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Phosphorus limitation's biochemical process that hinders nitrogen
fixation in the diazotrophic bacteria *Klebsiella variicola* W12

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Research Article

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Abstract

In both natural and agricultural environments, biological nitrogen (N) fixation (BNF) is essential to the supply of nitrogen. Using BNF may significantly reduce agroecosystems' reliance on chemical fertilizers, which can support sustainable agriculture. Although the underlying process is unclear, many field investigations have shown that BNF may be significantly inhibited in phosphorus (P)-deficient conditions. **Supplies and Procedures:** In order to understand how P availability impacts N fixing, comparative proteomics and lipidomics analyzes were performed on a diazotrophic bacteria, *Klebsiella variicola* W12, under P-deficient and P-replete circumstances. **Results:** *K. variicola* W12's N fixing activity was significantly suppressed under P deficit. The bacteria replaced glycerophospholipids in its membrane with P-free ornithine lipids in response to P constraint, lowering the cellular need for P. Comparative proteomics revealed that whereas nitrogenase and N-fixation-related transcriptional regulators NifL and NifA were downregulated, P restriction led to the overexpression of the PhoBR two-component system and a variety of organic and inorganic P uptake and transport systems. **Conclusion:** These findings offered molecular proof of how P availability controls BNF and demonstrated lipid remodeling as an adaptation mechanism for N₂-fixing bacteria to thrive under P stress. Thus, a conceptual model of N-P interaction at the level of microbial metabolism is put forward. Our research emphasizes the significance of controlling P availability to optimize the potential of BNF in agroecosystems for agricultural sustainable production and offers a simple yet tenable explanation for how P insufficiency reduces BNF seen in the field.

KEYWORDS

P deficit, ornithine lipids, nitrogen fixation, N-P coupling, and proteomics

1 | INTRODUCTION

In aquatic and terrestrial ecosystems, primary production is often limited by nitrogen (N) and phosphorus (P), two nutrients that are vital for plants and phytoplanktons (Elser et al., 2007). Every year, a significant amount of N and P fertilizers are applied to agricultural fields worldwide in order to relieve the N and P shortage and increase crop yield. As a result, N and P fertilization worsens the depletion of the world's finite P resources in addition to having detrimental environmental consequences such soil acidification, water eutrophication, and N₂O emissions. Through biological N₂ fixation (BNF), which is mediated by N₂-fixing bacteria, ecosystems may get significant N to overcome N deficit, in contrast to P supply, which only depends on the use of P fertilizers. According to estimates, BNF is a significant source of N supply even in fertilized agricultural environments, contributing 40-100 Tg N annually to terrestrial ecosystems alone (Ladha et al., 2016; Vitousek et al., 2013). Asymbiotic N₂ fixers are

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found in many natural and agricultural habitats, including associate and free-living diazo-trophs affiliating within α -, β -, and γ -proteobacteria (Furnkranz et al., 2008; Gaby & Buckley, 2011; Han et al., 2019; Shi et al., 2021). Asymbiotic diazotrophs can survive and fix N_2 without forming a symbiotic structure with plants, despite having a lower rate of N_2 fixation than symbiotic systems. Because of their widespread distribution and high adaptability to a variety of habitats, they play a significant role in N input in ecosystems (Reed et al., 2011). For instance, nonsymbiotic N_2 fixation is thought to have contributed around 24% of the N in crop biomass. ecology of agriculture (Ladha et al., 2016). According to Van Deynze et al. (2018), a five-year field research revealed that isolated indigenous maize cultivated in N-depleted fields in Mexico may provide 29%-82% of the plant N obtained from nitrogen fixation of diazotrophs, which were enhanced by carbohydrate-rich mucilage of aerial roots. The contribution of nonsymbiotic N_2 fixation to the N supply in the rainforest may be even more than anticipated when taking into account the diazotrophs living in the plant phyllosphere, according to more recent research on forest ecosystems (Furnkranz et al., 2008; Madhaiyan et al., 2015). However, many laboratory studies and field observations have shown that P deficit may inhibit BNF. For example, poor primary production in the eastern tropical North Atlantic Ocean (Mills et al., 2004) and central Atlantic Ocean (Sanudo-Wilhelmy et al., 2001) was shown to be mostly caused by P constraint on nitrogen fixation. Because P depletion may significantly lower BNF-derived N input into the environment, reducing P application has been suggested as a crucial strategy to limit cyanobacterial blooms in freshwater ecosystems (Wang et al., 2018). It has been well shown that in terrestrial ecosystems, such as tropical forests (Vitousek & Hobbie, 2000) and temperate grasslands (Reed et al., 2007), BNF rates decline with decreased P availability. P fertilization, on the other hand, may increase plant production and N_2 fixation (Almeida et al., 2000; Liese et al., 2017; Sanudo-Wilhelmy et al., 2001; Tang et al., 2017). P shortage decreases nodule counts and the dry weight of the plant shoot in addition to suppressing nodule nitrogenase activity for symbiotic N_2 fixation (Drevon & Hartwig, 1997; Høgh-Jensen et al., 2002) (Liese et al., 2017). All of these findings imply that P is more important than N in determining the ecosystem's production since it may help the ecosystem get over its N shortage. But it's unclear exactly how P availability influences BNF. P fertilization is thought to improve rhizodeposition, which raises the diazotroph community's activity and abundance (Crews et al., 2001; Reed et al., 2010; Vitousek, 1998). P availability has a significant impact on an organism's development and metabolism at the cellular level. Through metabolic switching and physiological adaptation, microbes may react quickly to P shortage (Giovannoni et al., 2014; Karl, 2014; Swan et al., 2013). Marine bacteria in oligotrophic environments have evolved defense mechanisms against P deprivation, such as the expression of high-affinity phosphate transporters or the use of other organic P sources, such as phosphonate (Karl, 2014). In order to reduce their cellular P quotas, marine bacteria may potentially adapt to oligotrophic conditions by streamlining, or simplifying, their genomes (Giovannoni et al., 2014; Swan et al., 2013). Apart from nucleic acids, phospholipids in the membrane, such as phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), are a significant source of cellular P, which may make up as much as 20% of the P content in bacteria (Van Mooy et al., 2008). By substituting membrane phospholipids with a range of nonphospholipids, such as aminolipids and glycolipids, many microbes, including terrestrial bacteria, can reduce the P cost in their membrane under low phosphate availability (Carini et al., 2015; Sohlenkamp & Geiger, 2016; Van Mooy et al., 2009). Although bacteria can better adapt to P deprivation when phospholipids in the membrane are replaced with P-free lipids, their effects on diazotrophs and nitrogenase activity have not been shown. Therefore, the effects of P shortage on cell development and nitrogenase activity were assessed in this work using the soil diazotrophic bacteria *Klebsiella variicola* W12. To learn more about the possible processes by which P availability influences N fixing, comparative proteomics and lipidomics analyzes were performed on the strain. Since its discovery in 2004, the species *K. variicola* has often been discovered in close proximity to plants, including bananas, rice, sugar cane, and maize (Rosenblueth et al.,

