Phosphoketolase overexpression increases biomass and lipid yield from methane in an obligate methanotrophic biocatalyst.

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Abstract

Microbial conversion of methane to high-value bio-based chemicals and materials offers a path to mitigate GHG emissions and valorize this abundant-yet - underutilized carbon source. In addition to fermentation optimization strategies, rational methanotrophic bacterial strain engineering offers a means to reach industrially relevant titers, carbon yields, and productivities of target products. The phosphoketolase pathway functions in heterofermentative bacteria where carbon flux through two sugar catabolic pathways to mixed acids (lactic acid and acetic acid) increases cellular ATP production. Importantly, this pathway also serves as an alternative route to produce acetyl-CoA that bypasses the CO₂ lost through pyruvate decarboxylation in the Embden-Meyerhof-Parnas pathway. Thus, the phosphoketolase pathway can be leveraged for carbon efficient biocatalysis to acetyl-CoA-derived intermediates and products. Here, we show that the industrially promising methane biocatalyst, *Methylobacterium buryatense*, encodes two phosphoketolase isoforms that are expressed in methanol- and methane-grown cells. Overexpression of the PktB isoform led to a 2-fold increase in intracellular acetyl-CoA concentration, and a 2.6-fold yield enhancement from methane to microbial biomass and lipids compared to wild-type, increasing the potential for methanotroph lipid-based fuel production. Off-gas analysis and metabolite profiling indicated that global metabolic rearrangements, including significant increases in post-translational protein acetylation and gene expression of the tetrahydromethanopterin-linked pathway, along with decreases in several excreted products, coincided with the superior biomass and lipid yield observed in the engineered strain. Further, these data suggest that phosphoketolase may play a key regulatory role in methanotrophic bacterial metabolism. Given that acetyl-CoA is a key intermediate in several biosynthetic pathways, phosphoketolase overexpression offers a viable strategy to enhance the economics of an array of biological methane conversion processes.

Keywords: methane biocatalysis, methanotroph, phosphoketolase, biogas, greenhouse gas mitigation

1. Introduction

Methane (CH_4) is the second most abundant greenhouse gas and has significantly more global warming potential compared to the most abundant greenhouse gas, carbon dioxide. CH_4 emissions are largely produced from anthropogenic sources, being a primary component of natural gas and anaerobic digestion-derived biogas. In addition, CH_4 discharged from ruminant livestock and landfills further contribute to global CH_4 emissions^{1,2}. Rising atmospheric CH_4 levels necessitate the development of novel technologies to mitigate GHG emissions. Methane biocatalysis using natural CH_4 -consuming bacteria (methanotrophs) offers a scalable and selective route to utilize an overabundant, often wasted carbon source, while also mitigating greenhouse gas emissions^{3,4}.

Methanotrophs use CH_4 as their sole carbon and energy source, and play a key role in the biogeochemical cycle by serving as a CH_4 sink^{5,6}. These organisms hold great promise for the capture and conversion of methane from stranded natural gas, anaerobic-digestion-derived biogas, and other anthropogenic CH_4 sources, but are currently limited by poor product titers, yields, and productivities associated with inefficient microbial methane utilization and inefficient carbon conversion^{7,8,4,9}. Recent development of genetic tools for use in industrially relevant methanotrophs has facilitated rational strain engineering of these organisms for the conversion of CH_4 to fuels and chemicals^{8,10-12}. Leveraging these tools to develop a biocatalyst with increased methane conversion efficiency would dramatically enhance process economics, allowing CH_4 bioconversion to be competitive with conventional sugar upgrading processes.

Phosphoketolases (PKTs) have been used for engineering carbon-efficient processes from sugars due to their intrinsic ability to bypass glycolytic pyruvate decarboxylation¹³. These enzymes catalyze the irreversible cleavage of xylulose-5-phosphate and/or fructose-6-phosphate to acetyl-phosphate (acetyl-P) and glyceraldehyde-3-phosphate or erythrose-4-phosphate, respectively^{14,15}. The acetyl-P produced by PKT is readily converted to acetyl-CoA with complete carbon conservation (Figure 1). Although PKT engineering strategies offer the advantage of complete carbon conversion, this occurs at the expense of reducing equivalents and ATP generated in downstream glycolytic biochemical conversions that are bypassed by the PKT pathway. PKTs have been characterized in obligate and heterofermentative bacteria and some species of fungi where they enable an alternative pathway for sugar-phosphate degradation. Interestingly, PKT orthologs have been observed in the genomes of an array of C1-utilizing microbes¹⁶, and it has been postulated that the PKT pathway may serve an important metabolic role in methanotrophic, methylotrophic, and autotrophic bacteria^{16,17}.

