbes displayed highly heterogeneous C−D intensities. Related C−D ratios ranged from 3% (black spectra) to 25% (red spectra), indicating the very different abilities of soil microbes in dissolving fixed P.

Figure 5b shows Raman-mapping images of extracted soil bacteria constructed with C−H (I) and C−D (II) bands as well as photomicrographic images (III and IV) after in situ

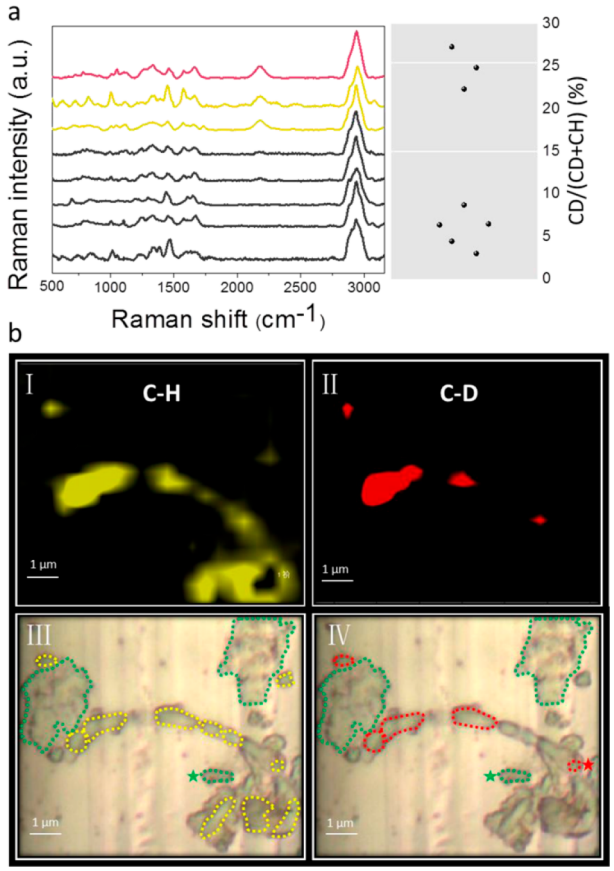

Figure 5. (a) Single-cell Raman spectra (left) and C−D ratios (right) of soil bacteria incubated in bioavailable P-free soil amended with 50% D_2O for 24 h. (b) Raman-mapping images, based on the C−H (I, yellow) and C−D bands (II, red), of soil bacteria incubated in bioavailable P-free soil and corresponding photomicrographic images (III and IV). The yellow and red dotted circles in III and IV outline bacteria with C−H and C−D bands, respectively. Green dotted circles outline abiotic particles without C−H bands.

incubation in soil. Photomicrographic images of bacteria extracted from soil are much more complex than those of soil bacteria incubated in artificial media because of the possible presence of abiotic particles such as soil residues (Figure 5b, III and IV). To discriminate bacteria and the more important PSB from abiotic particles, Raman images based on C−H bands, representing all bacteria, and C−D bands, representing PSB, were applied. The C−H Raman image (Figure 5b, I) clearly revealed that the regions outlined by yellow dotted lines in the photomicrographic image (III) were from bacteria, whereas those outlined by green dotted lines (III) were from abiotic soil residues, apparent because of the absence of C−H bands and thus shown as dark in the C−H Raman image (I). Even for the bacterium-like soil particle labeled with a green star in the photomicrograph (III), its darkness in the C−H Raman image demonstrated that it was not a bacterium. The C−D Raman image (II) further revealed PSB, outlined by red dotted lines in the photomicrograph (IV). The relatively larger yellow region as compared with the red region indicated that only a portion of the bacteria extracted from the soil were active in solubilizing P. The *pqqC* genes from the same bacterial sample were also amplified and could clearly be visualized in a gel image (Figure S2), thereby confirming the presence of PSB in the bacteria extracted from

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soil. These results demonstrated that single-cell Raman–D_2O combined with Raman mapping can accurately discern and locate PSB from complex soil microbial communities and eliminate interferences from abiotic soil residues.

CONCLUSIONS

This work is the first demonstration that single-cell Raman spectroscopy coupled with D_2O labeling can be used to probe both inorganic- and organic-P-solubilizing bacteria from complex soil communities in a culture-independent way. This approach is based on the findings that P is indispensable for microbial activities, measured as a C–D Raman band, and that PSB are more metabolically active than non-PSB when only inorganic or organic forms of fixed P are supplied. On the basis of these findings, C–D ratios were demonstrated as reliable indicators of PSB that can even semiquantitatively reveal microbial activities releasing fixed P. By using this indicator, Raman imaging further discerned and located PSB in a mixed bacterial culture as well as in more complex soil communities at the single-cell level. The heterogeneous activities of soil bacteria solubilizing fixed P were also revealed at the high-resolution single-cell level. Considering that isotope-labeled substrates of interest are not always accessible, the Raman–D_2O approach opens a window to unravel highly diverse functional or active bacteria and their ecological roles in ecosystems, thereby advancing the discovery and utilization of new microbial resources.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b04820.

Experimental description of genetic investigation of inorganic-phosphorus-solubilizing bacteria from artificial media and soil via the *pqqC* gene, precision calculation of Raman results, and C–D ratios before and after normalization (PDF)

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Notes

The authors declare no competing financial interest.

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