



# Genome-Wide Analyses of Proteome and Acetylome in *Zymomonas mobilis* Under N<sub>2</sub>-Fixing Condition

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*Zymomonas mobilis*, a promising candidate for industrial biofuel production, is capable of nitrogen fixation naturally without hindering ethanol production. However, little is known about the regulation of nitrogen fixation in *Z. mobilis*. We herein conducted a high throughput analysis of proteome and protein acetylation in *Z. mobilis* under N<sub>2</sub>-fixing conditions and established its first acetylome. The upregulated proteins mainly belong to processes of nitrogen fixation, motility, chemotaxis, flagellar assembly, energy production, transportation, and oxidation–reduction. Whereas, downregulated proteins are mainly related to energy-consuming and biosynthetic processes. Our acetylome analyses revealed 197 uniquely acetylated proteins, belonging to major pathways such as nitrogen fixation, central carbon metabolism, ammonia assimilation pathway, protein biosynthesis, and amino acid metabolism. Further, we observed acetylation in glycolytic enzymes of central carbon metabolism, the nitrogenase complex, the master regulator NifA, and the enzyme in GS/GOGAT cycle. These findings suggest that protein acetylation may play an important role in regulating various aspects of N<sub>2</sub>-metabolism in *Z. mobilis*. This study provides new knowledge of specific proteins and their associated cellular processes and pathways that may be regulated by protein acetylation in *Z. mobilis*.

**Keywords:** *Zymomonas mobilis*, 4D label-free proteomics, nitrogen fixation, post-translational modification, lysine acetylome

## INTRODUCTION

*Zymomonas mobilis*, a Gram-negative facultative anaerobic bacterium, is a promising candidate for industrial biofuel production with a suite of desirable characteristics (Doelle et al., 1993; Sprenger, 1996; Kalnenieks, 2006; Panesar et al., 2006; Bai et al., 2008; Yang et al., 2009, 2016; He et al., 2014; Martien et al., 2019; Todhanakasem et al., 2020). *Zymomonas mobilis* is capable to fix dinitrogen (N<sub>2</sub>) efficiently without affecting ethanol production, which further increases its economic and environmental importance (Kremer et al., 2015; Palamae et al., 2020). In comparison with other free-living diazotrophic bacteria, *Z. mobilis* has just a Mo-dependent nitrogen-fixing system. The nitrogen-fixing island of the ZM4 strain, for example, is a 23-kb chromosomal region that contains the transcriptional regulator *nifA*, the *rnf* operon encoding

the Rnf electron transport complex, the nitrogenase operon, and two operons involved in nitrogenase maturation (Tsoy et al., 2016; Tatli et al., 2019).

Aside from the conserved genetic structure of the nitrogen-fixing island, it is also very important to decipher the regulatory mechanisms underlying the nitrogen fixation. This will not only help to better understand nitrogen fixation but is also crucial for developing more efficient nitrogen-fixing pathways. Such novel pathways will help in reducing the use of chemical fertilizers and replacing the nitrogen source in the fermentation industry. Nitrogen fixation in free-living diazotrophic bacteria is regulated at diverse levels, including transcriptional regulation by NifA (or VnfA) and its regulator NifL (Dixon, 1998; Lei et al., 1999; Schmitz et al., 2002; Martinez-Argudo et al., 2004b; Perry et al., 2005), post-transcriptional regulation by non-coding RNAs (Wang et al., 2009; Zhan et al., 2016, 2019), translational regulation by a conformational switch in NifL (Martinez-Argudo et al., 2004a), and various post-translational modifications (PTMs). In addition, nitrogen fixation is also regulated by sensing oxygen, ammonium, cofactors, or signal messengers (Dixon and Kahn, 2004; Masepohl and Hallenbeck, 2010; Xie et al., 2010; Zhang et al., 2012; Hoffmann et al., 2015).

The PTM-based regulation of nitrogen fixation is of great importance. For instance, phosphorylation can activate the Ntr two-component system, which enhances nitrogen fixation (Jiang et al., 1998; Marx et al., 2016). ADP-ribosylation, which is mediated by DraT (ADP-ribosyltransferase) and DraG (ADP-ribosyl glycohydrolase), is another prevalent regulatory mechanism in diazotrophs (Huergo et al., 2012; Nordlund and Högbom, 2013; Moure et al., 2015; Bueno Batista and Dixon, 2019). Furthermore, uridylylated PII protein (GlnK)-mediated physiological switch-off of nitrogenase activity *via* inhibition of the Rnf1 electron transport system is also a known regulatory mechanism (Sarkar et al., 2012; Bueno Batista and Dixon, 2019). The *Rhodospirillum rubrum* mutants of GlnD (a bifunctional uridylyltransferase/uridylyl-removing enzyme) affected nitrogen fixation in various ways (Zhang et al., 2005). These studies emphasize the significance of uridylylation in regulation of nitrogen fixation.

Protein acetylation is one among many PTMs used to regulate cellular processes across all domains of life. Acetylation can dramatically change the protein's function by altering its properties such as solubility, hydrophobicity, surface properties, and stability. This, in turn, may influence protein conformation and interactions with other macromolecules such as DNA, other proteins, substrates, and cofactors, among others (Glozak et al., 2005; Yang and Seto, 2008; Xiong and Guan, 2012). While protein acetylation is well known for its regulatory role in eukaryotes (Hebbes et al., 1988; Glozak et al., 2005; Kim et al., 2006; Choudhary et al., 2009; Zhao et al., 2010; Drazic et al., 2016; Ree et al., 2018), research about the role of this PTM in prokaryotes is relatively a recent scientific endeavor (Yu et al., 2008; Hentchel and Escalante-Semerena, 2015; Ouidir et al., 2016; Wolfe, 2016; Carabetta and Cristea, 2017; Nakayasu et al., 2017; Ren et al., 2017; Liimatta et al., 2018; Liu et al., 2018a; Reverdy et al., 2018; Christensen et al., 2019a,b; VanDrisse and Escalante-Semerena, 2019). So far, we know little about

the role of acetylation in regulation of nitrogen fixation. A series of investigations, however, revealed that GlnR mediates an autofeedback-loop to regulate glutamine synthase activity *via* lysine-acetylation (You et al., 2016, 2017; Liu et al., 2018b). Based on these findings, acetylation is speculated to regulate the nitrogen fixation.

However, the regulation of N-fixation in *Z. mobilis* is not fully understood. Unlike many other diazotrophs, ZM4 does not appear to encode NifL transcriptional regulator or DraT/DraG-mediated ADP-ribosylation system. In addition, it employs a NtrX-NtrY instead of a NtrB-NtrC two-component system to regulate the nitrogen fixation (Yang et al., 2018). These genomic traits indicate that there are specific regulations for nitrogen fixation in ZM4. At post-translational level, a recent phosphoproteomic study in *Z. mobilis* has revealed that various proteins involved in nitrogen fixation such as NifA, NtrC, PII proteins, Rnf, Hfq, and nitrogenase, were significantly phosphorylated (Tatli et al., 2019). However, ZM4's proteomic profiling and other PTMs under nitrogen-fixing conditions are yet to be described. Therefore, in the present study, we conduct 4D label-free proteomics in *Z. mobilis* strain ZM4 under N<sub>2</sub>-fixing and non-fixing conditions. We describe the first genome-wide proteome and acetylome in this ethanogenic bacterium.