PKT pathway engineering has been successfully used to increase yield of an array of acetyl-CoA-derived products in diverse microbial biocatalysts, including

*Corynebacterium glutamicum*¹⁸, *Saccharomyces cerevisiae*^{13,19-23}, *Aspergillus nidulans*²⁴ and *Escherichia coli*²⁵ by providing a larger pool of acetyl-CoA precursor; however, they have yet to be leveraged to improve CH₄ biocatalysis. Herein, we overexpressed native methanotroph PKTs as a strategy to increase methane conversion efficiency to biomass in an obligate methanotrophic bacterium. Overexpression of the PktB isoform in the gammaproteobacterial methanotroph, *Methylobacillus buryatense*, resulted in global metabolic alterations, ultimately increasing the biomass and lipid yield from CH₄ by 2.6- and 2-fold, respectively, compared to wild-type in the engineered strain. These results represent the most significant yield improvement via PKT pathway engineering in any organism reported to date, and also support a novel function for PKT in methanotroph post-translational regulation.

Figure 1. Carbon flux through the PKT pathway bypasses pyruvate decarboxylation. CH₄ is assimilated to fructose-6-phosphate (F6P) in gammaproteobacterial methanotrophs, which enters the Embden-Meyerhof-Parnas (EMP) pathway. F6P and/or xylulose-5-phosphate (X5P) can be converted to intermediates of the ribulose phosphate (RuMP) pathway and acetyl-P (AcP) by phosphoketolase (PKT, red arrows). AcP is readily converted to acetyl-CoA (AcCoA) by the phosphotrasacetylase (Pta), bypassing the CO₂ lost in the decarboxylation of pyruvate to AcCoA. FPkt, F6P phosphoketolase; XPkt, X5P phosphoketolase; G3P, glyceraldehyde-3-phosphate; E4P, erythrose-4-phosphate; Ack, acetate kinase; Acs, acetyl-CoA synthase. Adapted from¹³

2. Materials and methods

2.1 Microbial strains and cultivation

Methylobacillus buryatense 5GB1S¹⁰ and engineered strains (Table S1) were routinely cultivated in NMS2 medium at 30°C with orbital shaking at 225 rpm as

previously described⁸. Strains were grown in sealed 150 mL glass serum bottles (Kimble Chase, Vineland, NJ) with 20% (v/v) CH₄ in air, or 500 mL baffled flasks supplemented with 1% CH₃OH (v/v). *Escherichia coli* Zymo 5a (Zymo Research, Irvine, CA) was used for cloning and plasmid propagation. Polymerase chain reactions were performed using Q5 High-Fidelity Polymerase from New England Biolabs and primers (Table S2) purchased from Integrated DNA Technologies (Coralville, IO).

2.2 Plasmid construction

Plasmids for constitutive phosphoketolase expression were constructed by ligating the methanol dehydrogenase promoter region (P_{mxa}), *pktA* (METBUDDRAFT_0184) or *pktB* (METBUDRAFT_1551), and the *Escherichia coli* *rrnBT1T2* terminator into the pAWP78 IncP-based vector¹⁰ to generate pPKTA and pPKTB using 2X Gibson Assembly Mix from New England Biolabs (Ipswich, MA) following the manufacturers protocol. The pPKTA and pPKTB vectors were confirmed by sequence analysis (Genewiz, South Plainfield, NJ). These vectors were transformed into *M. buryatense* via biparental mating using *E. coli* S17-1 as previously described⁸. Positive transformants selected on NMS2 agar containing 50 µg/mL of kanamycin were confirmed using primers oCAH042 and oCAH043 in polymerase chain reactions.

