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# Biochemical mechanism of phosphorus limitation impairing nitrogen fixation in diazotrophic bacterium *Klebsiella variicola* W12

Li-Mei Zhang<sup>1,3</sup>  | Eleonora Silvano<sup>2</sup> | Branko Rihtman<sup>2</sup> |  
 Maria Aguilo-Ferretjans<sup>2</sup> | Bing Han<sup>1,3</sup> | Wei Shi<sup>1,3</sup> | Yin Chen<sup>2</sup> 

<sup>1</sup>State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China

<sup>2</sup>School of Life Sciences, University of Warwick, Coventry, UK

<sup>3</sup>University of Chinese Academy of Sciences, Beijing, China

## Correspondence

Limei Zhang, State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, 100085 Beijing, China.  
 Email: [zhanglm@rcees.ac.cn](mailto:zhanglm@rcees.ac.cn)

Yin Chen, School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK.  
 Email: [y.chen.25@warwick.ac.uk](mailto:y.chen.25@warwick.ac.uk)

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## Abstract

**Introduction:** Biological nitrogen (N) fixation (BNF) plays a key role in nitrogen supply in agricultural and natural ecosystems. Harnessing BNF can substantially reduce dependence on chemical fertilizer in agroecosystems and hence can contribute to sustainable agriculture. However, a number of field studies have demonstrated that BNF can be largely suppressed in phosphorus (P)-deficient environments, while the underlying mechanism is not well understood.

**Materials & Methods:** In this study, comparative proteomics and lipidomics analyses were conducted on a diazotrophic bacterium *Klebsiella variicola* W12 under P-deficient and P-replete conditions to gain insight into how P availability affects N fixation.

**Results:** Under P deficiency, N fixation activity of *K. variicola* W12 was severely repressed. In response to P limitation, the bacterium synthesized P-free ornithine lipids to replace glycerophospholipids in its membrane to reduce cellular demand for P. Comparative proteomics showed that P limitation resulted in upregulation of the PhoBR two-component system, a range of organic and inorganic P uptake and transport systems, while nitrogenase and N-fixation-related transcriptional regulators NifL and NifA were downregulated.

**Conclusion:** These results revealed lipid renovation as an adaptation strategy for N<sub>2</sub>-fixing microbes to survive under P stress and provided biochemical evidence on how P availability regulates BNF. A conceptual model of N-P coupling at the microbial metabolism level is therefore proposed. Our study provides a simple yet plausible explanation of how P deficiency suppresses BNF observed in the field and highlights the importance of regulating P availability to maximize the potential of BNF in agroecosystems for agriculture sustainable production.

## KEYWORDS

nitrogen fixation, N-P coupling, ornithine lipids, P deficiency, proteomics

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## 1 | INTRODUCTION

Nitrogen (N) and phosphorus (P) are two essential nutrients for plants and phytoplanktons and are often limiting factors for primary productivity in aquatic and terrestrial ecosystems (Elser et al., 2007). In agricultural lands, a large quantity of N and P fertilizers are applied globally each year to alleviate the limitation of N and P to improve crop productivity. As a consequence, N and P fertilization not only causes negative environmental effects, such as water eutrophication, N<sub>2</sub>O emission and soil acidification, but also exacerbates the exhaustion of limited P resources in the world. Unlike P supply which solely relies on the application of P fertilizers, ecosystems can acquire considerable N to overcome N deficiency through biological N<sub>2</sub> fixation (BNF), which is mediated by N<sub>2</sub>-fixing microbes. It has been estimated that BNF contributes 40–100 Tg N to the terrestrial ecosystems alone every year and is an important source of N supply even in fertilized agricultural ecosystems (Ladha et al., 2016; Vitousek et al., 2013).

Asymbiotic N<sub>2</sub> fixers, including associate and free-living diazotrophs affiliating within  $\alpha$ -,  $\beta$ - and  $\gamma$ -proteobacteria, are widely present in various natural and agricultural ecosystems (Furnkranz et al., 2008; Gaby & Buckley, 2011; Han et al., 2019; Shi et al., 2021). Although the asymbiotic N<sub>2</sub> fixation rate is lower compared with the symbiotic system, asymbiotic diazotrophs can survive and fix N<sub>2</sub> without forming a symbiotic structure with plants, and therefore play an important role in N input in ecosystems due to their wide distribution and high adaptability to diverse habitats (Reed et al., 2011). For example, it is estimated that about 24% of N in crop biomass originated from nonsymbiotic N<sub>2</sub> fixation in the agricultural ecosystem (Ladha et al., 2016). A 5-year field study suggested that the isolated indigenous maize grown in N-depleted field in Mexico can supply 29%–82% of the plant N derived from nitrogen fixation of diazotrophs, which were enriched by carbohydrate-rich mucilage of aerial roots (Van Deynze et al., 2018). More recent studies in forest ecosystem also suggested that the contribution of nonsymbiotic N<sub>2</sub> fixation to N supply in the rainforest is even higher than expected when considering the diazotrophs inhabiting the plant phyllosphere (Furnkranz et al., 2008; Madhaiyan et al., 2015). However, a number of field observations and laboratory experiments have shown that BNF can be inhibited upon P deficiency. For instance, P limitation on nitrogen fixation was shown to be the main reason for low primary productivity in the central Atlantic Ocean (Sanudo-Wilhelmy et al., 2001) and the eastern tropical North Atlantic Ocean (Mills et al., 2004). In freshwater ecosystems, reduction of P application was proposed as an important measure to control cyanobacterial blooms because P depletion can greatly reduce BNF-derived N input into the ecosystem (Wang et al., 2018). In terrestrial ecosystems, it has been widely reported that BNF rates decrease with reduced P supply, for example, in tropical forests (Vitousek & Hobbie, 2000) as well as in temperate grassland (Reed et al., 2007). In contrast, P fertilization can improve N<sub>2</sub> fixation and plant productivity (Almeida et al., 2000; Liese et al., 2017; Sanudo-Wilhelmy et al., 2001; Tang et al., 2017). For symbiotic N<sub>2</sub> fixation, P deficiency not only suppresses the nodule nitrogenase activity (Drevon & Hartwig, 1997; Høgh-Jensen et al., 2002) but also reduces nodule numbers and dry weight of the plant shoot

(Liese et al., 2017). All of these observations suggest that P plays a critical role in determining the productivity of the ecosystem in comparison with N, as it could facilitate the ecosystem to overcome N limitation. However, the underlying mechanisms by which P availability affects BNF are not entirely clear. It is postulated that P fertilization may enhance rhizodeposition, which in turn increases the abundance and activity of the diazotroph community (Crews et al., 2001; Reed et al., 2010; Vitousek, 1998).