2004). According to many research (Chelius & Triplett, 2000; Dong et al., 2003; Rosenblueth et al., 2004), plant-borne *K. variicola* strains (previously known as *K. pneumoniae*) may fix N₂ in conjunction with plants. The *K. variicola* strain W12 utilized in this investigation was first isolated from the cotton rhizosphere and has a high nitrogenase activity that is stable in the lab (Ge et al., 2018; Zhang et al., 2002).

2 | MATERIALS AND METHODS

2.1 | Bacterial growth and nitrogenase activity assays

In Egypt, *Klebsiella variicola* W12 was isolated from cotton rhizosphere soil. The strain was regularly grown in the lab on a low-N medium with 10 g sucrose, 5 g malic acid, 0.1 g K₂HPO₄ • H₂O, 0.4 g KH₂PO₄ • H₂O, 0.2 g MgSO₄ • 7H₂O, 0.1 g NaCl, 0.02 g CaCl₂, 0.01 g FeCl₃, 0.002 g NaMoO₄ • H₂O, 0.2 g peptone, and 15 g agar at pH 7.0 (Zhang et al., 2002). A little amount of peptone (0.2 g/L) added to the medium significantly increases bacterial growth while having no effect on nitrogenase activity (Zhang et al., 2002). A single colony was taken from a low-N agar plate and inoculated into a 5-ml low-N liquid medium using the same ingredients, with the P concentration lowered to 100 µM, to create an inoculum culture. In three biological replicates, 1% (v/v) of the inoculum culture (i.e., 200 µl) with an OD₅₄₀ of 0.9 (2.1×10^{10} colony-forming unit/ml) was added to 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. By adding varying quantities of 10 or 50 mM phosphate stock solution containing K₂HPO₄ • H₂O and KH₂PO₄ • H₂O in the same molar ratio as in the medium above, the phosphate concentration was changed. The cell density was evaluated immediately after inoculation, and it was less than 0.05 (OD₅₄₀). After that, the serum vials were collected at 24, 36, and 48 hours after being statically incubated at 30° C. At each sampling point, the optical density (OD) of the bacterial culture was measured. Subsamples of 5–10 ml of culture were taken out for cellular proteome analyzes, and 1–2 ml of culture was utilized for lipid extraction. Based on a preliminary growth dynamic experiment that demonstrated that phosphate addition <100 µM suppressed the growth of *K. variicola* W12 and that the bacterium reached a stationary phase after 24 and 48 hours under P-sufficient and P-deplete conditions, respectively, the gradients of phosphate addition and sampling time points were selected. As previously mentioned, the acetylene reduction test was used to evaluate nitrogenase activity (Hardy et al., 1968). In short, the identical serum vials holding 20 ml of the low-N medium with phosphate addition gradients were filled with 1% (v/v) of the inoculum culture. After that, the serum vials were statically incubated at 30° C. 10% of the headspace gas was substituted with acetylene (99.99% v/v, Air Liquide) after incubation for 24, 36, and 48 hours. After acetylene was introduced, 200 µl of gasses were taken from the headspace of each serum vial to measure the ethylene concentration using an Agilent gas chromatograph (HP7890B; Agilent) at 0, 10, 20, and 30 minutes. The concentration of ethylene at 30 minutes was used to calculate nitrogenase activity, which was then normalized at equal OD₅₄₀ by dividing the OD₅₄₀ value and represented as the rate of product (ethylene) generation (nmol C₂H₄ OD₅₄₀-1 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:

Depending on each sample's OD₅₄₀ value, eight to fifteen milliliters of bacterial cultures in three biological replicates were employed at each time point. Centrifugation at 4° C for 15 minutes at 2000g was used to condense the cells, which were then pummeled at 12,000g. The cell pellets were loaded onto a precast Tris-Bis NuPAGE gel (Invitrogen) using 1× MOPS running solution (Invitrogen) after being gently dissolved in 100 µl of 1× lithium dodecyl sulfate loading buffer (Invitrogen). To purify polypeptides in the polyacrylamide gel by eliminating impurities, sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed for around five minutes (Christie-Oleza et al., 2012). Trypsin (Roche) proteolysis was used to remove and digest poly-acrylamide gel bands containing the cellular proteome, as previously reported (Christie-Oleza et al., 2012). Formic acid-acetonitrile (5%:25%, v/v) was used to extract the resultant tryptic peptides, which were then resuspended in

acetonitrile-trifluoroacetate (2.5%:0.05%, v/v). Using an Acclaim PepMap RSLC C18 reverse-phase column (Thermo Fisher Scientific) and an Ultimate 3000 LC system, tryptic peptides were isolated by nanoLC. An Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) operating in electrospray ionization mode was used to gather MS/MS spectra. In a two-hour run, survey scans of peptides ranging from 350 to 1500 mass-to-charge (m/z) were obtained for every sample. With a total of around 10 G MS/MS data, this produced 27 mass spectra profiles (3 phosphate concentrations $\times$ 3 time points $\times$ 3 biological replicates).

2.3 | MS/MS data search and statistical analyses

The MaxQuant software program was used to search compiled MS/MS raw data against the *K. variicola* genome (Cox & Mann, 2008). Samples were matched across runs and the default parameters were used. Differentially expressed proteins with a false discovery rate (FDR) of 0.01/0.05 and s0 of 2 were identified using the software program Perseus (v1.6.5.0) (Tyanova & Cox, 2018). The default settings were used to impute missing data, and the two-sample Student's t test was used for statistical analysis.

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

As previously mentioned, a modified Folch technique was used to extract lipids (Smith et al., 2019). In summary, 1.7–3.5 ml of bacterial cultures, based on the OD540 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. The cultures were then resuspended in 0.5 ml LC-MS grade methanol (Sigma-Aldrich). Chloroform and methanol were used to extract the lipids, and a Techne sample concentrator (Techne) was used to dry them under N₂. LC-MS grade solvents comprising 900 μ l acetonitrile and 100 μ l chloroform:methanol (1:1) were used to resuspend lipid pellets. The lipid extracts were kept at –80° C prior to further test, and all chemical agents employed for lipid extraction were precooled before use. A BEH amide XP column (Waters; particle size 2.5 μ m, 3.0 mm $\times$ 150 mm) and an Ultimate 3000 HPLC system (Thermo Fisher Scientific) were used to separate these lipid samples using HPLC. At a flow rate of 150 μ l/min, the column was kept at 30° C. Samples were run on a 15-minute gradient from 95% (v/v in water) acetonitrile to 28% (w/v in water) 10 mM ammonium acetate at pH 9.2, with a 10-minute equilibration period in between. A Bruker Amazon SL ion trap mass spectrometer (Bruker) was used to gather mass spectra in both positive and negative electrospray ionization modes. The Bruker Compass software program was used for data analysis. DataAnalysis was used to identify peaks and characterize lipid classes, while QuantAnalysis was used to quantify the relative abundance of ornithine lipids to glycerophospholipids.

2.5 | Genome sequencing of *K. variicola* W12

The Trizol reagent (Thermo Fisher Scientific) was used to extract the genomic DNA of *K. variicola* from one milliliter of liquid culture. DNA was supplied to MicrobesNG for sequencing utilizing the Illumina MiSeq technology. The QUAST tool (Gurevich et al., 2013; Seemann, 2014) was used to assemble the sequencing reads, which were then annotated by Prokka (Seemann, 2014) and added to the JGI IMG database with an accession number (GOLD Analysis Project ID Ga0373462).

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

Targeting the *olsF* gene, which codes for the bifunctional acyltransferase (*OlsF*), primers *OlsFF*1298 (5' –CGCACTATCGTATCTGT–3') and *OlsFR* 1633 (5' –GACCATCCACGCAGTTAT–3') were created to assess how *olsF* transcription responded to phosphate addition in *K. variicola* W12. For proteomics and lipidomics, the bacteria was grown under the identical conditions as previously mentioned. As directed by the manufacturer, DNA was extracted using DNase (Promega) and RNA was isolated using the E.Z.N.A.® Total RNA Kit (Omega Bio-Tek). 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'), PCR amplification was used to demonstrate the lack of DNA in RNA samples. Superscript III reverse transcriptase (Invitrogen) was used to create complementary DNA (cDNA) using random hexamer primers at a concentration of 0.01 $\mu\text{g } \mu\text{l}^{-1}$ for each reaction. Using the primer set OlsFF1298-OlsFR1633 and a thermal cycle program of 95° C for 2 minutes and 35 cycles of 95° C for 30 seconds, 60° C for 30 seconds, and 72° C for 30 seconds, the first-strand cDNA was further quantified by quantitative-PCR (Q-PCR). The signal was recorded at 90° C. 12.5 μl of 2 $\times$ SYBR Premix Ex Taq (TaKaRa Bio Inc.), 2 μl of cDNA template, and 0.5 μl (10 μM) of each primer were included in the qPCR reactions (25 μl). Ten-fold serial dilutions of a plasmid template containing an olsF gene fragment were used to create standard curves. Using the primers BACT1369F (5' -CGG TGA ATA CGTTCY CGG-3'), PROK1541R (5' -AAG GAG GTG ATC CRG CCG CA-3'), and the TaqMan probe TM1389F (5' -CTT GTACAC CGC CCG TC-3'), the transcripts of the 16S rRNA gene were quantified using a TaqMan assay to determine the relative abundance of the olsF gene under various P levels (Suzuki et al., 2000). Analysis of statistics SPSS 19 (IBM Co.) was used to conduct statistical analyzes. The statistical differences between the three phosphate levels at each sample time point in terms of cell density, nitrogenase activity, and ornithine lipid content were assessed using one-way analysis of variance.