## MATERIALS AND METHODS

### Strain and Growth Conditions

A single colony of *Z. mobilis* (sub sp. mobilis ZM4 strain) was inoculated to 20 ml of the rich media (RM; 20 g/l glucose, 10 g/l yeast extract, 1 g/l KH<sub>2</sub>PO<sub>4</sub>, 1 g/l peptone, and 1 g/l MgSO<sub>4</sub>•7H<sub>2</sub>O) and then cultured for 20 h (OD<sub>600</sub> ~1.5 to 1.9) in a 50-ml screw-cap tube. Then 10 ml of the cultures were transferred in 100 ml of RM and grown for 20 h in a 250-ml Duran bottle. Cells were harvested by centrifuging at 1216 RCF and 4°C for 10 min (Sorvall BIOFUGE Stratos, Thermo Scientific), washed twice with autoclaved ddH<sub>2</sub>O, and suspended in 100 ml autoclaved ddH<sub>2</sub>O. Twelve milliliters of the suspended cells were inoculated in 120 ml of nitrogen-fixing media, the modified ZYMM (Kremer et al., 2015), in a 300-ml serum bottle, followed by flushing nitrogen gas (N<sub>2</sub>, with the purity of 99.999%) at 1 MPa for 30 min. Then the serum bottle was sealed and incubated at 30°C with shaking at 150 rpm. Twenty-four-hour cultures (N-fixing) were centrifuged at 1751 RCF and 4°C for extraction of cellular proteins. The modified ZYMM used in this study was similar to that of Kremer et al. (2015), with the exception that ammonium molybdate was replaced with sodium molybdate. For non-fixing condition, the ZM4 strain was grown in non-nitrogen-fixing media (10 mM ammonium chloride instead of N<sub>2</sub> was used in the modified ZYMM), and samples were taken at exponential phase (8 h culture).

### Protein Extraction and Digestion

The samples were sonicated three times on ice in lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail, 3 μM trichostatin A, and 50 mM nicotinamide) using a high-intensity ultrasonic

processor (Scientz). Debris was removed by centrifugation (12,000 g at 4°C for 10 min). Supernatants were collected and precipitated with cold 20% trichloroacetic acid for 2 h on ice. Samples were centrifuged (12,000 g at 4°C for 5 min), supernatants were discarded, and the pellets were washed three times with cold acetone. The protein samples were redissolved in 8 M urea, and protein concentration was determined with a BCA Kit (Beyotime) as per the manufacturer's instructions.

For trypsin digestion, the protein samples (after washed with cold acetone) were redissolved in buffer (100 mM TEAB, pH 8.0). Then trypsin was added at a 1:50 ratio and samples were incubated overnight. Trypsin was added to the samples again at a 1:100 ratio, and the samples were incubated for 4 h. Finally, the samples were reduced with 5 mM DTT (for 45 min at 37°C) and alkylated with 15 mM iodoacetamide (for 45 min at RT in the dark).

## Enrichment of Acetylated Lysine Containing Peptides

To enrich the acetylated peptides, the tryptic peptides were dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0), and samples were incubated (overnight at 4°C) with pre-washed antibody beads (PTM-104, PTM Bio) with gentle shaking. The beads were then washed four times with NETN buffer and twice with water. The bound peptides were eluted from the beads with 0.1% trifluoroacetic acid. Finally, the eluted fractions were combined and vacuum-dried. For LC-MS/MS analysis, the resulting peptides were desalted with C<sub>18</sub> ZipTips (Millipore) according to the manufacturer's instructions.

## LC-MS/MS Analysis

The tryptic peptides were dissolved in buffer A (0.1% formic acid in water) and were directly loaded onto a reversed-phase analytical column (25-cm length, 75 μm i.d.). Peptides were eluted at a constant flow rate of 400 nl/min on a nanoElute UHPLC system (Bruker Daltonics), using a 3-step linear gradient: from 6 to 22% buffer B (0.1% formic acid in acetonitrile) over 43 min, from 22 to 30% B in 13 min, climbing to 80% B in 2 min, and then holding at 80% B for the last 2 min. The mass spectrometry was performed with timsTOF Pro (Bruker Daltonics) instrument with an electrospray voltage of 1.60 kV. Precursors and fragments were analyzed at the TOF detector with an MS/MS scan range from 100 to 1700 m/z. The timsTOF Pro was operated in parallel accumulation serial fragmentation (PASEF) mode. Precursors with charge states 0–5 were selected for fragmentation, and 10 PASEF-MS/MS scans were acquired per cycle. The dynamic exclusion was set to 30 s.

## Database Search and Data Analysis

The resulting MS/MS data were processed using MaxQuant software v.1.6.6.0.<sup>1</sup> Tandem mass spectra were searched against

the database (*Zymomonas mobilis* subsp. *Mobilis* strain ATCC\_31821\_264203\_PR\_20190830) and concatenated with reverse decoy database. Trypsin/P was specified as a cleavage enzyme allowing up to four missing cleavages. The mass tolerance for precursor ions was set as 40 ppm in the First search and 40 ppm in the Main search, while the mass tolerance for fragment ions was set as 0.04 Da. Carbamidomethyl on Cys was specified as fixed modification, while acetylation on protein N-terminal, oxidation on Met, and acetylation on Lys were specified as variable modifications. FDR was adjusted to <1%. The intensity values for each acetylated site were averaged across the biological replicates for each growth condition. A particular comparison ratio of average scores between the two conditions was considered as a fold change of comparison at a specific acetylated site. Acetylated sites that were not quantified in at least two biological replicates of each condition in a relevant comparison were excluded from analyses. Data were subjected to log<sub>2</sub> transformation. A two-tailed student's T-test was performed for each comparison at a specific acetylated site. A value of  $p < 0.05$  was considered significant. All value of  $ps$  were then converted to  $-\log_{10}$  scores.

## Bioinformatics Annotation of Peptides

All identified peptides were subjected to Gene Ontology (GO) annotations and enrichment at the UniProt-GOA database.<sup>2</sup> Proteins were classified by GO annotations under three categories (biological process, cellular component, and molecular function). For each category, a two-tailed Fisher's exact test was employed, and a GO term with a value of  $p < 0.05$  was considered as significant. For annotation of metabolic pathways, the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa and Goto, 2000; Tanabe and Kanehisa, 2012) was used. Protein interactions networks were generated using the STRING database with an interaction confidence score of 0.7 (Szklarczyk et al., 2015). Subcellular localization of proteins was predicted using CELLO v.2.5 (Yu et al., 2006). Significant values of  $p$  for each analysis were filtered and transformed into  $-\log_{10}$  scores for use in graphics. The 3D structures of proteins were predicted using SWISS-MODEL server.<sup>3</sup>