2.3 Real-time PCR

Total RNA was isolated from logarithmically growing cells (OD₆₀₀ = 1.0 using TriZol reagent (ThermoFisher) followed by removal of contaminating DNA by performing treatment with Turbo DNase (Life Technologies, Carlsbad, CA. cDNA was synthesized from 1 µg total RNA using iScript reverse transcriptase (Biorad, Hercules, CA) following the manufacturer's protocol. Real-time PCR was performed by using SYBR IQ master mix and a BioRad iCycler iQ Real-Time PCR System (BioRad). The primers used for the real-time RT-PCR are listed in Table S2. The cycle threshold (Ct) value for each gene was determined and normalized to the *rpoD* housekeeping sigma factor gene. Relative expression between two samples was calculated by using the $\Delta\Delta C_t$ method²⁴. Growth in CH₃OH or CH₄ as the carbon source did not result in any significant changes ($P > 0.05$) in *rpoD* mRNA expression.

2.4 SDS-PAGE

Cell-free extracts were prepared by sonication of logarithmically growing (20% CH₄) wild-type and engineered strains of *M. buryatense* in sample buffer (50 mM Tris pH 8.0 containing 100 mM NaCl). Protein concentrations in clarified extracts were determined with the Pierce 660nm Protein Assay Reagent (Life Technologies) using bovine serum albumin as a protein standard and normalized by the addition of sample buffer. Samples were mixed with 3X Red loading buffer (New England Biolabs, Ipswich, MA) containing 2 mM 2-mercaptoethanol. 5 µg total protein was loaded into an Any kD Mini-PROTEAN TGX Precast protein gel (Biorad) and electrophoresed at 150V. Proteins were visualized by Coomassie blue staining.

2.5 Phosphoketolase enzyme assay

Phosphoketolase activity in whole-cell lysates prepared from logarithmically growing wild-type (20% CH₄), pPKTA, and pPKTB strains was measured using a hydroxamate assay that detects acetyl phosphate^{26,27}. Briefly, cells (OD₆₀₀ = 1.0) were pelleted, resuspended in 50 mM potassium phosphate buffer (pH 6.5) and disrupted by sonication on ice. 100 µL reactions in reaction buffer [30 mM potassium phosphate (pH 6.5), L-cysteine hydrochloride (2 mM), sodium fluoride (20 mM), sodium iodoacetate (10 mM), thiamine pyrophosphate (1 mM), and fructose 6-phosphate (30 mM) (Sigma)] were initiated by the addition of 25 µL (10 µg total protein) of whole-cell extracts. Reactions were incubated at 37°C for 1 h. After incubation, 50 µL of 2 M hydroxylamine hydrochloride (pH 7.0) was added to the reaction and incubated at room temperature for 10 min. Reactions were then terminated by adding 300 µL of a 1:1 mixture of 2.5% ferric chloride in 2M hydrochloric acid and 10% trichloroacetic acid. A color change due to ferric-hydroxamate formation was measured at A_{505 nm}, and the concentration of acetyl-P formed during the initial reaction was calculated by regression analysis compared to known standards.

2.6 CH₄ fermentations

Seed cultures were started from plate biomass and grown in 1 L flasks (30°C, 500rpm) containing NMS2 medium supplemented with 2X KNO₃ (20 mM) with a continuous gas flow (20% CH₄ in air). Batch CH₄ fermentations were performed in a 0.5 L Biostat-Q plus bioreactor (Sartorius, Gottingen, Germany) containing NMS2 medium supplemented with 8X KNO₃ (80 mM), 2X phosphate buffer (4.6 mM), and 4X trace element solution to support high cell growth at 30°C as previously described⁸. Seed cultures were diluted to a final OD₆₀₀ = 0.5-1.0. Mixing was achieved by using a bottom marine impeller at 500 rpm. A continuous flow rate of 300 ccm 20% (v/v) methane in air was maintained. Culture samples were taken at the indicated times and growth was determined as OD₆₀₀ or dry cell weight after freeze-drying. The percent CH₄ and CO₂ in the off-gas was measured every 10 min for the duration of bioreactor fermentations by using BlueSens gas detectors (Herten, Germany). CH₄ consumption was determined by calculating the difference between off-gas detected before and after inoculation. % CH₄ consumption was converted to weight based on CH₄ density (0.000656 g/mL) and the flow rate (60 mL CH₄/min). The percent excreted CO₂ in the off-gas was converted to weight based on CO₂ density (0.00196 g/mL) and the gas flow rate (300 mL/min).