3 | RESULTS AND DISCUSSION

3.1 | Growth and N₂-fixing activity of W12 is repressed in response to P deficiency

W12 was cultivated on a low-N medium supplemented with three phosphate concentrations (0, 0.05, and 2 mM) in order to examine the effect of P availability on N₂-fixing activity. After the culture without extra phosphate amendment (0 mM), the cell density (OD₅₄₀) reached 0.53 after 48 hours, indicating that a minimal quantity of P in the inoculum was enough to maintain its development. On the other hand, when more phosphate was added to the medium at concentrations of 0.05 and 2 mM, the bacteria grew much better; after 48 hours, the OD₅₄₀ value was 0.79 and 0.89, respectively ($p < 0.05$; Figure 1a). In comparison to the control (5.3–13.1 nmol C₂H₄/OD₅₄₀/ml/h) ($p < 0.05$; Figure 1b), nitrogenase activity was considerably (>3-fold) greater in cultures supplemented with phosphate (33.8–47.2 and 43.3–58.0 nmol C₂H₄/OD₅₄₀/ml/h under 0.05 and 2 mM phosphate level, respectively). After 36 hours, nitrogenase activity and cell density stopped rising in cultures with low phosphate levels (0 and 0.05 mM), whereas they continued to rise until 48 hours in cultures supplemented with 2 mM phosphate. Our laboratory culture findings were consistent with earlier field research demonstrating that P availability may influence diazotrophs' N₂-fixing activity (Hogh-Jensen et al., 2002; Reed et al., 2007; Wang et al., 2018; Zheng et al., 2016).

3.2 | Lipidomic response of W12 to P limitation

We examined the intact membrane lipids of *K. variicola* W12 from the culture modified with three levels of phosphate (0, 0.05, and 2 mM) in order to provide insight into the lipidomic response of this diazotroph to P stress (Figure 2). PG and PE, two main phospholipids that are often found in bacteria like *Escherichia coli*, are produced by the bacterium when P is enough (Lopez-Lara & Geiger, 2017). However, a novel lipid with a m/z ratio of 649.5 was seen to elute at 13 minutes when the bacteria was grown under low phosphate conditions (0 and 0.05 mM phosphate addition). The existence of an ornithine head group was shown by further fragmentation by MS_n, and the fragmentation pattern is comparable with that of bacterial ornithine lipids (Smith et al., 2019; Vences-Guzman et al., 2015; Zhang et al., 2009). Betaine lipids were not found, which is consistent with the involvement of the btaAB genes.

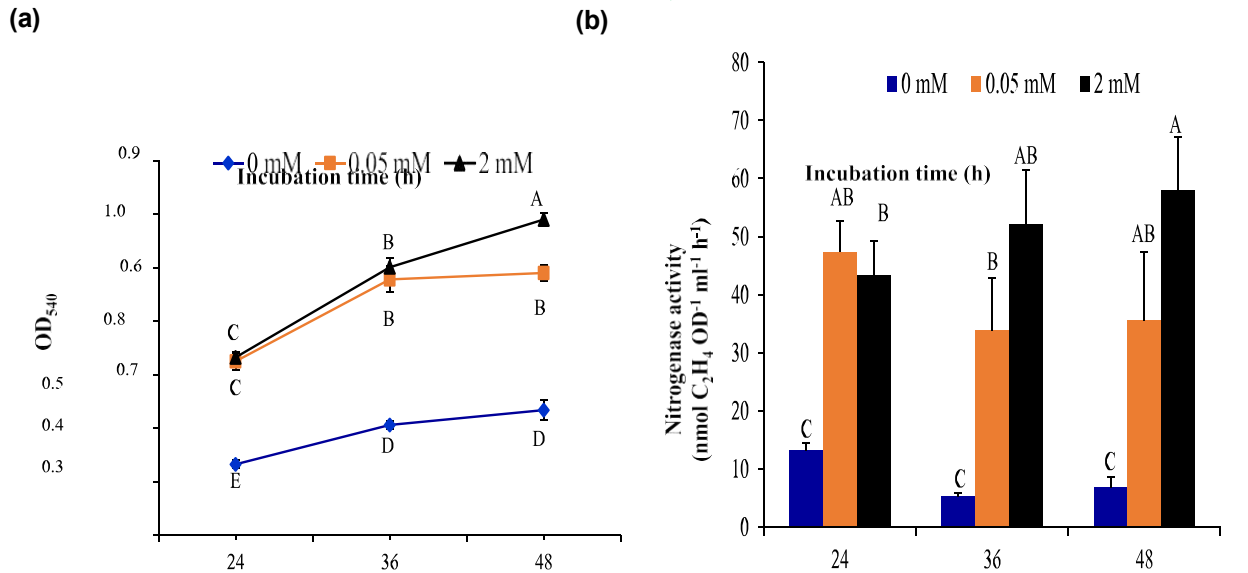


FIGURE 1: Nitrogenase activity in *Klebsiella variicola* W1 in response to P restriction. *K. variicola* was cultivated in a specified medium with and without phosphate amendments of 0.05 and 2 mM, and cell growth was tracked (a). The acetylene reduction test was used to assess nitrogenase activity at 24, 36, and 48 hours. The results were standardized at equal OD₅₄₀ (b). The standard deviation from three biological replicates is shown by error bars. The distinct letter denotes a significant variation between the treatment and across the incubation time < 0.05. OD, optical density. (a)