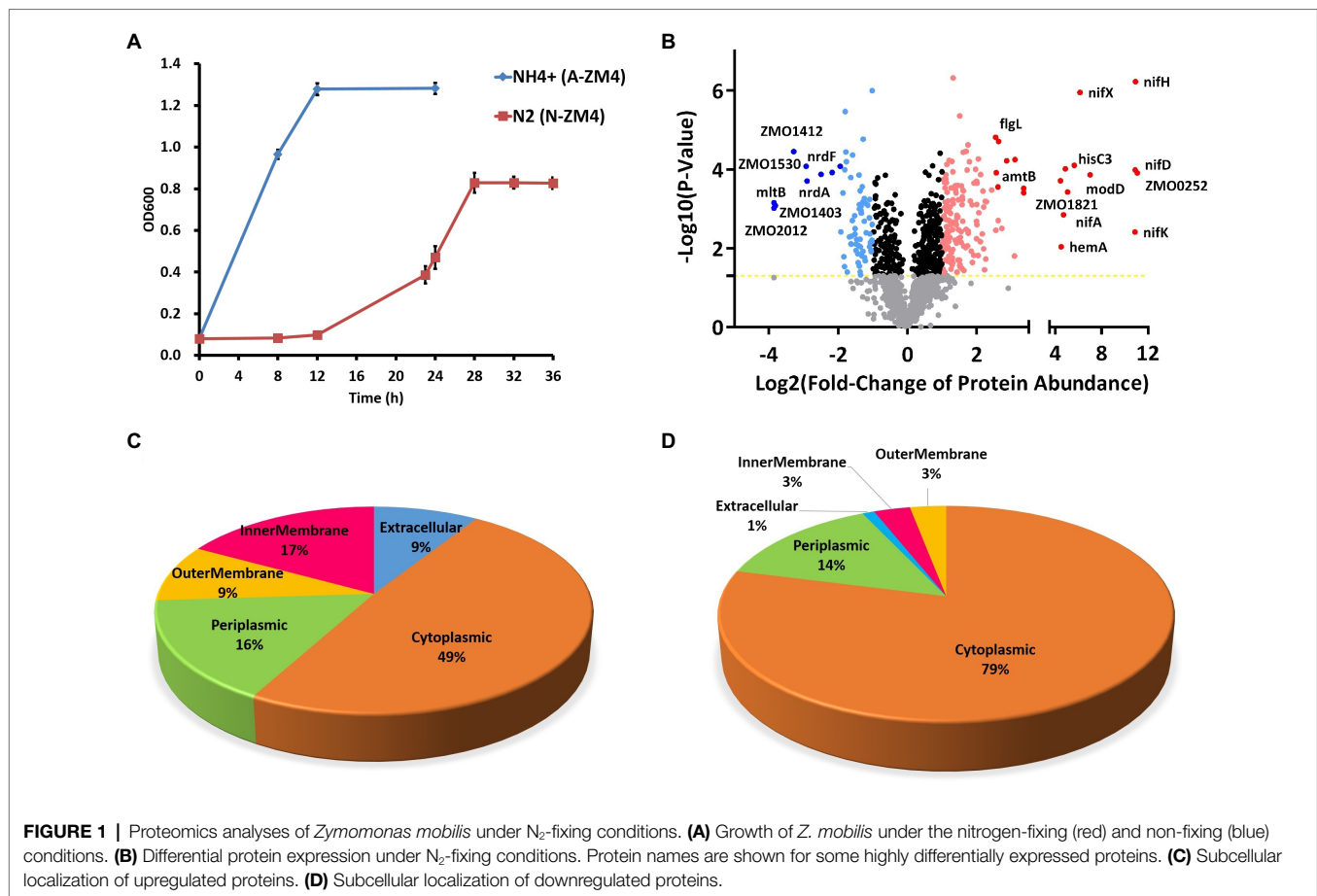
## RESULTS

### 4D Label-Free Quantitative Proteomics of *Zymomonas mobilis* Under N<sub>2</sub>-Fixing and Non-fixing Conditions

Cells grown under the N<sub>2</sub>-fixing (N-ZM4) and non-fixing (A-ZM4) conditions (Figure 1A) were harvested during exponential growth and lysed for high-resolution LC-MS/MS analysis. The resulting identified peptides were quantitatively compared by label-free quantitation. We identified a total of 1,449 proteins, of which 1,389 proteins were quantifiable (Supplementary Table S1). Of the quantifiable proteins, 242

<sup>2</sup><http://www.ebi.ac.uk/GOA/>

<sup>3</sup><https://swissmodel.expasy.org/>



showed significant differences between nitrogen-fixing (N-ZM4) and non-fixing (A-ZM4) group (Figure 1B). Among these, 170 proteins were upregulated (fold change > 2 with  $p < 0.05$ ), and 72 were downregulated (fold change < 0.5 with  $p < 0.05$ ) under the nitrogen-fixing condition. All of these proteins were chromosome encoded except four proteins (three upregulated, and one downregulated) that were encoded by native plasmid pZM39 (GenBank ID NZ\_CP023719.1). One of the pZM39-encoded upregulated proteins (Q8GF46) was an autotransporter domain-containing protein, and two (Q8GF51 and D3G2C3) were uncharacterized proteins. Whereas, the single pZm01-encoded downregulated protein (Q8GF26) was a putative partitioning protein ParB.

Various proteins associated with N<sub>2</sub>-fixation were highly upregulated under N<sub>2</sub>-fixing condition, including nitrogenase complex (NifH, NifD, NifK, and NifX), the regulators of N<sub>2</sub>-fixation (NifA, GlnB, and GlnA), Rnf electron transport complex (RnfH, RnfC, and RnfD), nitrogen regulation sensor (NtrY), sigma factor (RpoN), ammonium transporter (AmtB), and molybdenum transport system protein (ModD).

Various proteins related to energy production and transportation of energy molecules were also upregulated (Supplementary Table S1). These include leavansucrases SacB and SacC, sugar transporters (Glf, OprB1, and ZMO0293), ATPase (ZMO0637), and a predicted major intrinsic protein

(ZMO0252) that facilitates the passive transport of water, small carbohydrates, and other molecules. Similarly, TonB-dependent receptors and their associated proteins (ExbD and ExbB), which mediate substrate-specific transport across the outer membrane were also upregulated. The ABC transporter proteins ZMO0910, ZMO0254, ZMO1017, and ZMO1018 were also upregulated.

Proteins involved in motility, chemotaxis, and flagellar assembly were also found upregulated. Among other upregulated proteins the alcohol dehydrogenase was also included, implying that N<sub>2</sub>-fixation does not affect ethanol production. Proteins related to ferrous Fe<sup>2+</sup> ion transport and its insertion into protoporphyrin IX, iron-sulfur cluster assembly, magnesium transporter, and potassium transporter were also upregulated. Proteins involved in the oxidation-reduction process and suppression of deoxyribonucleotides synthesis, for example NrdR, were upregulated too.

Downregulated proteins were mainly associated with energy-consuming processes and biosynthesis of secondary metabolites. Examples include blockage of malonyl-CoA-based biosynthesis, sesquiterpenoid and triterpenoid biosynthesis, biotin biosynthesis, folate biosynthesis, and ammonia synthesis from glycine. Conversion of Fe<sup>2+</sup> to Fe<sup>3+</sup> ions was also inhibited (downregulated bacterioferritin) during N<sub>2</sub>-fixation. Whereas, Fe<sup>2+</sup> ion utilizing proteins were upregulated.



To further analyze which cellular areas were affected by differential protein expression, we performed subcellular localization analysis of differentially expressed proteins. Among the upregulated proteins, 49% were localized to cytoplasmic, 16% to periplasmic, 17% to the inner membrane, 9% to the outer membrane, and 9% to extracellular categories (Figure 1C). Whereas, localization of downregulated proteins resulted in 79% to cytoplasmic, 14% to periplasmic, 3% to the inner membrane, 3% to the outer membrane, and 1% to extracellular categories (Figure 1D).

## Functional Enrichment of Differentially Expressed Proteins

We then further focused on differentially expressed proteins and performed functional enrichment of associated GO terms and pathways, and visualized significantly enriched GO terms and KEGG pathways under both conditions.