At the cellular level, P availability has powerful influences on the growth and metabolism of organisms. Microbes can respond rapidly to P deficiency via metabolic switch and physiological adaptation (Giovannoni et al., 2014; Karl, 2014; Swan et al., 2013). In oligotrophic oceans, marine microbes have developed strategies to cope with P deficiency by expressing high-affinity phosphate transporters or using alternate organic P sources (e.g., phosphonate) (Karl, 2014). Marine microbes can also adapt to oligotrophic environments by streamlining genomes (i.e., simplifying the genome) to minimize their cellular P quotas (Giovannoni et al., 2014; Swan et al., 2013). In addition to nucleic acids, phospholipids in the membrane, for example, phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), represent a major reservoir for cellular P, which can account for up to ~20% of the P content in bacteria (Van Mooy et al., 2008). Many microbes, including terrestrial bacteria, can minimize the P cost in their membrane under low phosphate availability by replacing membrane phospholipids with a variety of nonphospholipids, such as aminolipids and glycolipids (Carini et al., 2015; Sohlenkamp & Geiger, 2016; Van Mooy et al., 2009). Although the replacement of phospholipids with P-free lipids in the membrane allows bacteria to better adapt to P deficiency, its impact on diazotrophs and nitrogenase activity has not been reported.

Therefore, in this study, a soil diazotrophic bacterium *Klebsiella variicola* W12 was used to evaluate the influences of P deficiency on cell growth and nitrogenase activity. Comparative proteomics and lipidomics analyses were conducted on the strain to gain insight into the potential mechanisms of how P availability affects N fixation. The species *K. variicola* was established in 2004 and has been frequently found in close association with plants (e.g., banana, rice, sugar cane and maize) (Rosenblueth et al., 2004). A number of studies have reported that the plant-borne *K. variicola* strains (formerly classified as *K. pneumoniae*) are able to fix N<sub>2</sub> in association with plants (Chelius & Triplett, 2000; Dong et al., 2003; Rosenblueth et al., 2004). The *K. variicola* strain W12 used in this study was originally isolated from the rhizosphere of cotton and has high nitrogenase activity, which can be stably maintained in the laboratory (Ge et al., 2018; Zhang et al., 2002).

## 2 | MATERIALS AND METHODS

### 2.1 | Bacterial growth and nitrogenase activity assays

*Klebsiella variicola* W12 was isolated from the rhizosphere soil of cotton in Egypt. The strain was routinely cultivated in the laboratory

on a low-N medium containing (per litre) 10 g sucrose, 5 g malic acid, 0.1 g  $K_2HPO_4 \cdot H_2O$ , 0.4 g  $KH_2PO_4 \cdot H_2O$ , 0.2 g  $MgSO_4 \cdot 7H_2O$ , 0.1 g NaCl, 0.02 g  $CaCl_2$ , 0.01 g  $FeCl_3$ , 0.002 g  $NaMoO_4 \cdot H_2O$ , 0.2 g peptone and 15 g agar at pH 7.0 (Zhang et al., 2002). The addition of a low dose of peptone (0.2 g/L) in the medium does not suppress the nitrogenase activity, but greatly enhances bacterial growth (Zhang et al., 2002). Inoculum culture was prepared by picking up a single colony from a low-N agar plate and inoculated into a 5-ml low-N liquid medium with the same ingredients, except that the P concentration was reduced to 100  $\mu M$ . To determine the impact of P availability on nitrogen fixation, 1% (v/v) of the inoculum culture (i.e., 200  $\mu l$ ) with an  $OD_{540}$  of 0.9 ( $2.1 \times 10^{10}$  colony-forming unit/ml) was transferred to a 120 ml serum vials containing 20 ml of the low-N medium with a gradient of phosphate at 0, 0.05 and 2 mM, respectively, in three biological replicates. The concentration of phosphate was adjusted by adding different volumes of 10 or 50 mM phosphate stock solution comprising  $K_2HPO_4 \cdot H_2O$  and  $KH_2PO_4 \cdot H_2O$  in the same molar ratio as in the medium above. Immediately after inoculation, the cell density was measured, which was  $<0.05$  ( $OD_{540}$ ). The serum vials were then incubated statically at 30°C and sampled at 24, 36 and 48 h. Subsamples of 5–10 ml of culture were withdrawn for cellular proteomics analyses and 1–2 ml of culture was used for lipid extraction, and the optical density (OD) of the bacterial culture was recorded at each sampling point. The gradients of phosphate addition and sampling time points were chosen based on a preliminary growth dynamic experiment showing that phosphate addition  $<100 \mu M$  suppressed the growth of *K. variicola* W12, and that the bacterium arrived at a stationary phase after 24 and 48 h under P-sufficient and P-deplete condition, respectively.

Nitrogenase activity was measured using the acetylene reduction assay as described previously (Hardy et al., 1968). Briefly, 1% (v/v) of the inoculum culture was transferred to the same serum vials containing 20 ml of the low-N medium with phosphate addition gradients as described above. The serum vials were then incubated statically at 30°C. After incubation for 24, 36 and 48 h, 10% of the headspace gas was replaced with acetylene (99.99% v/v, Air Liquide). Then, 200  $\mu l$  gases were sampled from the headspace of each serum vial to determine the concentration of ethylene using an Agilent gas chromatograph (HP7890B; Agilent) at 0, 10, 20 and 30 min after acetylene was added. Nitrogenase activity was determined based on the concentration of ethylene at 30 min and then normalized at equal  $OD_{540}$  by dividing  $OD_{540}$  value and were expressed as the rate of product (ethylene) formation ( $nmol C_2H_4 OD_{540}^{-1} ml^{-1} h^{-1}$ ).

## 2.2 | Preparation of proteome samples, trypsin in-gel proteolysis and nano-liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) analysis

Eight to fifteen millilitres of bacterial cultures in three biological replicates were used for each time point, depending on the  $OD_{540}$  value of each sample. Cells were condensed by centrifugation at 4°C for 15 min at 2000g and then pelleted at 12,000g. The cell pellets

were carefully dissolved in 100  $\mu l$  1 $\times$  lithium dodecyl sulphate loading buffer (Invitrogen) before loading on a precast Tris-Bis NuPAGE gel (Invitrogen) using 1 $\times$  MOPS running solution (Invitrogen). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was run for approximately 5 min to purify polypeptides in the polyacrylamide gel by removing contaminants (Christie-Oleza et al., 2012). Polyacrylamide gel bands containing the cellular proteome were excised and digested by trypsin (Roche) proteolysis as described previously (Christie-Oleza et al., 2012). The resulting tryptic peptides were extracted using formic acid-acetonitrile (5%:25%, v/v) before being resuspended in acetonitrile-trifluoroacetate (2.5%:0.05%, v/v). Tryptic peptides were separated by nanoLC using an Ultimate 3000 LC system with an Acclaim PepMap RSLC C18 reverse-phase column (Thermo Fisher Scientific). MS/MS spectra were collected using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) in electrospray ionization mode. Survey scans of peptides from 350 to 1500 mass-to-charge ( $m/z$ ) were collected for each sample in a 2-h run. This resulted in 27 mass spectra profiles (3 phosphate concentrations  $\times$  3 time points  $\times$  3 biological replicates) with a total of  $\sim 10$  G MS/MS data.