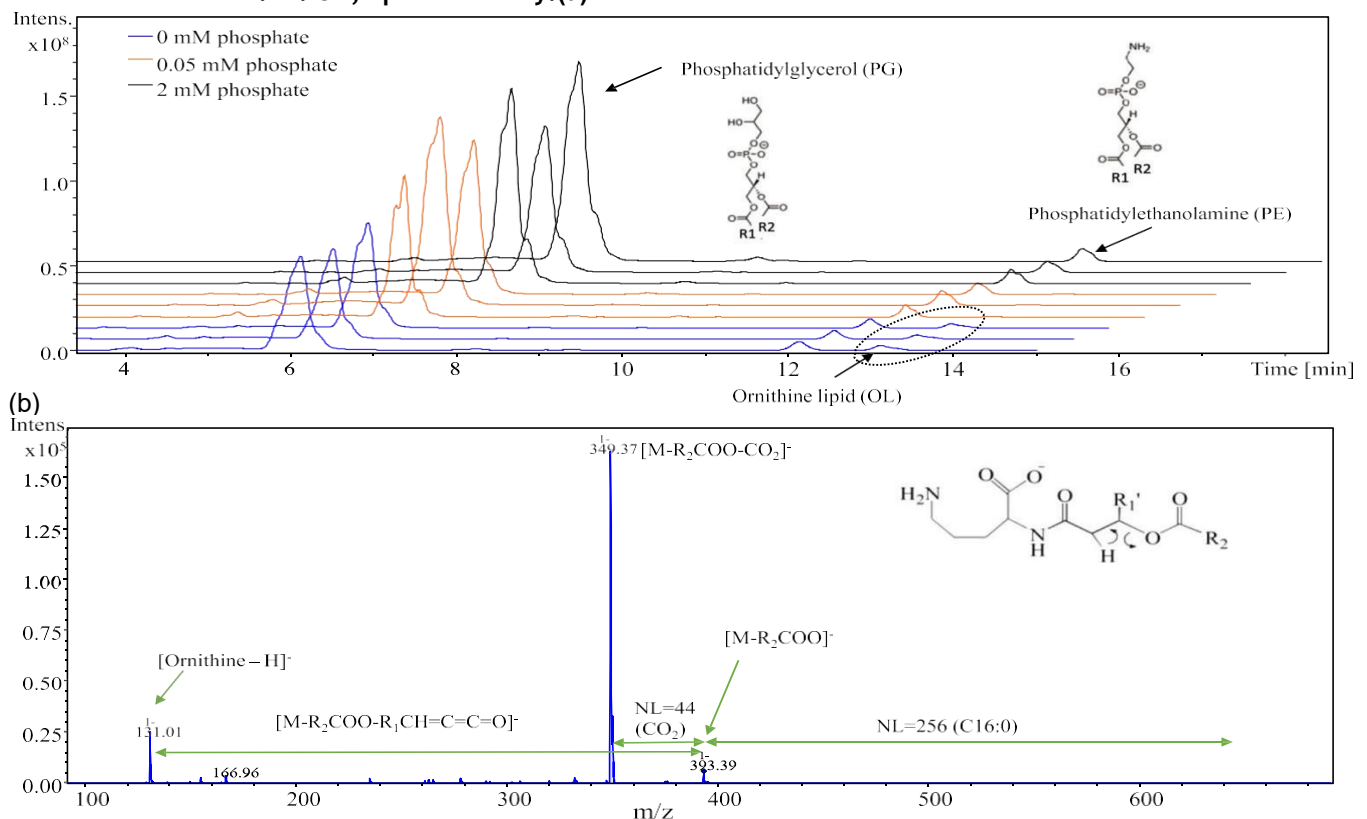
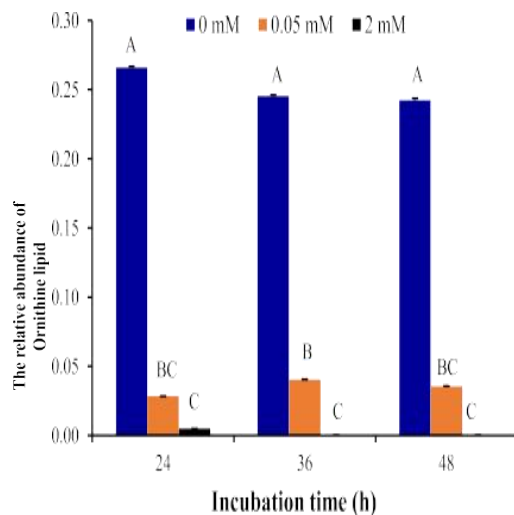


FIGURE 2: Lipidomics of *Klebsiella variicola* W12 under three phosphate addition levels: mass spectrum features. (a) Lipid profiles of *K. variicola* W12 cultivated at various phosphate addition levels using high-performance liquid chromatography-mass spectrometry (MS). Three separate biological replicates' lipid profiles were examined. The elution times of the three main lipids in this bacterium—PG, PE, and OL—are shown by the arrows. (b) MS fragmentation of m/z 649.5 ions that eluted in the negative mode at 13 minutes. The genome of *K. variicola* lacks NL