The significantly enriched areas for upregulated proteins (Figure 2A) under the N<sub>2</sub>-fixing condition were associated with motility of cell (flagellum-dependent) or subcellular component and their localization, nitrogen fixation, molecular transportation, and response to stress or external stimuli. Nitrogen fixation is a stressful condition and significantly enriched GO terms associated with response to stress or external stimuli can be seen here. The chemotaxis and cell motility are thus apparent. Various GO terms associated with nitrogen fixation process such as nitrogenase complex and its associated proteins like molybdenum-iron transporters, proton-transporting two-sector ATPase complex, heme, and porphyrin-containing compound biosynthesis, nitrogen compound metabolic process, regulation of nitrogen utilization, and transport of nitrogen compound were significantly enriched. Whereas, nitrogen compound metabolic process, nucleotide biosynthetic process, carboxylic acid metabolism, oxoacid metabolism, cellular amide

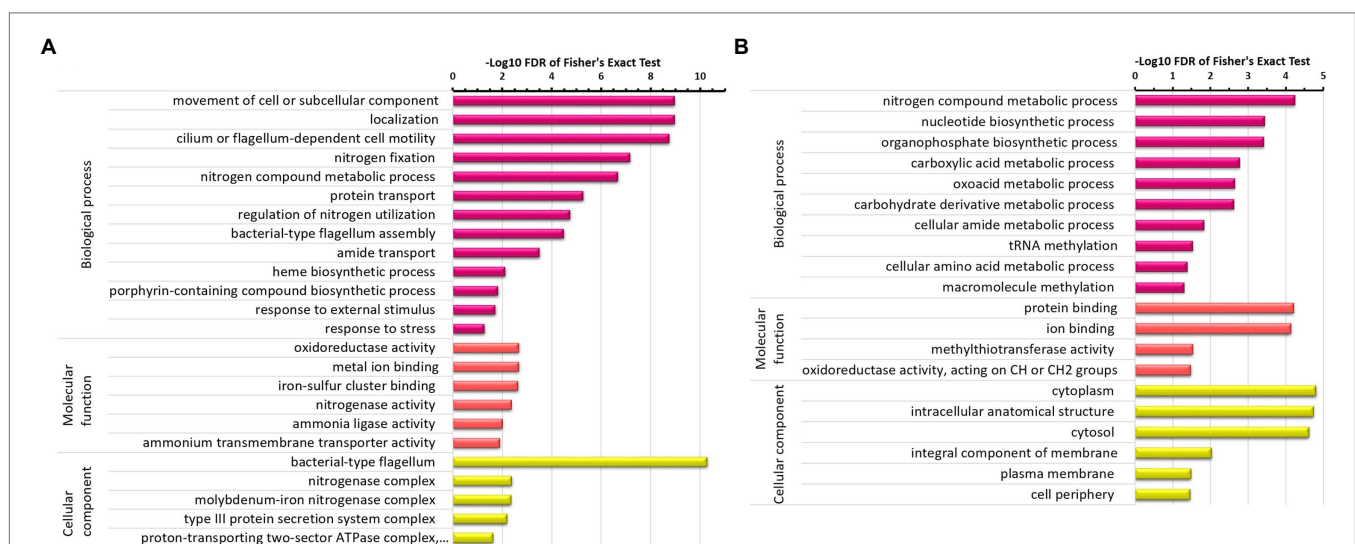
metabolism, amino acid metabolism, and macromolecule methylation were significantly enriched areas for downregulated proteins (Figure 2B).

Major KEGG pathways that were significantly enriched for upregulated proteins (Figure 3A) under the N<sub>2</sub>-fixing condition included the flagellar assembly, nitrogen metabolism, chloroalkane, and chloroalkene degradation, two-component system, folate biosynthesis, pantothenate and CoA biosynthesis, porphyrin and chlorophyll metabolism, and microbial metabolism in diverse environments. Some significantly enriched KEGG pathways for downregulated proteins (Figure 3B) were pyrimidine and purine metabolism, biosynthesis of secondary metabolites, carbon metabolism, and biosynthesis of amino acids.

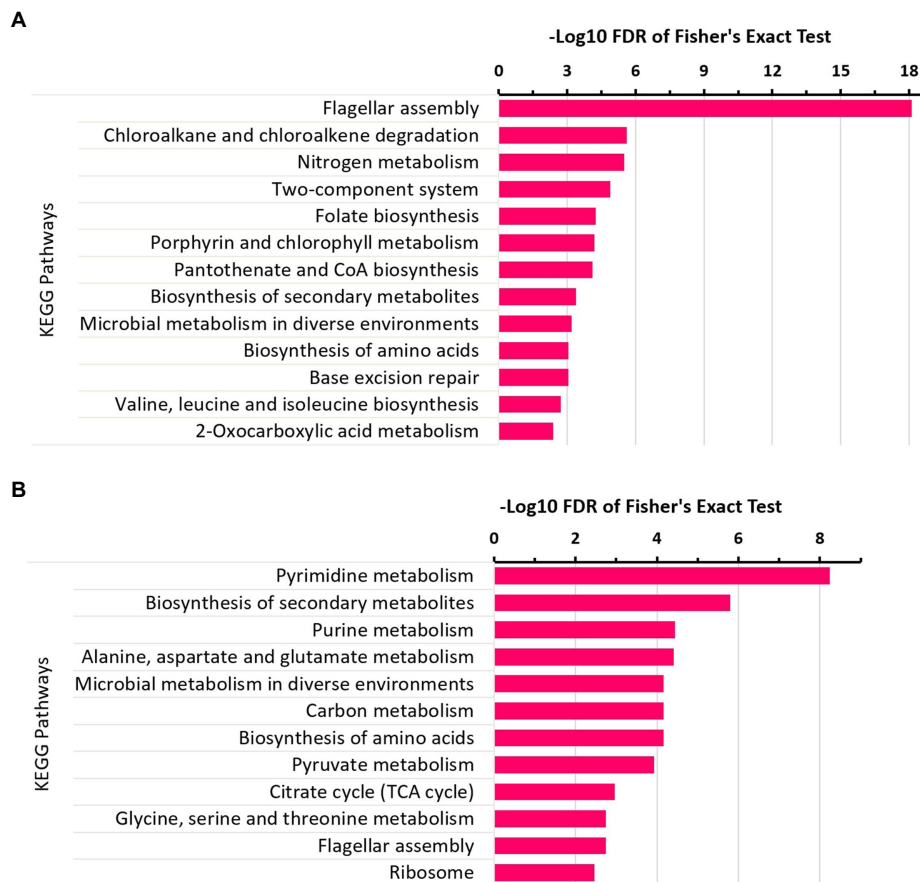
## Acetylome Analysis of *Zymomonas mobilis*

The result of the above proteomics analysis revealed that ZMO1821, a protein located in ZM4's nitrogen-fixing island, was upregulated along with other nitrogen-fixing associated proteins (Figure 1B). This protein was predicted as a SIR2 family protein that likely functions as a deacetylase. Since acetylation widely occurs in bacteria, we next conducted acetylome analyses of ZM4 under the nitrogen-fixing condition.

For acetylome analyses, the tryptic peptides obtained from each of the replicates were enriched for acetylated lysine residues and were then subjected to a high-resolution LC-MS/MS analysis on a timsTOF Pro instrument. The resulting identified peptides were quantitatively compared by label-free quantitation. We identified a total of 2,935 acetylation sites that were confidently localized ( $\geq 75\%$  probability) across the samples (Supplementary Table S2). Of these, 1,591 sites had quantification information in at least two replicates of the comparison, and these sites were sorted into 663 unique acetylated proteins (Supplementary Table S3). In each protein, the number of



**FIGURE 2 |** GO terms enrichment analysis of differentially expressed proteins in *Z. mobilis* under N<sub>2</sub>-fixing condition. **(A)** GO terms enrichment analysis of upregulated proteins under the N<sub>2</sub>-fixing condition. **(B)** GO terms enrichment analysis of downregulated proteins under the N<sub>2</sub>-fixing condition.



**FIGURE 3 |** KEGG pathways enrichment analysis of differentially expressed proteins in *Z. mobilis* under N<sub>2</sub>-fixing condition. **(A)** KEGG pathways enrichment analysis of upregulated proteins under the N<sub>2</sub>-fixing condition. **(B)** KEGG pathways enrichment analysis of downregulated proteins under the N<sub>2</sub>-fixing condition.

acetylated sites varied from 1 to 16. Acetylome of *Z. mobilis* encompassed 37% of all of its proteins.

