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Metabolic role of pyrophosphate-linked phosphofructokinase pfk for C1 assimilation in Methylophilus microbium alcaliphilum 20Z

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Abstract
Background: Methanotrophs is a promising biocatalyst in biotechnological applications with their ability to utilize single carbon (C1) feedstock to produce high-value compounds. Understanding the behavior of biological networks of methanotrophic bacteria in different parameters is vital to systems biology and metabolic engineering. Interestingly, methanotrophic bacteria possess the pyrophosphate-dependent 6-phosphofructokinase (PPi-PFK) instead of the ATP-dependent 6-phosphofructokinase, indicating their potentials to serve as promising model for investigation the role of inorganic pyrophosphate (PPi) and PPi-dependent glycolysis in bacteria. Gene knockout experiments along with global-omics approaches can be used for studying gene functions as well as unraveling regulatory networks that rely on the gene product.

Results: In this study, we performed gene knockout and RNA-seq experiments in Methylophilus microbium alcaliphilum 20Z to investigate the functional roles of PPi-PFK in C1 metabolism when cells were grown on methane and methanol, highlighting its metabolic importance in C1 assimilation in Methylophilus microbium 20Z. We further conducted adaptive laboratory evolution (ALE) to investigate regulatory architecture in pfk knockout strain. Whole-genome resequencing and RNA-seq approaches were performed to characterize the genetic and metabolic responses of adaptation to pfk knockout. A number of mutations, as well as gene expression profiles, were identified in pfk ALE strain to overcome insufficient C1 assimilation pathway which limits the growth in the unevolved strain.

Conclusions: This study first revealed the regulatory roles of PPi-PFK on C1 metabolism and then provided novel insights into mechanism of adaptation to the loss of this major metabolic enzyme as well as an improved basis for future strain design in type I methanotrophs.

Keywords: Adaptive laboratory evolution, C1 assimilation, Methanotroph, Pyrophosphate-linked phosphofructokinase, Pyrophosphate metabolism

Background
Methanotrophs, prokaryotes that use C1 compounds, such as methane or methanol, as the sole carbon and energy source, have drawn increasing attraction as promising industrial biocatalysts for bioconversion of methane and methanol to commercially relevant products [1–4]. In order to develop more efficient biocatalyst for C1 bioconversion, broadening knowledge on C1 metabolism is crucial. Multiple advantageous metabolic capabilities including high growth rate, well-established genetic toolbox, and availability of multi-omics studies made type I methanotrophs more attractive recently [1]. Type I methanotrophs mainly avail RuMP cycle for C1 assimilation in which formaldehyde condensed with
ribulose monophosphate to produce fructose-6-phosphate, which is in turn incorporated into glycolytic pathways, such as Embden–Meyerhof–Parnas (EMP) and Entner–Doudoroff (ED) pathways [4, 5]. Some gammaproteobacterial methanotrophs use the EMP pathway as the main assimilation pathway for C1 substrates, which improves achievable carbon and energy yields compared to the ED pathway [6]. However, our fundamental knowledge of C1 assimilation of methanotrophs is still limited, particularly for pMMO regulation, energy mechanisms, carbon flux regulation, and more. In general, metabolic pathways downstream of the primary methane assimilation are poorly understood, and such that knowledge on how methanotrophic bacteria adapt to different cultivation conditions are very restricted [1]. These gaps in knowledge should be investigated and be addressed in order to improve metabolic engineering strategies [1, 4]. *Methylotovinicrobium alcaliphilum* 20Z, formerly known as *Methylomicrobium alcaliphilum* 20Z, is a model obligate methanotroph and it has been studied and developed to serve as an advantageous industrial biocatalyst for methane bioconversion [7–9]. Systems biology oriented knowledge-bases in accompany with the repositories of conventional genetic toolboxes in this model provide opportunities for a better understanding of central metabolism on different nutritional conditions for methanotrophs and further to guiding metabolic engineering strategy to produce relevant biochemical products [10].

It has been reported that intracellular inorganic pyrophosphate (PPI) concentrations in methanotrophs are 20 times higher than in *Escherichia coli* and low ATP concentration was observed in the methanotrophs [11]. Moreover, extremely low inorganic pyrophosphatase activity has been detected [12], suggesting an important role of PPI and PPI-dependent enzymes in the methanotrophic metabolism. Interestingly, *M. alcaliphilum* 20Z has pyrophosphate-linked phosphofructokinase (PPI-PFK) for glycolysis instead of the classical glycolytic enzyme ATP-dependent 6-phosphofructokinase (ATP-PFK). Among several PPI-dependent enzymes in methanotrophs, this enzyme could be a model to elucidate possible roles of PPI and PPI-dependent glycolysis in microorganisms [6, 12]. PPI-PFK is the starting point of the EMP pathway, and it catalyzes interconversion between fructose 6-phosphate (F6P) and fructose 1,6-bisphosphate (FBP), which might involve in PPI recycling to economize ATP synthesis as well as control the PPI level. Thus, in-depth investigation of PPI-PFK as well as other PPI-dependent enzymes can uncover more complex metabolic roles of PPI-pools, its biosynthesis, and cell energetics in methanotrophs. The utility of gene knock-out approach has been proven useful to investigate the structure and dynamics of metabolic networks, particularly by observing responses to knockouts of metabolic enzymes and global regulators. Such that, the primary objective of this study is a comprehensive characterization of PPI-PFK in *M. alcaliphilum* 20Z with genetic modification, transcriptomic analysis, and network analysis with a genome-scale model.