(neutral loss), OL (ornithine lipids), PE (phosphatidylethanolamine), and PG (phosphatidylglycerol) in betaine lipid production (Riekhof et al., 2005). In bacteria, two routes for the production of ornithine lipids have been found. The first step in *Sinorhizobium meliloti* and *Ruegeria pomeroyi* is carried out by the N-acyltransferase OlsB, which adds a 3-hydroxy fatty acyl group to the α -amino group of ornithine. An O-acyltransferase called OlsA uses the lyso-ornithine intermediate as a substrate to create ornithine lipids (Gao et al., 2004; Smith et al., 2019; Weissenmayer et al., 2002). Nevertheless, the *K. variicola* W12 genome lacks the *olsB/A* genes. Rather, the bifunctional acyltransferase OlsF, which was recently identified in *Serratia proteamaculans* for ornithine lipid production, is present in the genome (Vences-Guzman et al., 2015). The quantity of total ornithine lipids in *K. variicola* W12 is maximum when no extra phosphate is introduced to the culture, according to relative quantification of these lipids (Figure 3a). 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), the *olsF* gene transcripts in the culture were consistently significantly higher under low P addition levels (0 and 0.05 mM phosphate) than at high P addition levels (2 mM phosphate). All of these point to *K. variicola* W12's ability to produce ornithine lipid under P deprivation via the acyltransferase OlsF. According to a recent research, non-P betaine lipids dramatically rose in the most severely P-deficient soils, while phospholipids made up over 90% of total polar lipids in P-sufficient soils (Warren, 2020). For asymbiotic diazotrophs and other soil microbes under P-deplete stress, substitution of phospholipids with non-P polar lipids, such as ornithine and betaine lipids, may be a crucial mechanism. The lipidomics and genomics analyzes in this study indicated a distinct pathway of ornithine lipid biosynthesis via bifunctional acyltransferase OlsF in diazotrophic microbes, despite the fact that a similar lipid remodeling mechanism has been discovered in cyanobacteria (Pereira et al., 2016) and rhizobia (Zavaleta-Pastor et al., 2010).

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

We analyzed the complete cellular proteome of the bacterium grown under three phosphate amendment levels (0, 0.05, and 2 mM) at three time intervals (24, 36, and 48 h) in order to provide insight into the cellular response of *K. variicola* to P limitation. For every treatment and sampling time point, three biological replicates were used; only proteins present in all three biological replicates were kept for further statistical analysis. Under these circumstances, 1347 proteins were found out of 5435 protein-coding genes in the genome of *K. variicola* W12 (Supporting Information: Table 1). The conventional nitrogen fixation gene cluster *nifJHDKTYENXUSVWZMFLABQ*, which is identical to that of *K. pneumoniae*, is present in the genome of *K. variicola* W12 (Rubio & Ludden, 2008). Nitrogen fixation-related proteins, including dinitrogenase (*NifDK*), dinitrogenase reductase (*NifH*), and the N₂ fixation transcriptional regulators *NifA* and *NifL*, were markedly elevated in cultures supplemented with extra phosphate supplementation as opposed to those without. **a)**



(b)