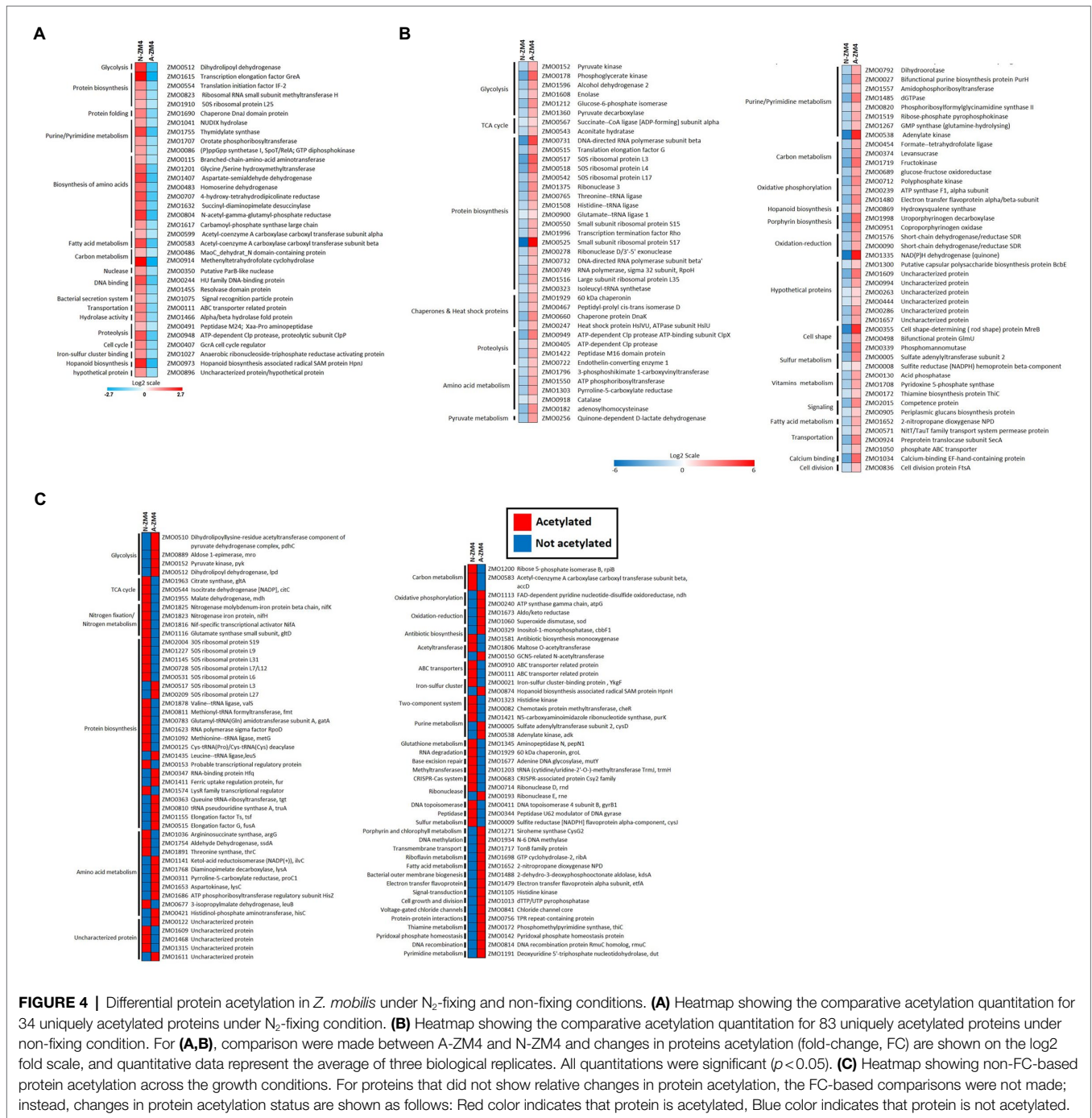
## Differential Protein Acetylation

To get insights into differential protein acetylation across the N<sub>2</sub>-fixing and non-fixing conditions, we compared the protein acetylation data among the different growth conditions. Our results showed that 197 proteins were uniquely acetylated at 229 sites across the growth conditions (**Supplementary Table S4**). Among these, 117 proteins (at 137 sites) showed relative changes in protein acetylation, and we made fold-change (FC) comparisons for these proteins (**Figures 4A,B**). In FC-based comparison, 34 proteins were uniquely acetylated (at 39 sites) under the N<sub>2</sub>-fixing condition (**Figure 4A**), while 83 proteins were uniquely acetylated (at 98 sites) under the non-fixing condition (**Figure 4B**). For proteins that did not show relative changes in protein-acetylation (i.e., significant acetylation was observed under the one growth condition only), the FC-based comparisons were not made, and protein-acetylation was simply considered as present or absent for such proteins. A total of 92 sites showed non-FC-based pattern of protein-acetylation across the growth conditions (**Figure 4C**). The master regulator of the nitrogen-fixing island (NifA), the nitrogenase

subunits (NifH and NifK), and ammonia assimilatory protein glutamate synthase were found acetylated under the nitrogen-fixing condition (**Figure 4C**).

## Functional Enrichment of Differentially Acetylated Proteins

To further explore which GO terms and pathways are significantly represented by these differentially acetylated proteins, we executed enrichment analysis of GO terms and KEGG pathways associated with differentially acetylated proteins. This allowed us to further narrow down our focus around the areas that were significantly affected by protein acetylation across the experimental conditions. Under the N<sub>2</sub>-fixing condition, the significantly enriched GO terms were nitrogen compound metabolic process, gene expression, protein metabolism, amide metabolism, carboxylic acid metabolism, and amino acid metabolism (**Figure 5A**). While the significantly enriched GO terms under the non-fixing condition were nitrogen compound metabolism, nucleobase-containing compounds metabolism, translation, ATP metabolic process, amide biosynthetic process, RNA metabolic process, organophosphate biosynthetic process, translation, and phosphorylation (**Figure 5B**). Whereas, the pathways such as microbial metabolism in diverse environments,



**FIGURE 4 |** Differential protein acetylation in *Z. mobilis* under N<sub>2</sub>-fixing and non-fixing conditions. **(A)** Heatmap showing the comparative acetylation quantitation for 34 uniquely acetylated proteins under N<sub>2</sub>-fixing condition. **(B)** Heatmap showing the comparative acetylation quantitation for 83 uniquely acetylated proteins under non-fixing condition. For **(A,B)**, comparison were made between A-ZM4 and N-ZM4 and changes in proteins acetylation (fold-change, FC) are shown on the log<sub>2</sub> fold scale, and quantitative data represent the average of three biological replicates. All quantitations were significant (*p* < 0.05). **(C)** Heatmap showing non-FC-based protein acetylation across the growth conditions. For proteins that did not show relative changes in protein acetylation, the FC-based comparisons were not made; instead, changes in protein acetylation status are shown as follows: Red color indicates that protein is acetylated, Blue color indicates that protein is not acetylated.