2.7 HPLC analysis

HPLC was used to detect formate, acetate, and methanol in culture supernatants. At indicated intervals, growth was measured spectrophotometrically and a 1mL sample was taken for HPLC analysis. The culture supernatant was filtered using a

0.2 μ m syringe filter and then a 0.1mL injection was separated using a model 1260 HPLC (Agilent, Santa Clara, CA) and a cation H HPx-87H column (Bio-Rad). A 0.6mL/min flow rate at 55°C with 0.01N sulfuric acid as the mobile phase was used. DAD detection was measured at 220nm and referenced at 360nm, and metabolite concentrations were calculated by regression analysis compared to known standards.

2.8 Intracellular Acetyl-CoA Quantification

Intracellular concentrations of acetyl-CoA were measured by using a Acetyl-Coenzyme A Assay Kit (Sigma) following the manufacturers protocol. Briefly, 10mL of wild-type and pPKTB cultures grown with a continuous gas flow (20% CH₄ in air) was pelleted and immediately frozen at -80°C. Cell pellets were thawed on ice and resuspended in 500 μ L assay buffer followed by 3 x 30s rounds of sonication on ice. Clarified lysates were deproteinized by using Amicon Ultra 0.5 mL 3K devices (ThermoFisher). 50 μ L deproteinized sample was added per well and pmol acetyl-CoA per well was calculated by regression analysis based on known standards. The sample data were normalized to dry cell weight by converting culture densities to dry cell weight based on culture volume and OD₆₀₀ using the equation $DCW\ (g/L) = 0.3479 \cdot OD_{600} + 2.702$ developed by comparing culture density to DCW in bioreactor fermentations.

2.9 FAME analysis

Whole biomass lipid content was measured by gas chromatography as fatty acid methyl esters (FAMES) after transesterification of total chloroform:methanol (2:1, v/v)-extracted lipid, as described previously⁸. Detected FAME species were identified via comparison to known standards.

2.10 Metabolite profiling

Intracellular and excreted metabolites were analyzed by Metabolon, Inc. (Durham, NC) from cultures grown in 1 L flasks (30°C, 500rpm) with a continuous gas flow (20% CH₄ in air). At OD₆₀₀ = 1.0, cultures were pelleted at 4300 rpm for 5 min at 4°C. Culture supernatant and pelleted biomass were then transferred to 2.0 mL screw-top vials and flash frozen in liquid nitrogen. Sample analyses were conducted as described previously²⁸. In brief, samples were extracted and split into equal parts for analysis on the LC/MS/MS, and Polar LC platforms. Ions were matched to an in-house library of standards for metabolite identification and for metabolite quantitation by peak area integration. Welch's two-sample *t*-tests and Principal Component Analysis (PCA) were used to analyze the data. For all analyses, following normalization to protein measured by Bradford, missing values, if any, were imputed with the observed minimum for that particular compound. The statistical analyses were performed on natural log-transformed data and were considered significant if $p < 0.05$.

3. Results and Discussion

3.1. *Methylobacterium buryatense* expresses two phosphoketolase isoforms.

PKT orthologs have been observed in the genomes of an array of methanotrophic and methylotrophic bacteria¹⁶; however, their role in CH₄ metabolism remains elusive. *M. buryatense* encodes two putative PKT isoforms, PktA and PktB, that share 63.7% amino acid identity (Figure S1). To establish baseline expression of these isoforms, we evaluated transcription of the native PKTs and associated pathway genes during growth on either CH₄ or CH₃OH. Real-time PCR analysis indicated that *M. buryatense* expresses a complete phosphoketolase pathway, including *pktA*, *pktB*, acetate kinase *ack*, phosphotransacetylase *pta*, and the acetyl-CoA synthase *acs* genes, when grown with CH₃OH or CH₄ as the sole carbon source (Figure S2). Interestingly, the PKT pathway genes were highly upregulated (100-1000 fold) in CH₄-grown cells compared to CH₃OH-grown cells (Figure 2). These data provide support for an alternative route of carbon flux in CH₄-grown *M. buryatense*, which should be considered in future metabolic models and flux analyses. Additionally, the conserved nature of the PKT pathway in an array of methanotrophic, methylotrophic, and autotrophic organisms supports that this pathway likely plays an important role in C1 metabolism.