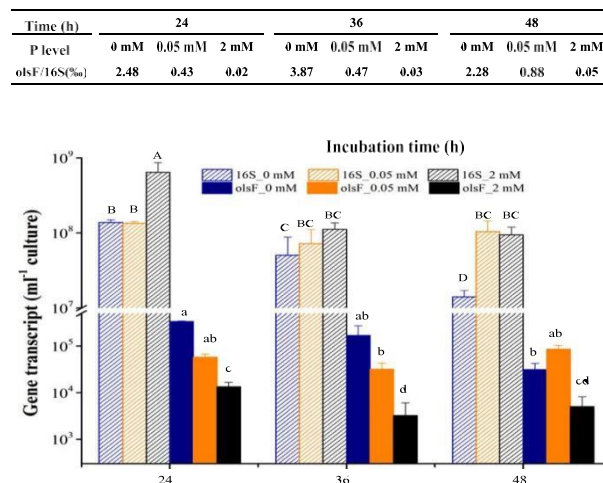


FIGURE 3: Klebsiella variicola W12's lipidomics reaction to phosphorus restriction. (a) Phosphorus stress causes the production of ornithine lipids. The ratio of ornithine lipids to glycerophospholipids (PE + PG) was used to represent the concentration of ornithine lipid. A significant difference between the treatment and throughout the incubation period at $p < 0.05$ is indicated by the distinct letter. (b) *K. variicola* W12's 16S rRNA and *olsF* gene transcript abundance at three different phosphate addition levels. For the 16S rRNA gene and the *olsF* gene, respectively, different capital and lowercase letters indicate significant variations between the treatments and during the incubation period at $p < 0.05$. The ratios of *olsF* gene transcripts to 16S rRNA gene transcripts at various p levels are shown in the numbers above the figure. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and ribosomal RNA (rRNA) with phosphate between 24 and 48 hours (0.05 and 2 mM) (Figure 4). This is consistent with the studies of nitrogenase activity, which demonstrate a significant rise in nitrogenase activity upon P addition (Figure 1). However, in cultures treated with phosphate, the two-component regulator PhoBR and several proteins involved in the transport and subsequent metabolism of organic and inorganic P were markedly downregulated; this was reversed when no more phosphate was added (Figure 4). These include phosphonate transporters, alkaline phosphatase (PhoA), C-P lyase (Phn), and the ABC transporter for phosphate (PstSABC). In a similar vein, diazotrophic cyanobacteria overcome P deprivation by increasing the expression of genes encoding phosphatase (*phoD*) and P transporter (*pstS*) (Pereira et al., 2016; Wang et al., 2018). In comparison to cultures with lower P (0 and 0.05 mM), the whole cellular proteome in cultures with a 2 mM phosphate amendment differs dramatically (Figure 5). The bacteria overcomes N restriction when P is replenished because nitrogenase activity is much greater (Figure 1). Thus, in order to achieve optimal cell growth, the cells upregulate the biosynthesis of ribosomes and amino acids (Supporting Information: Table 3). A conceptual model to explain how P regulates nitrogen fixation in nonsymbiotic diazotrophs the data presented in our study thus supports a plausible model to explain the upregulation of nitrogenase activity when P is replete (Figure 6). During P and N (ammonium) dual limitation, *K. variicola* maintains a modest nitrogenase activity while devoting more energy for obtaining organic and inorganic phosphate from the medium since both nitrogen fixation and P uptake are energy-demanding activities for microbial cells. The minimal nitrogenase activity seen in the culture without phosphate supplement (Figure 1) and the notable overexpression of phosphate transporters, PhoBR two-component systems, and a variety of organic phosphorus metabolic proteins (Figure 4 and Supporting Information: Table 3) provide evidence for this. The cells also replace membrane phospholipids with P-free ornithine lipids in response to P restriction (Figures 2 and 3). The cells begin to downregulate genes necessary for P absorption while focusing more energy on nitrogen fixation as P constraint becomes less acute (i.e., at 0.05 mM). This is seen from the markedly increased nitrogenase activity (Figure 1).

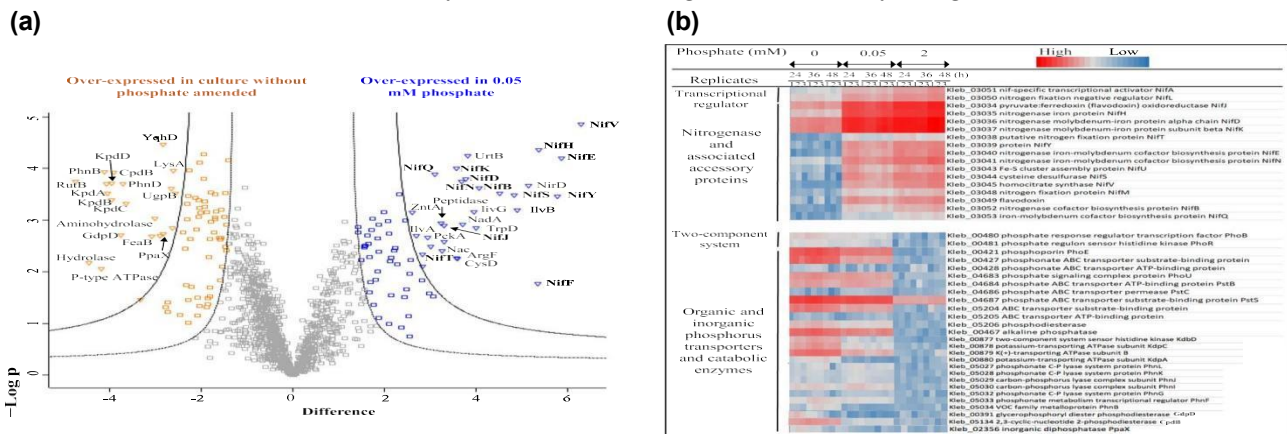


FIGURE 4: Comparative proteomics studies of *Klebsiella variicola* W12 under phosphorus stress. (a) A volcano map showing differentially expressed proteins at $FDR < 0.05$ (dashed line) and $FDR <$

0.01 (solid line). Samples collected at 36 hours from cultures treated with 0.05 mM phosphate were compared to those without any phosphate additions (0 mM). Blue and orange highlights indicate proteins that are increased with phosphate addition (0.05 mM) or without phosphate addition, respectively. FDR < 0.01 is represented by triangles, whereas FDR < 0.05 is represented by squares. Supporting Information contains the list of proteins and annotations. Table 2. (b) A heatmap showing the proteins involved in the metabolism of organic and inorganic phosphorus as well as nitrogen fixation. Three biological replicates that were taken from cultures at 24, 36, and 48 hours, respectively, were used to analyze the cellular proteome. Different phosphate concentrations (0, 0.05, and 2 mM) were added to the bacterial cultures. False discovery rate, or FDR.