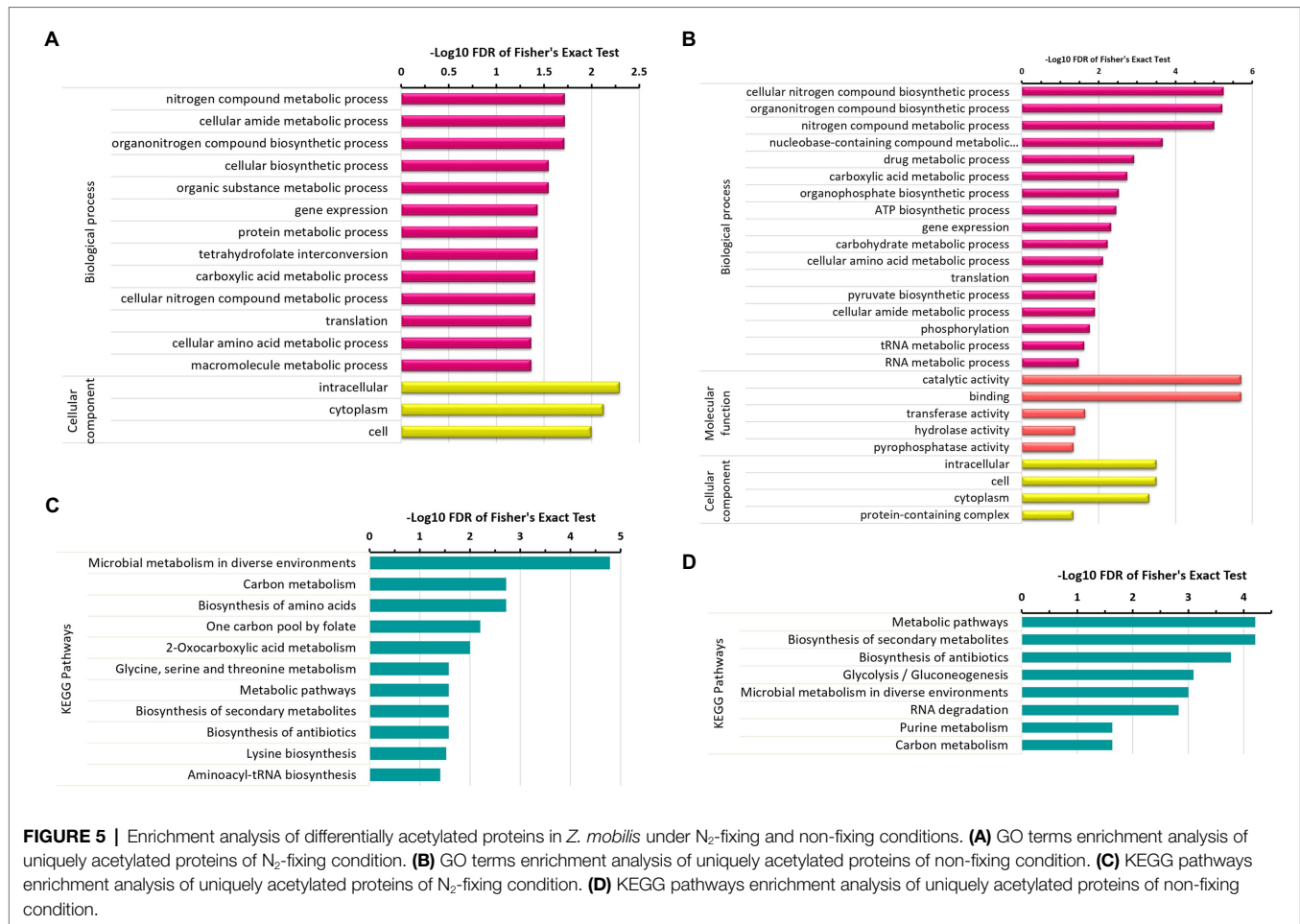
carbon metabolism, amino acid metabolism, one carbon pool by folate, and biosynthesis of secondary metabolites were significantly enriched under the N<sub>2</sub>-fixing condition (Figure 5C). While the significantly enriched pathways under the non-fixing condition were biosynthesis of antibiotics, glycolysis/gluconeogenesis, RNA degradation, and purine metabolism (Figure 5D).

To further explore the connections among all differentially acetylated proteins, we reconstructed protein interaction networks. Under the N<sub>2</sub>-fixing condition, there were 82 nodes

with 136 predicted physical interactions (Figure 6A). Whereas, differentially acetylated proteins under the non-fixing condition formed 126 nodes that were connected by 434 predicted physical interactions (Figure 6B).

### Changes in Regulation and Acetylation of Proteins Associated With Central Carbon Metabolism and Nitrogen Fixation Pathway

The pathway microbial metabolism in diverse environments includes nitrogen fixation and central carbon metabolism, and

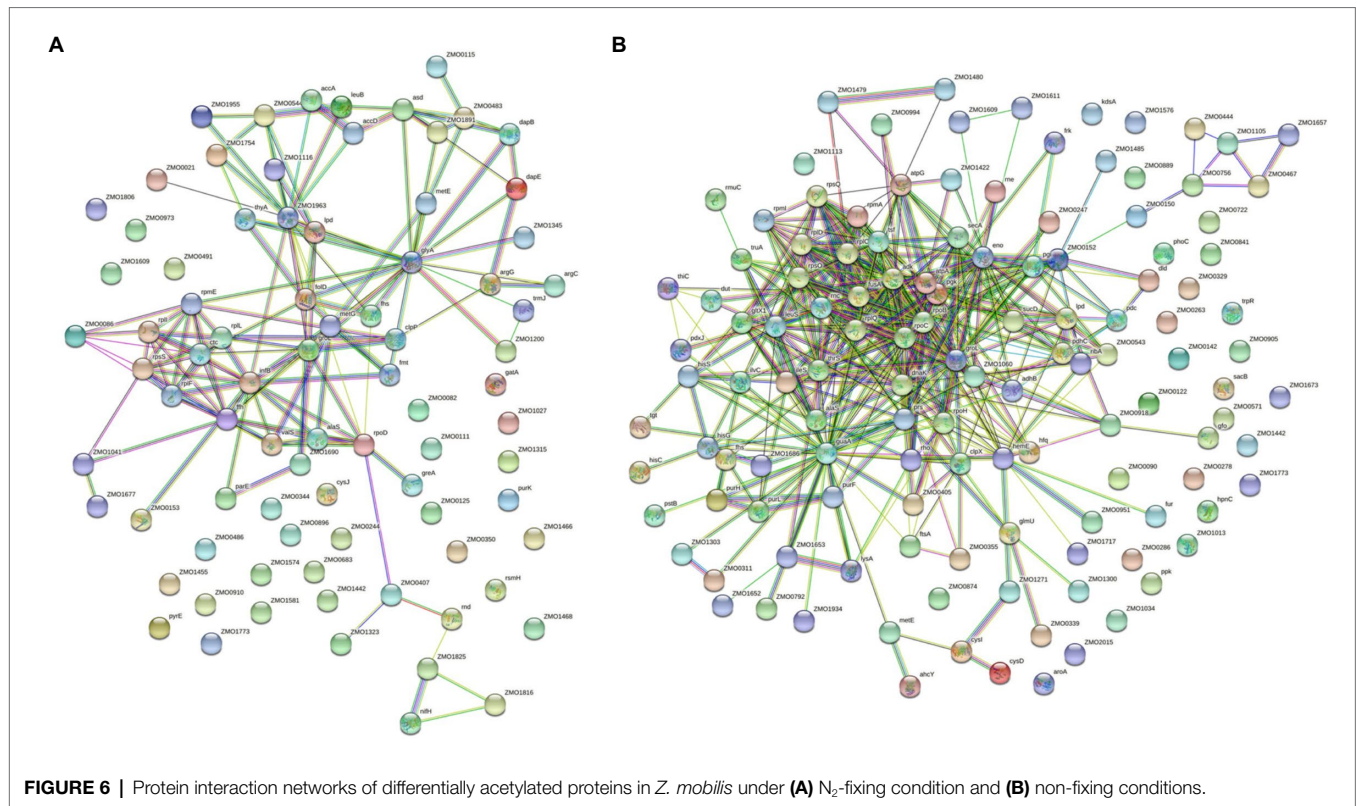


it was significantly enriched under nitrogen-fixing conditions. We, therefore, analyzed the nitrogen fixation in connection to central carbon metabolism for changes in regulation and acetylation of associated proteins (Figure 7). Further, to get a thorough understanding of nitrogen fixation in *Z. mobilis* at PTMs level, we also compared our results for protein phosphorylation under the N<sub>2</sub>-fixing condition with that of the previous study (Tatli et al., 2019) in *Z. mobilis*. The central carbon metabolism in *Z. mobilis* includes Entner–Doudoroff (ED) pathway, glycolysis, and incomplete TCA cycle (Figure 7A), which is linked to nitrogen fixation (Figures 7B,C; Bueno Batista and Dixon, 2019; Tatli et al., 2019) via ammonia assimilation pathway or GS/GOGAT cycle (Figure 7D; Bueno Batista and Dixon, 2019). Sugars are generally catabolized by *Z. mobilis* via the ED pathway into pyruvate and glyceraldehyde-3-phosphate (GA3P). The GA3P is ultimately converted into pyruvate by glycolysis. The *Z. mobilis* can convert >95% of the pyruvate into ethanol using pyruvate decarboxylase (Pdc) and alcohol dehydrogenases (AdhA and AdhB). We found that almost all of the proteins in ED pathway and glycolysis were not differentially expressed under the nitrogen-fixing condition. However, proteins such as phosphoglycerate kinase (Pkg), enolase (Eno), and pyruvate kinase (Pyk) were acetylated under the non-fixing condition. Pkg was acetylated at two positions (K244