## 2.3 | MS/MS data search and statistical analyses

Compiled MS/MS raw files were searched against the *K. variicola* genome using the MaxQuant software package (Cox & Mann, 2008). Default settings were used and samples were matched between runs. The software package Perseus (v1.6.5.0) was used to determine differentially expressed proteins with a false discovery rate (FDR) of 0.01/0.05 and  $s_0$  of 2 (Tyanova & Cox, 2018). Missing values were imputed using the default parameters and statistical analyses were performed using two-sample Student's *t* test.

## 2.4 | Lipidomics analyses of *K. variicola* growing under different phosphate levels

Lipids were extracted using a modified Folch method as described previously (Smith et al., 2019). Briefly, 1.7–3.5 ml of bacterial cultures, depending on the  $OD_{540}$  value, were collected in 2 ml glass Screw Top Vials (Thermo Fisher Scientific) by centrifugation at 4000g for 15 min for 1–2 times at 4°C and resuspended in 0.5 ml LC-MS grade methanol (Sigma-Aldrich). Lipid extraction was carried out using chloroform and methanol and dried under  $N_2$  using a Techne sample concentrator (Techne). Lipid pellets were resuspended in LC-MS grade solvents consisting of 100  $\mu l$  chloroform:methanol (1:1) and 900  $\mu l$  acetonitrile. All chemical agents used for lipid extraction were precooled before using, and the lipid extracts were stored at  $-80^\circ C$  before further assay.

These lipid samples were separated by HPLC using an Ultimate 3000 HPLC system (Thermo Fisher Scientific) coupled with a BEH amide XP column (Waters; particle size 2.5  $\mu m$ , 3.0 mm  $\times$  150 mm). The column was maintained at 30°C, with a flow rate of 150  $\mu l$ /min.

Samples were run on a 15-min gradient from 95% (v/v in water) acetonitrile to 28% (w/v in water) 10 mM ammonium acetate at pH 9.2, with 10 min equilibration between samples. Mass spectra were collected in both positive and negative electrospray ionization mode using a Bruker amazon SL ion trap mass spectrometer (Bruker). Data analyses were performed using the Bruker Compass software package using DataAnalysis for peak identification and characterization of lipid class, and QuantAnalysis for quantification of the relative abundance of ornithine lipids to glycerophospholipids.

## 2.5 | Genome sequencing of *K. variicola* W12

Genomic DNA of *K. variicola* was extracted from 1 ml of liquid culture using the Trizol reagent (Thermo Fisher Scientific). DNA was sent out for sequencing using the Illumina MiSeq platform at MicrobesNG. Sequencing reads were assembled using the QUAST tool (Gurevich et al., 2013; Seemann, 2014), annotated by Prokka (Seemann, 2014) and deposited to the JGI IMG database under an accession number (GOLD Analysis Project ID Ga0373462).

## 2.6 | Quantitative reverse transcription-PCR analysis of *olsF* gene in *K. variicola* W12

Primers OlsFF1298 (5'-CGCACTATCGCTACTGT-3') and OlsFR1633 (5'-GACCATCCACGCAGTTAT-3'), targeting the *olsF* gene, which encodes the bifunctional acyltransferase (OlsF), were designed to evaluate the response of *olsF* transcription to phosphate addition in *K. variicola* W12. The bacterium was cultivated under the same condition as described above for proteomics and lipidomics. RNA was extracted using E.Z.N.A.<sup>®</sup> Total RNA Kit (Omega Bio-Tek) and DNA was removed with DNase (Promega) according to the manufacturer's instructions. The absence of DNA in RNA samples was confirmed by the PCR amplification using the bacterial 16S ribosomal RNA (rRNA) gene primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). Complementary DNA (cDNA) was produced using Superscript III reverse transcriptase (Invitrogen) with random hexamer primers at a concentration of 0.01  $\mu\text{g } \mu\text{l}^{-1}$  for each reaction. The first-strand cDNA was further quantified by quantitative-PCR (Q-PCR) with the primer set OlsFF1298-OlsFR1633 using the thermal cycle programme: 95°C for 2 min and 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and the signal was recorded at 90°C. The qPCR reactions (25  $\mu\text{l}$ ) contained 12.5  $\mu\text{l}$  2 $\times$  SYBR Premix Ex Taq (TaKaRa Bio Inc.), 2  $\mu\text{l}$  of cDNA template, 0.5  $\mu\text{l}$  (10  $\mu\text{M}$ ) of each primer. Standard curves were generated using 10-fold serial dilutions of a plasmid template containing a fragment of the *olsF* gene. To calibrate the relative abundance of *olsF* gene transcript under different P levels, the transcripts of 16S rRNA gene were quantified using a TaqMan assay with the primers BACT1369F (5'-CGG TGA ATA CGTTCY CGG-3'), PROK1541R (5'-AAG GAG GTG ATC CRG CCG C A-3') and the TaqMan probe TM1389F (5'-CTT GTACAC CGC CCG TC-3') (Suzuki et al., 2000).

## 2.7 | Statistical analysis

Statistical analyses were performed using SPSS 19 (IBM Co.). One-way analysis of variance was used to evaluate the statistical difference in cell density, nitrogenase activity and ornithine lipid content among three phosphate levels at each sampling time point.