Figure 2. *Methylobacterium buryatense* expresses a complete phosphoketolase pathway.

Real-time PCR analysis of relative phosphoketolase pathway transcription comparing *M. buryatense* grown with CH₄ or CH₃OH as the carbon source. *pktA* (phosphoketase isoform A), *pktB* (phosphoketolase isoform B), *ack* (acetate kinase), *pta* (phosphotransacetylase), *acs* (acetyl-CoA synthase). Data represent the mean ± SD of 3 biological replicates.

3.2. Phosphoketolase pathway overexpression in *M. buryatense*.

The phosphoketolase pathway has been leveraged to enhance metabolic processes from sugars in several microbial biocatalysts¹³ and from CH₃OH in a synthetic *in vitro* pathway²⁹, but has yet to be explored in CH₄ bioconversion processes. Given that *M. buryatense* expresses all PKT pathway components, we examined overexpression of the PKT isoforms in order to achieve high carbon flux through the

pathway. PktA and PktB isoforms were overexpressed using an IncP-based vector, driven by the methanol dehydrogenase (P_{mxa}) promoter (Figure 3A), one of the most active promoters in *Methylomicrobium* species³⁰. These PKT expression vectors were conjugated into *M. buryatense* through biparental mating to generate the engineered pPKTA and pPKTB strains. Both strains exhibited significantly enhanced transcription and protein content of their respective PKT isoform during CH₄-dependent growth (Figure 3B and C), with an associated increase in PKT activity compared to wild-type *M. buryatense* (Figure 3D). Interestingly, although expression of the PKT isoforms was independently under the control of the P_{mxa} promoter, we observed higher transcription, protein expression, and associated PKT activity in the pPKTA strain, potentially due to differential post-transcriptional regulation between the two PKT isoforms. Growth kinetics were similar between wild-type and pPKTB, which both reached high cell density $OD_{600} \cong 20$ (Figure 4A). However, the pPKTA strain exhibited a growth defect after 48 h (Figure 4B), presumably due to either the high concentrations of acetate excreted into the medium or the increased PktA expression by this strain³¹. Given that PktA overexpression caused growth inhibition, we focused on the pPKTB strain for the remainder of our studies. The pPKTB strain displayed a 2-fold increase in intracellular acetyl-CoA concentration. This, coupled with the observed increase in acetate excretion (Figure 4B), indicates significant flux through the PKT pathway is occurring in this strain.

Figure 3. Overexpression of the native phosphoketolases in *M. buryatense*. A) Plasmids were constructed for high, constitutive expression of two PKT isoforms (PktA and PktB) under the control of the methanol dehydrogenase promoter (P_{mxa}). B) Real-time PCR analysis and C) SDS-PAGE analysis of whole-cell lysates confirming increased PKT transcription and protein expression by engineered strains, respectively. D) *In vitro* conversion of fructose-6-phosphate to acetyl-phosphate (AcP) by whole-cell lysates determined by using the hydroxymate method. Data in B and D represent the mean $\pm$ SD of 3 biological replicates.

Figure 4. Increased production of acetate and acetyl-CoA in phosphoketolase overexpressing strains. A) Growth and B) excreted acetate by wild-type (black circles) and engineered *M. buryatense* overexpressing PktA (light grey circles) or PktB (grey circles). C) Comparison of intracellular acetyl-coenzyme A (AcCoA) concentration in wild-type (black bar) and pPKTB (grey bar). Data represents the mean $\pm$ SD of 2-3 biological replicates. * $p < 0.05$.

3.3. Phosphoketolase overexpression increases biomass and lipid yield in bioreactor fermentations.

In stirred-tank bioreactors, pPKTB grew to higher cell density (10.3 g/L DCW) compared to wild-type (7.9 g/L DCW) after 96 h of growth (Figure 5A). Wild-type *M. buryatense* consumed CH₄ at a rate (0.063 g/h, Figure 5B and C) comparable to that previously reported for this organism⁷. Interestingly, despite superior biomass accumulation by pPKTB, its CH₄ assimilation rate was much lower (0.034 g/h, Figure 5B), significantly ($p < 0.01$) improving the biomass yield in this strain by 2.6-fold (Figure 5C; pPKTB, 1.1 g DCW/g CH₄ vs wild-type, 0.43 g DCW/g CH₄). A similar increase in pPKTB biomass yield was also observed in shake-flask experiments with 1% CH₃OH as the sole carbon source (Figure S3).