and K271), Eno at one (K402), and Pyk at three (K51, K182, and K386). The protein malate dehydrogenase (Mdh) was acetylated under the N<sub>2</sub>-fixing condition at one site (K380). Whereas, a phosphoglycerate mutase (GpmA) was acetylated under both nitrogen-fixing (K126) and non-fixing (K104) conditions. In the ethanol-producing pathway, AdhA was upregulated and the rest of the proteins were normally regulated under the nitrogen-fixing condition. However, acetylation was also observed under the non-fixing condition in proteins Pdc (K219) and AdhB (K160) of the ethanol fermentation pathway. Recently, protein phosphorylation was observed (Tatli et al., 2019) in nearly all the glycolytic enzymes of central carbon metabolism in *Z. mobilis*. Among those, the KHG/KDPG aldolase (Eda), Eno, GpmA, and Pdc were phosphorylated under nitrogen-fixing condition, while AdhB was phosphorylated under the non-fixing condition.

In TCA cycle, isocitrate dehydrogenase (CitC, ZMO0544) was upregulated, fumarate hydratase (ZMO1307) was downregulated, and the rest of the proteins were normally regulated under the nitrogen-fixing condition. Whereas, two proteins of the TCA cycle were found acetylated under the non-fixing condition, that is, aconitate hydratase (AcnA, ZMO0543) at K663, and succinate–CoA ligase (SucD, ZMO0567) at K293. Also, CitC and GltA (ZMO1963) from TCA cycle





were found acetylated at site K57 and K311 under the N<sub>2</sub>-fixing condition, respectively. In comparison, for phosphorylated-based PTM in *Z. mobilis*, only Mdh was reported phosphorylated among the TCA cycle proteins in the previous study (Tatli et al., 2019) under the nitrogen-fixing condition.

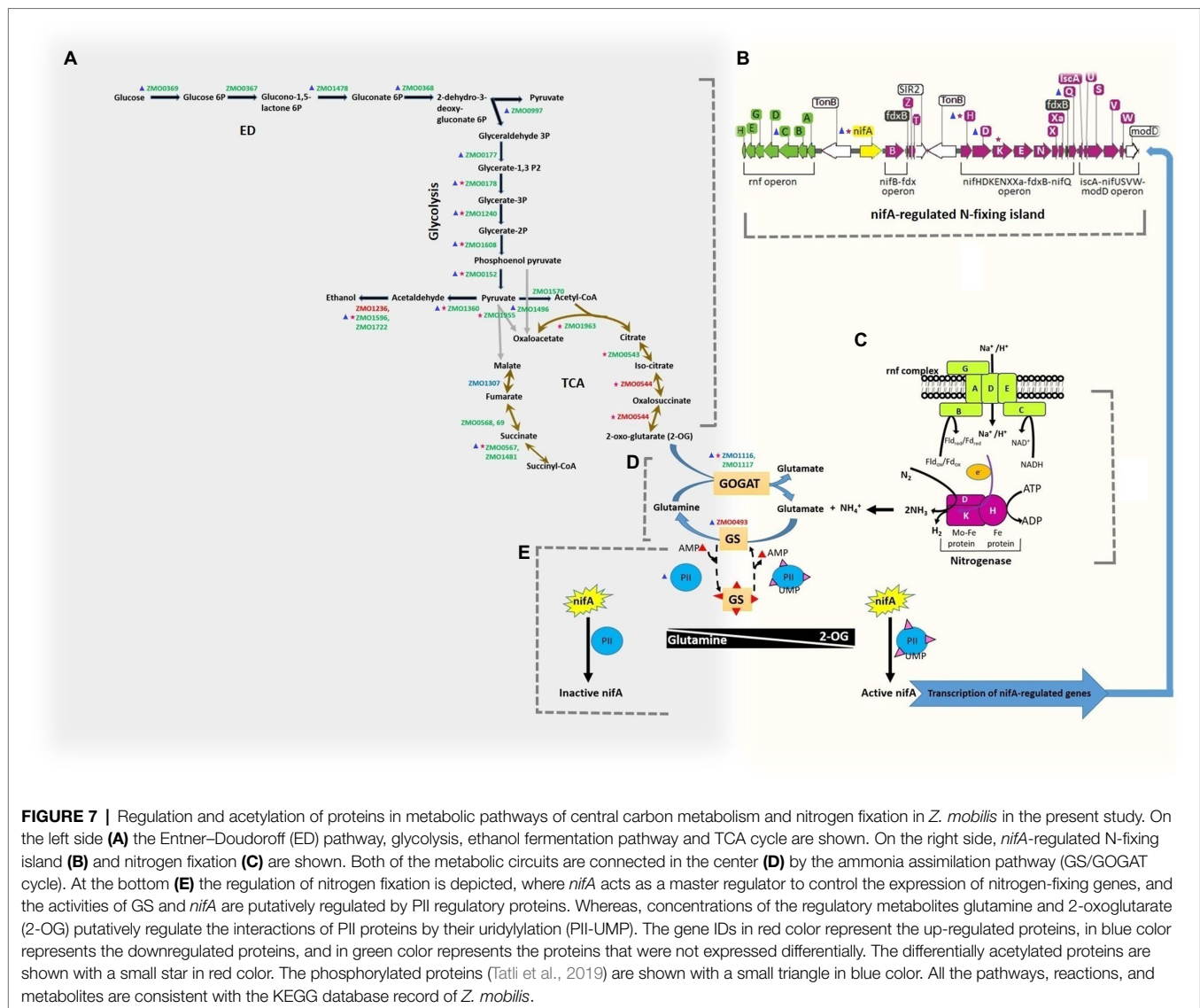
The ATP-dependent reduction of dinitrogen (N<sub>2</sub>) to bio-available ammonia (NH<sub>3</sub>) in *Z. mobilis* is carried out by molybdenum (Mo)-dependent nitrogenase complex encoded by the *nifH*, *nifD*, and *nifK* genes of N-fixing island (Figures 7B,C). We found that various proteins from the N<sub>2</sub>-fixing island such as NifA, NifH, NifD, NifK, NifX, ModD, RnfC, RnfD, and RnfH were highly upregulated under the nitrogen-fixing condition. However, only three N-fixing proteins, including NifA, NifH, and NifK, were significantly acetylated at K45, K12, and K395, respectively, under the N<sub>2</sub>-fixing condition. These acetylated lysine residues were found conserved by BLAST-p search in various diazotrophs species. To see if the acetylated residues of nitrogenase complex lie on the surfaces where the two nitrogenase proteins meet, we constructed hypothetical 3D structures of NifH and NifK of *Z. mobilis* and visualized the positions of acetylated K-residues, as shown in **Supplementary Figures S1, S2**. We also visualized the corresponding acetylated K-residues (for NifH and NifK) of *Z. mobilis* in the solved nitrogenase crystal structure of *Azotobacter vinelandii* (**Supplementary Figure S3**). The acetylated lysine of NifH (K12) was located inside its crystal structure near [4Fe-4S] clusters. However, it was not the point at which NifH met NifD or NifK. The acetylated lysine of NifK (K395) was located on the surface, and its corresponding K-residue

in the nitrogenase structure was not found interacting with the other two proteins of the nitrogenase complex. Whereas, phosphorylation in *Z. mobilis* has been reported previously (Tatli et al., 2019) in proteins of N-fixing island under the nitrogen-fixing (NifH, NifD, NifQ, and RnfC) and non-fixing (NifA) conditions.