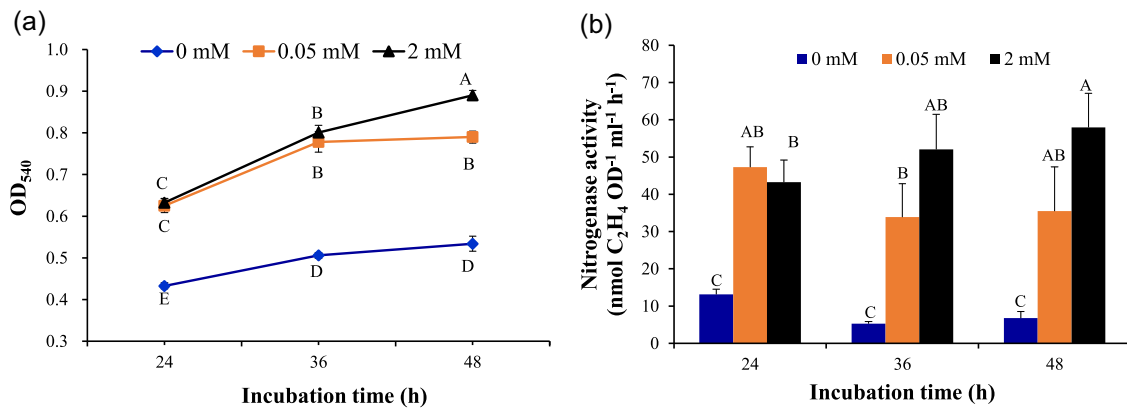
## 3 | RESULTS AND DISCUSSION

### 3.1 | Growth and N<sub>2</sub>-fixing activity of *K. variicola* W12 is repressed in response to P deficiency

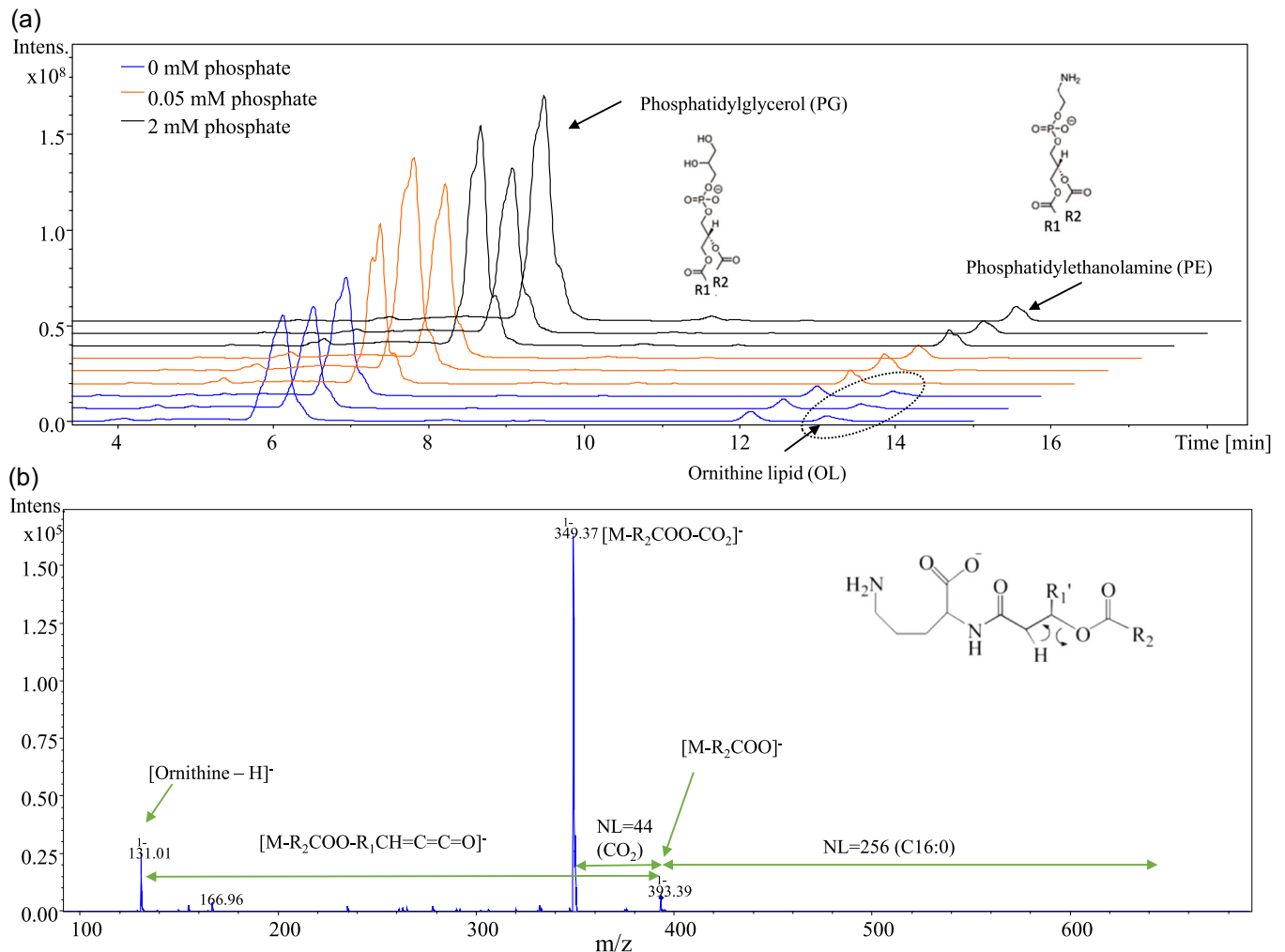
To investigate the impact of P availability on N<sub>2</sub>-fixing activity, *K. variicola* W12 was grown in a low-N medium supplemented with three concentrations of phosphate (0, 0.05 and 2 mM). The cell density (OD<sub>540</sub>) reached 0.53 within 48 h in the culture without additional phosphate amendment (0 mM), suggesting that a small amount of P present in the inoculum was sufficient to support its growth. In contrast, the bacterium grew significantly better when additional phosphate was supplied in the medium at the levels of 0.05 and 2 mM, with the OD<sub>540</sub> value at 0.79 and 0.89, respectively, after 48 h ( $p < 0.05$ ; Figure 1a). Nitrogenase activity was significantly higher (>3-folds) in cultures supplemented with phosphate (33.8–47.2 and 43.3–58.0 nmol C<sub>2</sub>H<sub>4</sub>/OD<sub>540</sub>/ml/h under 0.05 and 2 mM phosphate level, respectively) compared with that of the control (5.3–13.1 nmol C<sub>2</sub>H<sub>4</sub>/OD<sub>540</sub>/ml/h) ( $p < 0.05$ ; Figure 1b). Nitrogenase activity and cell density ceased to increase in cultures with low phosphate levels (0 and 0.05 mM) after 36 h, but kept increasing until 48 h in cultures supplemented with 2 mM phosphate. Our results using laboratory culture agreed with previous field studies showing that N<sub>2</sub>-fixing activity of diazotrophs can be modulated by P availability (Hogh-Jensen et al., 2002; Reed et al., 2007; Wang et al., 2018; Zheng et al., 2016).

### 3.2 | Lipidomic response of *K. variicola* W12 to P limitation

To gain an insight into the lipidomic response of this diazotroph to P stress, we analysed the intact membrane lipids of *K. variicola* W12 from the culture amended with three levels of phosphate (0, 0.05 and 2 mM) (Figure 2). When P is sufficient, the bacterium produces two major phospholipids, PG and PE, both of which are commonly found in bacteria such as *Escherichia coli* (Lopez-Lara & Geiger, 2017). However, when the bacterium was cultivated in low phosphate conditions (0 and 0.05 mM phosphate addition), a new lipid eluted at 13 min was observed with an  $m/z$  ratio of 649.5. Further fragmentation by MS<sup>n</sup> revealed the presence of an  $m/z$  of 131, indicating the presence of an ornithine head group and the fragmentation pattern is consistent with that of bacterial ornithine lipids (Smith et al., 2019; Vences-Guzman et al., 2015; Zhang et al., 2009). We did not detect betaine lipids, consistent with the fact that the *btaAB* genes involved



**FIGURE 1** Nitrogenase activity in response to P limitation in *Klebsiella variicola* W12. *K. variicola* was grown in a defined medium without (0 mM) and with 0.05 and 2 mM phosphate amendment and cell growth was monitored (a). Nitrogenase activity was measured at 24, 36 and 48 h using the acetylene reduction assay and normalized at equal OD<sub>540</sub> (b). Error bars denote standard deviation from three biological replicates. The different letter indicates a significantly different between the treatment and across the incubation time at  $p < 0.05$ . OD, optical density.