In order to accommodate the particulate methane monooxygenase responsible for methane activation, methanotrophs synthesize an elaborate intracellular membrane network that is a viable fuel molecule precursor¹². Given that we observed a 2-fold increase in lipid precursor acetyl-CoA levels, we evaluated the lipid composition of wild-type and pPKTB. Total lipid content was similar between the two strains (~9% based on DCW, Figure 5D), which primarily consisted of hexadecanoic and hexadecenoic acids (Table S3), resulting in ~2-fold improvement in lipid yield (Figure 5E).

Figure 5. Phosphoketolase overexpression increases methanotroph biomass and lipid yield from methane. A) Growth (DCW, dry cell weight) of wild-type (black circles/bars) and engineered (grey circles/bars) *M. buryatense* was measured over time in a 0.5 L gas bioreactor with continuous CH₄ feed (20% CH₄ in air). B) Total CH₄ consumed during 96 h of culture. C) Ratio of biomass DCW to total CH₄ consumption after 96 h. D) Total lipid content presented as fatty acid methyl esters (FAME) of wild-type (black bars) and engineered pPKTB (grey bars) *M. buryatense* dry cell weight (DCW). E) Ratio of FAME titer to total CH₄ consumption after 96 h. Data represent the mean $\pm$ SD of 2-3 biological replicates. * $p < 0.05$, ** $p < 0.01$.

3.4 Metabolite profiling reveals carbon flux alterations in the engineered methanotrophic strain.

Methanotrophs convert CH₄ to CH₃OH, which is further oxidized to formaldehyde (CH₂O). In gammaproteobacterial methanotrophs like *M. buryatense*, CH₂O is either assimilated into central carbon metabolism or further oxidized to formate (CHOOH) and CO₂ to produce NADH reducing equivalents via the tetrahydromethanopterin (H₄MPT)-linked dissimilatory pathway^{30,32}. A recent *in silico* metabolic flux model³³ suggests that complete carbon flux through the PKT pathway would increase CH₄ oxidation to CO₂ in order to supply NADH and compensate for the reducing

equivalents lost at the expense of increased flux through the redox-neutral PKT pathway (Figure 1). To test this hypothesis, we evaluated expression of the H₄MPT-linked formaldehyde activating enzyme *fae* and formate dehydrogenase *fdh* genes. As shown in Figure 6A, transcription of all *fae* and *fdh* genes was increased in the pPKTB strain compared to wild-type. Complete oxidation of CH₄ through the H₄MPT pathway is supported by a significant decrease in excreted formate by pPKTB (Figure 6B), which, instead of being excreted, is likely oxidized to CO₂ due to the increase in NAD-linked formate dehydrogenase expression in this strain. We next examined the potential impact of PKT overexpression on CO₂ evolution. We observed decreased CO₂ evolution from the pPKTB culture (Figure S4), but the ratio of evolved CO₂ to assimilated CH₄ was not significantly altered in pPKTB compared to wild-type in stirred-tank bioreactors (data not shown). In agreement with the metabolic flux model that PKT overexpression increases reducing equivalent production *via* the dissimilatory pathway³³, our data indicate that carbon typically excreted by wild-type cells is, instead, oxidized to CO₂ in an effort to produce NAD(P)H. However, in contrast to the model, PKT overexpression does not divert CH₄ flux through the dissimilatory pathway away from biomass. PKT overexpression resulting in increased biomass yield suggest this strain not only is more efficient at utilizing assimilated carbon, but may also possess increased energy efficiency mediated through unknown mechanisms.

Figure 6. PKT overexpression decreases flux through the tetrahydromethopterin (H₄MPT)-linked C1 transfer pathway. A) Real-time PCR analysis of H₄MPT pathway gene transcription in wild-type (black bars) and engineered (grey bars) strains. B) Excreted formate in culture supernatants detected by HPLC. *fae* (formaldehyde activating enzyme), *fdh* (formate dehydrogenase). Data represent the mean $\pm$ SD of at least 3 biological replicates. * $p < 0.05$.