The nitrogen fixation and central carbon metabolism are connected by the ammonia assimilation pathway or GS/GOGAT cycle (**Figure 7D**), where fixed nitrogen is ultimately assimilated into central metabolism. The nitrogen regulatory protein PII (ZMO0492) putatively regulates ammonia assimilation and nitrogen fixation (**Figure 7E**). We observed that proteins GS and PII were upregulated. Whereas, ammonia assimilatory protein glutamate synthase (GltD, ZMO1116) was found acetylated under the N<sub>2</sub>-fixing condition at a single site (K27). Protein phosphorylation has also been reported under the nitrogen-fixing condition in GS, GOGAT, and PII proteins in the previous study (Tatli et al., 2019).

## DISCUSSION

In this study, we conducted high throughput analyses of proteome and protein acetylation in *Z. mobilis* under N<sub>2</sub>-fixing and non-fixing conditions and established its first acetylome. We identified a total of 1,449 proteins, 242 of which were differentially and significantly regulated. Whereas, 229 sites of 197 proteins were uniquely acetylated across the growth conditions.



The genome-wide proteome analyses revealed that upregulated proteins were mostly involved in processes such as nitrogen fixation, motility, chemotaxis, flagellar assembly, energy production, transportation, and oxidation–reduction (Figures 2, 3). Since nitrogen fixation is a costly process that requires a supply of ATP and reducing power (Yan et al., 2010; Varley et al., 2015; Suyal et al., 2017), it is reasonable that the proteins involved in energy production or oxidation–reduction were upregulated under nitrogen fixation. Flagellar motility and chemotaxis have been demonstrated to be involved in energy taxis, a behavior that bacteria seek optimal conditions for metabolic activity, under the condition of nitrogen fixation (Xie et al., 2010; Ganusova et al., 2021). We hypothesize that the similar chemosensing and signaling machinery for nitrogen fixation exists in ZM4. Overall, our results show that *Z. mobilis* has the same molecular responses to nitrogen fixation as other diazotrophic organisms. A notable finding was that the ethanol-producing

protein alcohol dehydrogenase was upregulated, which is consistent with prior studies showing that *Z. mobilis* can fix nitrogen efficiently without affecting ethanol production (Kremer et al., 2015; Palamae et al., 2020; Alencar et al., 2021).

We established the first-ever acetylome of *Z. mobilis* under nitrogen fixation. As shown in Figure 7, the acetylated proteins were significantly enriched in pathways such as nitrogen fixation, central carbon metabolism, and ammonia assimilation (GS/GOGAT cycle). Acetylation is a complex mechanism that can both upregulate and/or downregulate the activities of targeted proteins (Bontemps-Gallo et al., 2018). Acetylation in bacteria has been shown to modulate cellular metabolic processes or pathways ranging from transcription and translation to central metabolism (Hu et al., 2010; Wang et al., 2010; Bernal et al., 2014; Wolfe, 2016; Carabetta and Cristea, 2017; Nakayasu et al., 2017). Protein acetylation appears to be a prevalent and conserved mechanism in bacteria (Gardner and Escalante-Semerena, 2008;

Wang et al., 2010; Crosby et al., 2012; Hayden et al., 2013; Tucker and Escalante-Semerena, 2013; Sang et al., 2016; Nakayasu et al., 2017; Bontemps-Gallo et al., 2018). Our results are consistent with these studies. Moreover, the nitrogen fixation and central carbon metabolism are linked by GS/GOGAT cycle (Bueno Batista and Dixon, 2019; Tatli et al., 2019), where fixed nitrogen is ultimately assimilated into central metabolism. Thus, the acetylome of *Z. mobilis* under the nitrogen-fixing condition provides a further extension that acetylation in bacteria can regulate both carbon and nitrogen metabolism. Furthermore, we also detected acetylation in the nitrogenase complex and the master regulator NifA under the nitrogen fixation. The acetylated lysine residues of these proteins were found to be conserved among the diazotrophs. Therefore, it is reasonable to speculate that protein acetylation plays a key role in regulating nitrogen metabolism and nitrogen fixation in *Z. mobilis*.

Therefore, an intriguing question arises: what enzymes can regulate the protein acetylation in *Z. mobilis*? By searching the ZM4 genome, three hypothetical SIR2 family proteins (Yang et al., 2018), a main lysine deacetylase family, were found. One of them is located on chromosome, and the other two are encoded by a native plasmid pZM32. Interestingly, the hypothetical chromosomal SIR2 encoding gene (*ZMO1821*) is located in the nitrogen-fixing island between *nifA* and nitrogenase genes (*nifH*, *nifD*, and *nifK*). Our proteomic analysis showed that the *ZMO1821* protein, NifA, and nitrogenase genes were upregulated under the nitrogen-fixing condition (**Figure 1B** and **Supplementary Table S1**). This result motivated us to investigate a lysine acetylome profiling in ZM4. Moreover, we find that the acetylation status of several putative acetyltransferases, such as *ZMO0150*, *ZMO1806*, and *ZMO1400*, was changed under nitrogen fixation. These acetyltransferases are most likely involved in reversible acetylation reactions with SIR2 deacetylases. Combined with the phosphorylation proteome profiling, NifA, NifH, and NifK were both phosphorylated and acetylated under the nitrogen-fixing condition (**Figure 7**). Thus, we assume there is a regulation interaction between the both PTMs. In other prokaryotes, the SIR2 family protein CobB and its orthologs have been extensively studied. They perform diverse functions but are not seem to be essential for life because their gene's deletion did not result in cellular death (Starai et al., 2002; Li et al., 2010, 2020; Liu et al., 2018a). However, we rarely know about deacetylases in diazotrophic organisms to date. We intend to investigate their roles in regulating nitrogen fixation and other metabolic pathways in the future.

## CONCLUSION

In summary, our work demonstrates the overall protein expression patterns and their PTM (acetylation)-based regulation under nitrogen fixation in *Z. mobilis*. Using a mass spectrometry-based proteomics approach, we performed a genome-wide analysis of proteome and protein acetylation in *Z. mobilis* and established its first acetylome under the N<sub>2</sub>-fixing and non-fixing

conditions. The findings that we have presented here provide new knowledge of specific proteins and their associated cellular processes and pathways that may be regulated by protein acetylation in *Z. mobilis*. Functional validation of these acetylated proteins should be the focus of future research. The results of this study will serve as a baseline for further research in the area.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ebi.ac.uk/pride/>, PXD027418.

## AUTHOR CONTRIBUTIONS

BW and MH designed the study. YH, MC, and BW prepared samples for analysis. AN, XG, BW, and SK analyzed the data. AN and SK wrote the manuscript. BW and MH critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2021.740555/full#supplementary-material>

**Supplementary Figure S1** | Hypothetical protein structure of NifH of *Zymomonas mobilis*. (A) Surface model. (B) Ribbon representation. (C) The acetylated residue K12 is shown in enlarged.

**Supplementary Figure S2** | Hypothetical protein structure of NifK of *Zymomonas mobilis*. (A) Surface model. (B) Ribbon representation. (C) The acetylated residue K395 is shown in enlarged.

**Supplementary Figure S3** | Ribbon representation of X-ray diffraction-based crystal structure of nitrogenase complex of *Azotobacter vinelandii* (PDB accession 4WZB). Corresponding K-residues for nitrogenase proteins of *Zymomonas mobilis* are highlighted.



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