**FIGURE 2** Mass spectrum characteristics of lipidomics of *Klebsiella variicola* W12 under three phosphate addition levels. (a) High-performance liquid chromatography-mass spectrometry (MS) lipid profiles of *K. variicola* W12 grown under different phosphate addition levels. Lipid profiles were analysed from three independent biological replicates. The arrows point to the elution time of three major lipids in this bacterium, PG, PE and OL. (b) MS fragmentation of  $m/z$  649.5 ions eluted at 13 min in the negative mode. NL, neutral loss; OL, ornithine lipids; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

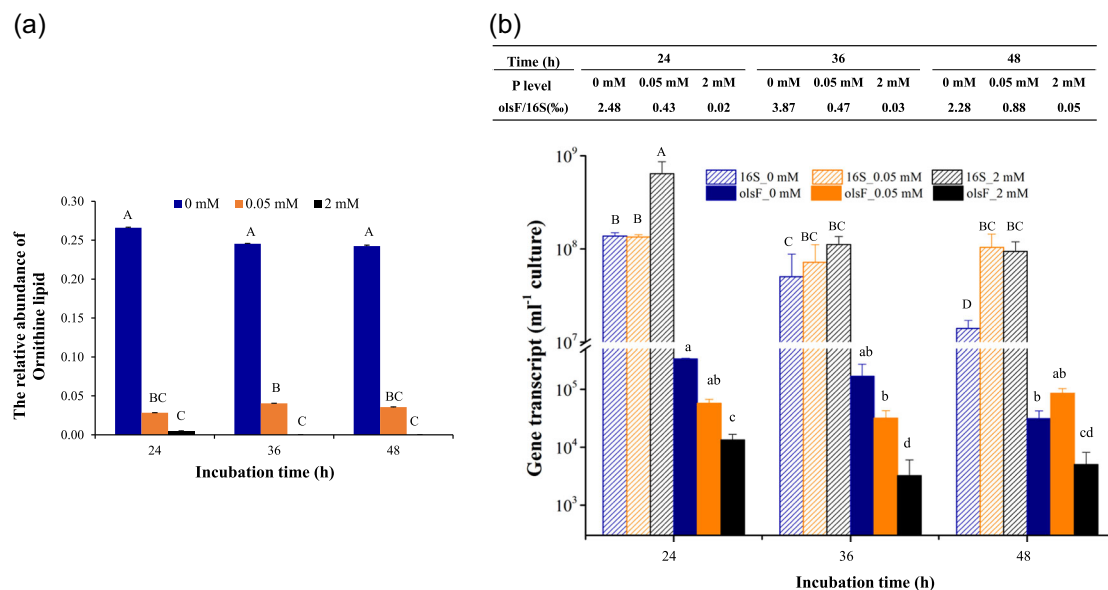
in betaine lipid biosynthesis are absent in the genome of *K. variicola* (Riekhof et al., 2005). Two pathways for ornithine lipid biosynthesis have been identified in bacteria. In *Sinorhizobium meliloti* and *Ruegeria pomeroyi*, an N-acyltransferase OlsB is responsible for the first step, adding a 3-hydroxy fatty acyl group to the  $\alpha$ -amino group of ornithine. The lyso-ornithine intermediate serves as the substrate for an O-acyltransferase OlsA to form ornithine lipids (Gao et al., 2004; Smith et al., 2019; Weissenmayer et al., 2002). However, the *olsB/A* genes are not present in the genome of *K. variicola* W12. Instead, the genome contains a bifunctional acyltransferase OlsF, which was recently characterized in *Serratia proteamaculans* for ornithine lipid biosynthesis (Vences-Guzman et al., 2015). Relative quantification of total ornithine lipids in *K. variicola* W12 confirmed that their abundance is highest when no additional phosphate was added to the culture (Figure 3a). Consistently, the *olsF* gene transcripts in the culture were significantly higher under low P addition levels (0 and 0.05 mM phosphate) than at high P addition levels (2 mM phosphate), with the highest ratios of *olsF* gene transcript to 16S rRNA gene transcript under 0 mM phosphate addition (2.28%–3.87%) ( $p < 0.05$ ; Figure 3b). Together, these suggest that *K. variicola* W12 can synthesize ornithine lipid via the acyltransferase OlsF during P deficiency. Coincidentally, a recent study suggested that phospholipids accounted for more than 90% of total polar lipids in P-sufficient soils, while non-P betaine lipids significantly increased in the most severely P-deficient soils (Warren, 2020). Substitution of phospholipids with non-P polar lipids such as ornithine and betaine lipids to reduce P requirements could be an important mechanism for asymbiotic

diazotrophs and other soil microorganisms under P-deplete stress. Although a similar lipid remodelling mechanism has been found in cyanobacteria (Pereira et al., 2016) and rhizobia (Zavaleta-Pastor et al., 2010), the lipidomics and genomics analyses in this study suggested a different pathway of ornithine lipid biosynthesis via bifunctional acyltransferase OlsF in diazotrophic microbes.

### 3.3 | Proteomic response of *K. variicola* W12 to P stress

To gain an insight into the cellular response of *K. variicola* to P limitation, we compared the total cellular proteome of the bacterium cultivated under three phosphate amendment levels (0, 0.05 and 2 mM) at three time points (24, 36 and 48 h). Three biological replicates were included for each treatment and sampling time point, and only those proteins that are found in all three biological replicates were retained for further statistical analyses. Out of 5435 protein-coding genes in the genome of *K. variicola* W12, a total of 1347 proteins was detected under these conditions (Supporting Information: Table 1).