To gain further insight into the increase in biomass yield, we examined intracellular and excreted metabolite profiles in wild-type and pPKTB *M. buryatense* actively growing on CH₄ (Figure 7). The pPKTB strain had several significantly increased protein acetylation markers, with a 46-fold increase in intracellular N-6-acetyllysine compared to wild-type cells (Table S3). These data further support that the pPKTB strain has significantly elevated levels of intracellular acetyl-CoA, which is the primary substrate for N-acetyl transferase-dependent acetylation. In addition, the CoA precursor, panthothenic acid was significantly increased in pPKTB, which has

been shown to increase intracellular acetyl-CoA levels when supplemented in *E. coli* growth medium³⁴. Other notable intracellular metabolites significantly altered in the pPKTB strain included the PKT cofactor thiamine diphosphate, sedoheptulose of the pentose phosphate pathway, and pyruvate. Further, nicotinic acid was 50-fold higher in pPKTB cells compared to wild-type, potentially due to increased de novo synthesis of pyridine nucleotides to overcome the limited reducing power in this strain. Compared to wild-type, several metabolites were found to be decreased in the culture supernatants of the pPKTB strain, including malate and succinate of the TCA cycle and xanthine/hypoxanthine of purine metabolism (Table S4). In contrast, both glutamate and glutamine were significantly higher in the pPKTB supernatant compared to wild-type, implicating a role for PKT in nitrogen assimilation.

Figure 7. Phosphoketolase overexpression results in alterations to both intracellular and excreted metabolites. Positively and negatively altered A) intracellular and B) excreted metabolites are presented as a volcano plot with p -value vs. the metabolite ratio of pPKTB to wild-type (WT) during growth on a continuous 20 % CH₄ in air gas stream.

In addition to biomass enhancements directly related to increased acetyl-CoA, a number of additional pathways could be altered in the pPKTB strain due to global regulatory alterations. For example, aerobic methanotrophs excrete a substantial amount of carbon as exopolymeric substance, which can account for upwards of 30% (g/g) of total assimilated carbon³⁵. Quantification of EPS and other excreted products not identified by metabolomics in this study may provide further insight into the redistribution of cellular carbon that resulted in the increased pPKTB biomass yield. We note that global metabolomic analyses indicate decreased abundance of several key metabolites, including formate, malate, and succinate. Thus, it is feasible that alterations in biomass yield are due to additive effects of global assimilated carbon redistribution.

4. Conclusions

Methanotrophic bacteria have the potential to play a significant role in the emerging bioeconomy, serving as biocatalysts for the conversion of natural gas- and anaerobic digestion-derived methane to an array of petrochemical replacements. Additionally, methanotrophs hold great promise as tools for mitigation of greenhouse gas emissions, serving as a critical methane sink in Earth's biogeochemical carbon cycle. However, to realize the full potential of these microbial catalysts, significant improvements in methane conversion efficiency are needed to achieve industrially relevant conversion metrics. To this end, we have demonstrated effective metabolic engineering strategies to increase intracellular acetyl-CoA levels and enhance yield from methane to microbial biomass and biomass-derived lipids via ectopic overexpression of PKT. The resultant biocatalyst demonstrated >2-fold biomass and lipid yield enhancement, underscoring the potential for targeted strain engineering strategies, and generating the most carbon-efficient methanotrophic biocatalyst reported to date.

In addition to the widespread applied implications of these findings, a number of fundamental findings have arisen from these data. Notably, the PKT pathway is significantly upregulated when *M. buryatense* is grown with CH₄ compared with CH₃OH as the sole carbon source, suggesting that this organism might possess CH₄-specific sensing mechanisms. Increased protein acetylation markers and significant differential metabolite distribution in the strain overexpressing PKT supports a fundamental role for this enzyme in global metabolic regulation. Given that protein acetylation is a post-translational regulator of several metabolic enzymes^{36,37}, it's possible that as yet unidentified global regulatory effects could contribute to the increased pPKTB biomass yield. Future analyses will target further elucidation of the interplay between PKT pathway flux and transcriptional and translational regulation; a more complete understanding of PKT-regulated methanotrophic metabolism will identify additional strain-engineering opportunities, targeting maximal carbon efficiency in these promising biocatalysts.

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