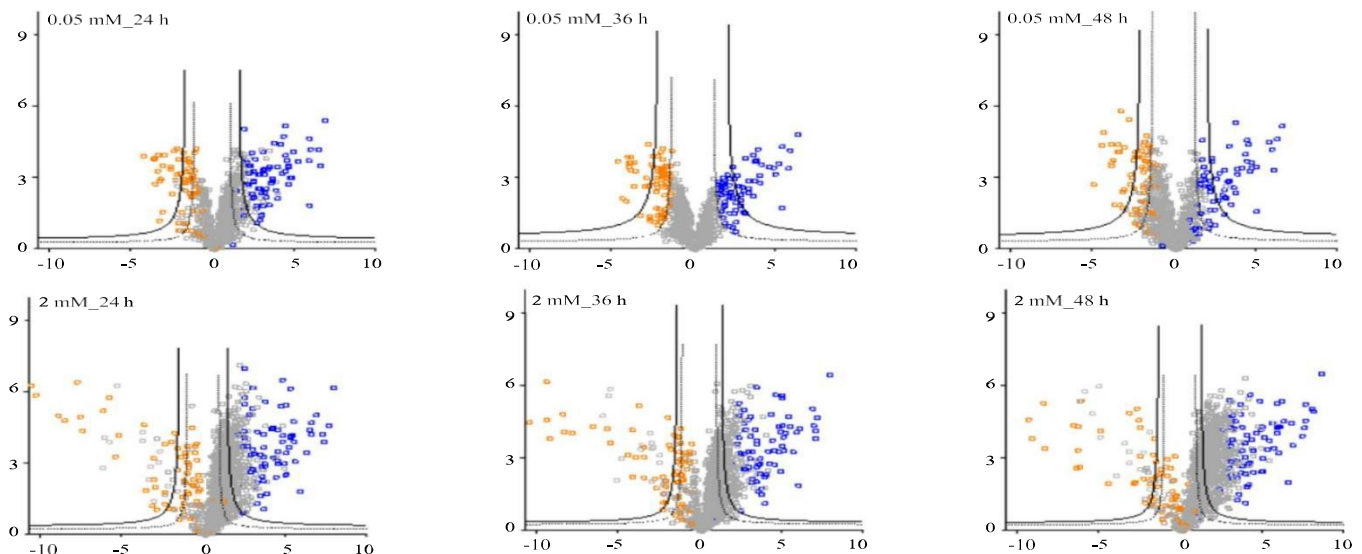


FIGURE 5: Volcano graphs that illustrate how the cellular proteome changes when phosphorus is added (0.05 and 2 mM). At 36 hours, samples were compared to those with no phosphate addition (0 mM). Figure 4 (FDR < 0.05) displays the same proteins, which are indicated in orange (overexpressed in no phosphate) or blue (overexpressed in 0.05 mM phosphate). FDR = 0.01 is represented by the solid lines, while FDR = 0.05 is represented by the dashed lines. Supporting Information contains a list of proteins having differential expression and FDR < 0.05.

Table 3.FDR, false discovery rate.

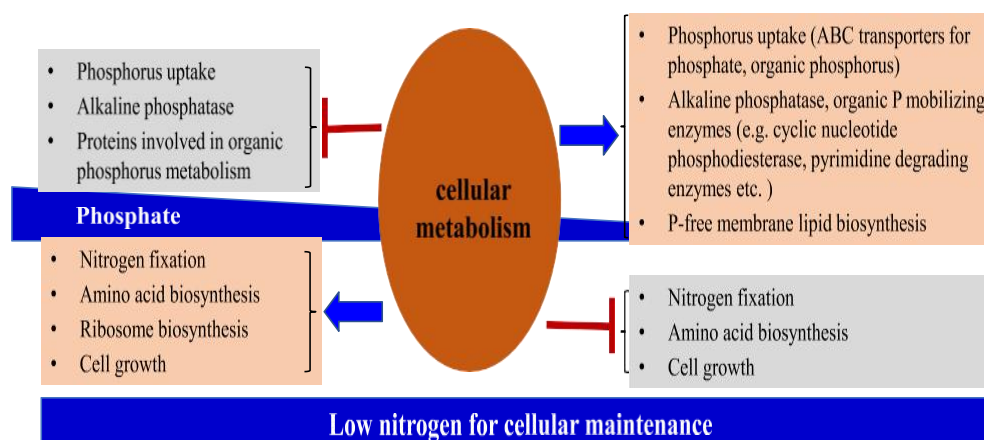


FIGURE 6: A functional conceptual model of *Klebsiella variicola* cellular metabolism under phosphorus shortage stress. The bacteria fixes dinitrogen gas at the expenditure of additional energy to create ammonium for cell development when phosphorus is abundant. Because phosphorus is necessary for the creation of macromolecules, cell development is impeded when phosphorus levels are low. Therefore, by producing ABC transporters for both organic and inorganic phosphorus as well as a variety of enzymes involved in organic phosphorus degradation, the bacterium turns off nitrogen fixing and directs cellular energy toward phosphorus acquisition. By producing substitute nonphosphorus ornithine lipids to replace membrane phospholipids, the bacteria further lowers the physiological need for phosphorus.

4 | CONCLUSIONS

Therefore, our research offers new information on how the nonsymbiotic diazotroph *K. variicola* responds to P shortage both physiologically and molecularly. Our lipidomics data showed that the nonsymbiotic diazotroph adapts to P shortage by substituting P-free ornithine lipids for membrane phospholipids. Additionally, our findings provide a comprehensive understanding of the molecular and metabolic processes by which P deprivation impacted BNF in *K. variicola*. Both natural and agricultural ecosystems may often overcome N constraint by obtaining significant N input via BNF (Furnkranz et al., 2008; Ladha et al., 2016; Madhaiyan et al., 2015; Van Deynze et al., 2018). Therefore, P availability is crucial in determining primary production in natural ecosystems via controlling nitrogenase activity and BNF. The model we presented here, which is based on cellular demand for N and P, may have global applicability since both N and P constraints are common in terrestrial ecosystems worldwide (Elser et al., 2007). According to this theory of N and P coupling, it would be beneficial for agricultural sustainable production and environmental health to decrease N fertilizer input while moderating P input in agroecosystems to maximize the potential of BNF in ecosystems themselves.

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