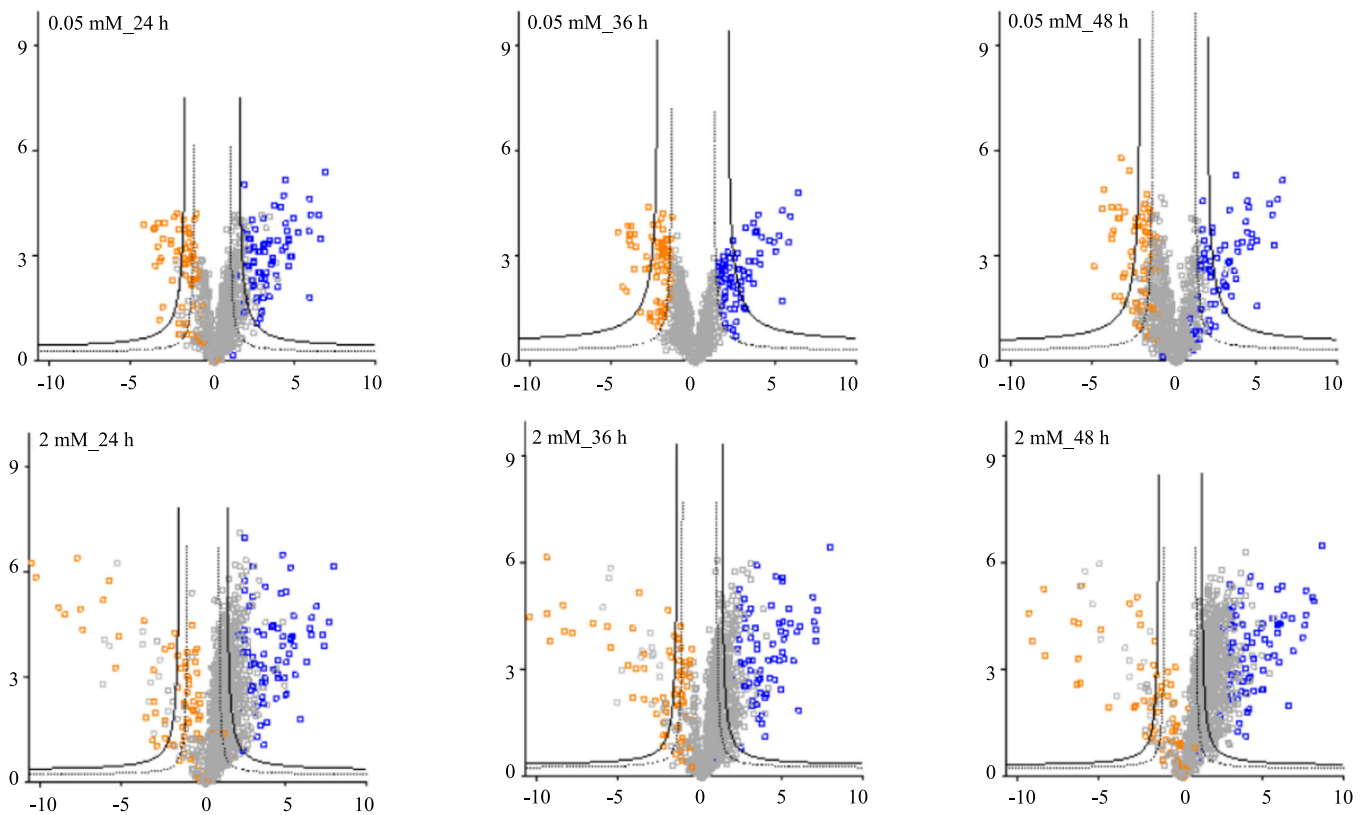
The genome of *K. variicola* W12 contains a canonical nitrogen fixation gene cluster *nifJHDKTYENXUSVWZMFLABQ*, which is identical to that of *K. pneumoniae* (Rubio & Ludden, 2008). Compared to cultures without additional phosphate amendment, proteins involved in nitrogen fixation, such as dinitrogenase (NifDK), dinitrogenase reductase (NifH) and the  $N_2$  fixation transcriptional regulators NifA and NifL, were significantly upregulated in cultures supplemented



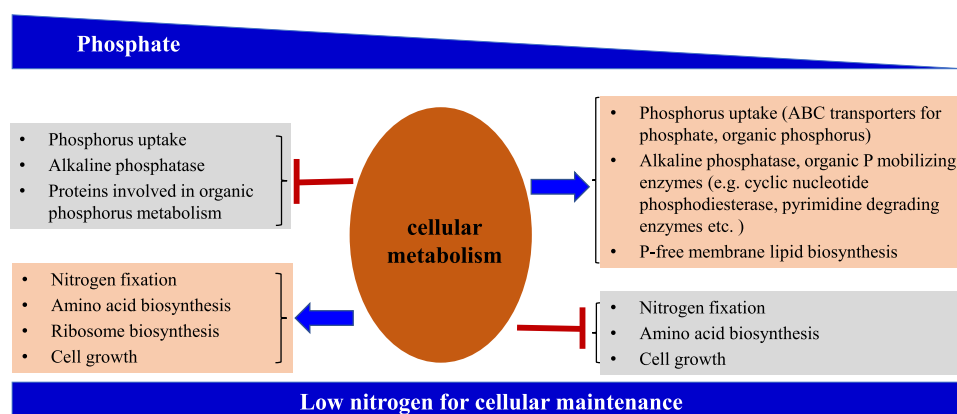
**FIGURE 3** Lipidomics response of *Klebsiella variicola* W12 to phosphorus limitation. (a) Ornithine lipids are produced in response to phosphorus stress. The concentration of ornithine lipid was expressed as the ratio of ornithine lipids to glycerophospholipids (PE + PG). The different letter indicates a significant difference between the treatment and across the incubation time at  $p < 0.05$ . (b) The abundance of 16S rRNA and *olsF* gene transcripts of *K. variicola* W12 under three phosphate addition levels. Different uppercase and lowercase letters indicate significant differences between the treatments and across the incubation time at  $p < 0.05$ , for 16S rRNA gene and *olsF* gene, respectively. The values above the figure show the ratios of *olsF* gene transcripts to 16S rRNA gene transcripts under different P levels. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; rRNA, ribosomal RNA.







**FIGURE 5** Volcano plots showing the progression of the cellular proteome in response to phosphorus addition (0.05 and 2 mM). Samples were compared to no phosphate addition (0 mM) at 36 h. The same proteins are shown in Figure 4 (FDR < 0.05), which are highlighted in blue (overexpressed in 0.05 mM phosphate) or orange (overexpressed in no phosphate). The solid lines represent FDR = 0.01 and the dashed lines represent FDR = 0.05. A list of proteins that are differentially expressed with FDR < 0.05 is presented in Supporting Information: Table 3. FDR, false discovery rate.



**FIGURE 6** A working conceptual model of cellular metabolism in *Klebsiella variicola* in response to phosphorous limitation stress. When phosphorus is plentiful, the bacterium fixes dinitrogen gas at the expense of more energy to produce ammonium for cell growth. When phosphorus is at scarce, cell growth is inhibited due to the lack of phosphorus for macromolecule biosynthesis. As such, the bacterium switches off nitrogen fixation and diverts cellular energy towards phosphorus acquisition by expressing ABC transporters for organic and inorganic phosphorus as well as a range of enzymes involved in organic phosphorus degradation. The bacterium further reduces cellular demand for phosphorus by synthesizing alternative nonphosphorus ornithine lipids to replace membrane phospholipids.

## 4 | CONCLUSIONS

Our work, therefore, provides novel insight into the physiological and molecular response of the nonsymbiotic diazotroph *K. variicola* to P deficiency. Our lipidomics data revealed that replacing membrane phospholipids with P-free ornithine lipids is an adaptation strategy of the nonsymbiotic diazotroph to P deficiency. Our data also provide a synergistic view of the biochemical and molecular mechanisms by which P deficiency affected BNF in *K. variicola*. In many cases, both natural and agricultural ecosystems can obtain considerable N input via BNF to overcome N limitation (Furnkranz et al., 2008; Ladha et al., 2016; Madhaiyan et al., 2015; Van Deynze et al., 2018). Through regulating nitrogenase activity and BNF, P availability, therefore, plays a critical role in determining primary productivity in natural ecosystems. Because both N and P limitations are widespread in terrestrial ecosystems on a global scale (Elser et al., 2007), the model that we proposed here based on cellular demand for N and P may be of global relevance. Based on this rationale of N and P coupling, moderate P input in agroecosystems to maximize the potential of BNF in ecosystems themselves but minimize N fertilizers input would be promising for agriculture sustainable production and environmental health.

### AUTHOR CONTRIBUTIONS

Li-Mei Zhang and Yin Chen designed the study. Li-Mei Zhang and Maria Aguilo-Ferretjans performed experiments with the assistance of Eleonora Silvano, Bing Han and Wei Shi. Eleonora Silvano, Branko Rihtman and Yin Chen analysed the data. Li-Mei Zhang and Yin Chen wrote the manuscript. All authors contributed to the final version of the manuscript.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### DATA AVAILABILITY STATEMENT

The genome sequence of *Klebsiella variicola* W12 has been submitted to JGI/IMG Database with ID209268.

### ETHICS STATEMENT

The authors confirm that they have adhered to the ethical policies of the journal.

### ORCID

Li-Mei Zhang  <http://orcid.org/0000-0002-7383-8475>

Yin Chen  <http://orcid.org/0000-0002-0367-4276>

### REFERENCES

- Almeida JPF, Hartwig UA, Frehner M, Nosberger J, Luscher A. Evidence that P deficiency induces N feedback regulation of symbiotic N<sub>2</sub> fixation in white clover (*Trifolium repens* L.). *J Exp Bot*. 2000;51:1289–97.
- Carini P, Van Mooy BAS, Thrash JC, White A, Zhao Y, Campbell EO, et al. SAR11 lipid renovation in response to phosphate starvation. *Proc Natl Acad Sci USA*. 2015;112:7767–72.
- Chelius MK, Triplett EW. Immunolocalization of dinitrogenase reductase produced by *Klebsiella pneumoniae* in association with *Zea mays* L. *Appl Environ Microb*. 2000;66:783–7.
- Christie-Oleza JA, Fernandez B, Nogales B, Bosch R, Armengaud J. Proteomic insights into the lifestyle of an environmentally relevant marine bacterium. *ISME J*. 2012;6:124–35.
- Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol*. 2008;26:1367–72.
- Crews TE, Kurina LM, Vitousek PM. Organic matter and nitrogen accumulation and nitrogen fixation during early ecosystem development in Hawaii. *Biogeochemistry*. 2001;52:259–79.
- Dong YM, Chelius MK, Brisse S, Kozyrovska N, Kovtunovych G, Podschun R, et al. Comparisons between two *Klebsiella*: the plant endophyte *K. pneumoniae* 342 and a clinical isolate, *K. pneumoniae* MGH78578. *Symbiosis*. 2003;35:247–59.
- Drevon JJ, Hartwig UA. Phosphorus deficiency increases the argon-induced decline of nodule nitrogenase activity in soybean and alfalfa. *Planta*. 1997;201:463–9.
- Elser JJ, Bracken MES, Cleland EE, Gruner DS, Harpole WS, Hillebrand H, et al. Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol Lett*. 2007;10:1135–42.
- Furnkranz M, Wanek W, Richter A, Abell G, Rasche F, Sessitsch A. Nitrogen fixation by phyllosphere bacteria associated with higher plants and their colonizing epiphytes of a tropical lowland rainforest of Costa Rica. *ISME J*. 2008;2:561–70.
- Gaby JC, Buckley DH. A global census of nitrogenase diversity. *Environ Microbiol*. 2011;13:1790–9.
- Gao JL, Weissenmayer B, Taylor AM, Thomas-Oates J, Lopez-Lara IM, Geiger O. Identification of a gene required for the formation of lysornithine lipid, an intermediate in the biosynthesis of ornithine-containing lipids. *Mol Microbiol*. 2004;53:1757–70.
- Ge AH, Fang P, Xiong C, Zhang LM, He JZ. Optimization of liquid inoculant of a nitrogen-fixing bacterium and its colonization on maize phyllosphere. *Microbiol China*. 2018;45:1303–13.
- Giovannoni SJ, Thrash JC, Temperton B. Implications of streamlining theory for microbial ecology. *ISME J*. 2014;8:1553–65.
- Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUASt: quality assessment tool for genome assemblies. *Bioinformatics*. 2013;29:1072–5.
- Han LL, Wang Q, Shen JP, Di HJ, Wang JT, Wei WX, et al. Multiple factors drive the abundance and diversity of the diazotrophic community in typical farmland soils of China. *FEMS Microbiol Ecol*. 2019;95(8):fiz113. <https://doi.org/10.1093/femsec/fiz113>
- Hardy RWF, Holsten RD, Jackson EK, Burns RC. The acetylene-ethylene assay for N<sub>2</sub> fixation: laboratory and field evaluation. *Plant Physiol*. 1968;43:1185–1207.
- Hogh-Jensen H, Schjoerring JK, Soussana JF. The influence of phosphorus deficiency on growth and nitrogen fixation of white clover plants. *Ann Bot-London*. 2002;90:745–53.
- Karl DM. Microbially mediated transformations of phosphorus in the sea: new views of an old cycle. *Annu Rev Mar Sci*. 2014;6:279–337.

- Ladha JK, Tirol-Padre A, Reddy CK, Cassman KG, Verma S, Powelson DS, et al. Global nitrogen budgets in cereals: a 50-year assessment for maize, rice, and wheat production systems. *Sci Rep*. 2016;6:19355. <https://doi.org/10.1038/srep19355>
- Liese R, Schulze J, Cabeza RA. Nitrate application or P deficiency induce a decline in *Medicago truncatula* N<sub>2</sub> fixation by similar changes in the nodule transcriptome. *Sci Rep*. 2017;7:46264. <https://doi.org/10.1038/srep46264>
- Lopez-Lara IM, Geiger O. Bacterial lipid diversity. *BBA Mol Cell Biol*. 2017;1862:1287–99.
- Madhaiyan M, Alex THH, Ngoh ST, Prithviraj B, Ji LH. Leaf-residing *Methylobacterium* species fix nitrogen and promote biomass and seed production in *Jatropha curcas*. *Biotechnol Biofuels*. 2015;8:222.
- Mills MM, Ridame C, Davey M, La Roche J, Geider RJ. Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. *Nature*. 2004;429:292–4.
- Pereira N, Shilova IN, Zehr JP. Molecular markers define progressing stages of phosphorus limitation in the nitrogen-fixing cyanobacterium, *Crocospaera*. *J Phycol*. 2016;52:274–82.
- Reed SC, Cleveland CC, Townsend AR. Functional ecology of free-living nitrogen fixation: a contemporary perspective. *Annu Rev Ecol Evol Syst*. 2011;42:489–512.
- Reed SC, Seastedt TR, Mann CM, Suding KN, Townsend AR, Cherwin KL. Phosphorus fertilization stimulates nitrogen fixation and increases inorganic nitrogen concentrations in a restored prairie. *Appl Soil Ecol*. 2007;36:238–42.
- Reed SC, Townsend AR, Cleveland CC, Nemergut DR. Microbial community shifts influence patterns in tropical forest nitrogen fixation. *Oecologia*. 2010;164:521–31.
- Riekhof WR, Andre C, Benning C. Two enzymes, BtaA and BtaB, are sufficient for betaine lipid biosynthesis in bacteria. *Arch Biochem Biophys*. 2005;441:96–105.
- Rosenblueth M, Martinez L, Silva J, Martinez-Romero E. *Klebsiella variicola*, a novel species with clinical and plant-associated isolates. *Syst Appl Microbiol*. 2004;27:27–35.
- Rubio LM, Ludden PW. Biosynthesis of the iron–molybdenum cofactor of nitrogenase. *Annu Rev Microbiol*. 2008;62:93–111.
- Sañudo-Wilhelmy SA, Kustka AB, Gobler CJ, Hutchins DA, Yang M, Lwiza K, et al. Phosphorus limitation of nitrogen fixation by *Trichodesmium* in the central Atlantic Ocean. *Nature*. 2001;411:66–9.
- Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*. 2014;30:2068–9.
- Shi W, Zhao HY, Chen Y, Wang JS, Han B, Li CP, et al. Organic manure rather than phosphorus fertilization primarily determined asymbiotic nitrogen fixation rate and the stability of diazotrophic community in an upland red soil. *Agr Ecosyst Environ*. 2021;319:107535. <https://doi.org/10.1016/j.agee.2021.107535>
- Smith AF, Rihtman B, Stirrup R, Silvano E, Mausz MA, Scanlan DJ, et al. Elucidation of glutamine lipid biosynthesis in marine bacteria reveals its importance under phosphorus deplete growth in Rhodobacteraceae. *ISME J*. 2019;13:39–49.
- Sohlenkamp C, Geiger O. Bacterial membrane lipids: diversity in structures and pathways. *FEMS Microbiol Rev*. 2016;40:133–59.
- Suzuki MT, Taylor LT, DeLong EF. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. *Appl Environ Microbiol*. 2000;66:4605–14.
- Swan BK, Tupper B, Sczyrba A, Lauro FM, Martinez-Garcia M, González JM, et al. Prevalent genome streamlining and latitudinal divergence of planktonic bacteria in the surface ocean. *Proc Natl Acad Sci USA*. 2013;110:11463–8.
- Tang YF, Zhang MM, Chen AL, Zhang WZ, Wei WX, Sheng R. Impact of fertilization regimes on diazotroph community compositions and N<sub>2</sub> fixation activity in paddy soil. *Agr Ecosyst Environ*. 2017;247:1–8.
- Tyanova S, Cox J. Perseus: a bioinformatics platform for integrative analysis of proteomics data in cancer research. *Methods Mol Biol*. 2018;1711:133–48.
- Van Deynze A, Zamora P, Delaux PM, Heitmann C, Jayaraman D, Rajasekar S, et al. Nitrogen fixation in a landrace of maize is supported by a mucilage-associated diazotrophic microbiota. *PLoS Biol*. 2018;16:e2006352. <https://doi.org/10.1371/journal.pbio.2006352>
- Van Mooy BAS, Fredricks HF, Pedler BE, Dyhrman ST, Karl DM, Koblížek M, et al. Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature*. 2009;458:69–72.
- Van Mooy BAS, Moutin T, Duhamel S, Rimmelin P, Van Wambeke F. Phospholipid synthesis rates in the eastern subtropical South Pacific Ocean. *Biogeosciences*. 2008;5:133–9.
- Vences-Guzman MA, Guan ZQ, Escobedo-Hinojosa WI, Bermudez-Barrientos JR, Geiger O, Sohlenkamp C. Discovery of a bifunctional acyltransferase responsible for ornithine lipid synthesis in *Serratia proteamaculans*. *Environ Microbiol*. 2015;17:1487–96.
- Vitousek PM. Foliar and litter nutrients, nutrient resorption, and decomposition in Hawaiian *Metrosideros polymorpha*. *Ecosystems*. 1998;1:401–7.
- Vitousek PM, Hobbie S. Heterotrophic nitrogen fixation in decomposing litter: patterns and regulation. *Ecology*. 2000;81:2366–76.
- Vitousek PM, Menge DNL, Reed SC, Cleveland CC. Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philos Trans R Soc B*. 2013;368(1621):20130119. <https://doi.org/10.1098/rstb.2013.0119>
- Wang S, Xiao J, Wan L, Zhou Z, Wang Z, Song C, et al. Mutual dependence of nitrogen and phosphorus as key nutrient elements: one facilitates *dolichospermum flos-aquae* to overcome the limitations of the other. *Environ Sci Technol*. 2018;52:5653–61.
- Warren CR. Soil microbial populations substitute phospholipids with betaine lipids in response to low P availability. *Soil Biol Biochem*. 2020;140:107655.
- Weissenmayer B, Gao JL, Lopez-Lara IM, Geiger O. Identification of a gene required for the biosynthesis of ornithine-derived lipids. *Mol Microbiol*. 2002;45:721–33.
- Zavaleta-Pastor M, Sohlenkamp C, Gao JL, Guan Z, Zaheer R, Finan TM, et al. *Sinorhizobium melloti* phospholipase C required for lipid remodeling during phosphorus limitation. *Proc Natl Acad Sci USA*. 2010;107:302–7.
- Zhang LM, Fang P, Ji TW. Adaptability of a nitrogen-fixing strain W12 to the diverse environmental factors. *J Zhejiang Univ*. 2002;28(6):664–8.
- Zhang X, Fergulson-Miller SM, Reid GE. Characterization of ornithine and glutamine lipids extracted from cell membranes of *Rhodobacter sphaeroides*. *J Am Soc Mass Spectr*. 2009;20:198–212.
- Zheng M, Li D, Lu X, Zhu X, Zhang W, Huang J, et al. Effects of phosphorus addition with and without nitrogen addition on biological nitrogen fixation in tropical legume and non-legume tree plantations. *Biogeochemistry*. 2016;131:65–76.

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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