Since regulation of cellular metabolism is pivotal in responding and adapting to changing environments, understanding the mechanisms of microbial adaptive evolution under such conditions in comparison to the wild-type and specific knock-out strains can shed light on functions of key metabolic genes and their effect propagating through the whole metabolic network. The deletion of such key regulator or metabolic enzyme could lead to redistribute of metabolic fluxes in the cell, which can settle down in using alternative pathways and regulation [13–15]. Adaptive laboratory evolution (ALE) is a powerful approach in which microbe is cultured continuously for many generations, subsequently improving its fitness through natural selection. The end-point mutants are then re-sequenced and characterized to identify genetic mutations and to investigate mechanistic insights with multiple-omics analysis [13–15]. ALE approach has been widely used not only to study the adaptation of the cells to shifting environmental conditions or toxic chemicals but also to investigate the adaptive responses to genetic perturbations such as loss of major metabolic enzymes [13–15]. This approach could provide valuable knowledge for both evolutionary and metabolic research. However, there reports on ALE in methanotrophs have been scarce. Thus, the study of PPI-PFK in the central metabolism of methanotrophic with genetic perturbation for pfk and following ALE experiments was performed in hope to understand the metabolic roles of pfk in methanotrophs. In addition, transcriptomic analysis and network analysis were performed to gain insights on the fundamental roles of PPI-PFK in C1 assimilation for methanotrophs.

**Results**

PPI-PFK is required for efficient methanotrophic growth of *M. alcaliphilum* 20Z

It was postulated that methane assimilation in *M. alcaliphilum* 20Z is coupled with the pyrophosphate-mediated glycolytic pathway where the major fraction of cellular pyruvate comes from the Embden–Meyerhof–Parnas (EMP) pathway [6] (Fig. 1a). In order to assess the metabolic role and its impact of PPI-PFK on methane and energy metabolism of *M. alcaliphilum* 20Z under different culture conditions, a pfk mutant strain was constructed using marker-exchange protocol [7, 10]. After isolation of the pfk mutant on the selective plate, it
was confirmed with Sanger sequencing and PCR [7, 10]. To evaluate the physiological effect of Ppi-PFK, the pfk mutant was then cultured in liquid culture using NMS medium and the growth rate of Δpfk was monitored on 2 different carbon sources, methane and methanol. During cells growth on both carbon sources, the Δpfk showed reduced growth with longer doubling time and lag phase compared to wild-type (Additional file 1: Figure S1A). This observation confirmed that Ppi-PFK is apparently involved in assimilating the carbon source and shed light on its possible role in methane and methanol metabolism of *M. alcaliphilum* 20Z. Interestingly, pfk knock-out

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**Fig. 1** Central metabolism of *M. alcaliphilum* 20Z with pyrophosphate-linked phosphofructokinase (Ppi-PFK) were deleted by the gene knockout, Ppi-PFK phosphorylates fructose 6-phosphate to fructose 1,6-bisphosphate in glycolysis pathway (a), Clusters of Orthologous Groups (COGs) analysis of differentially expressed genes when pfk was knockout on methane (b) and methanol (c).
decreased growth rate more severely on methanol (Additional file 1: Figure S1B), suggesting pfk might have different regulatory implication in utilizing different carbon sources.

In silico growth rate as well as metabolic flux distribution of wild-type and PPI-PFK knockout on methane and methanol was calculated with the genome-scale metabolic model (GSM) iLA407 and MCMC (Markov Chain Monte Carlo) sampling (Additional file 2: Table S1). The flux analysis with the GSM of *M. alcaliphilum* 20Z indicated that the metabolic reaction catalyzed by PPI-PFK can carry very high flux when cells were grown on both methane and methanol. Moreover in silico simulation with PPI-PFK deletion predicted a substantial decrease in the growth rate, agreeing with our experimental result (Additional file 1: Figure S1C, D).

**Transcriptome change in central metabolism of pfk knockout M. alcaliphilum 20Z on methane**

In order to further investigate the metabolic role of pfk in the central carbon metabolism, transcriptome analysis with RNA-seq was performed for *M. alcaliphilum* 20Z wild-type and Δpfk knock-out strains on methane. During growth on methane, the expression level of 898 genes was changed significantly when pfk was removed: 475 genes down-regulated and 423 genes up-regulated (Additional file 3: Table S2). Functional analysis with Clusters of Orthologous Groups (COG) highlighted that differentially expressed genes have functions statistically enriched in signal transduction mechanism, inorganic ion transport/metabolism and cell wall/membrane/envelope biogenesis, suggesting deletion of pfk has effects on a broad range of gene functions (Fig. 1b). Then closer attention was paid to the transcriptional response of metabolic genes in the central metabolic pathways of *M. alcaliphilum* 20Z grown on methane. As a severe growth decrease of the pfk null mutant has implied on methane, a significant number of metabolic genes of central metabolic pathways showed differential expression (Additional file 3: Table S2). In agreement with this observation from RNA-seq analysis, RT-qPCR also showed the downregulation of several key genes in C1 assimilation pathway (Additional file 1: Figure S2A and S2B). In particular, the expression level of *pmo*, which encoded methane monooxygenase, was extremely down-regulated which was 1.4- to 2.5-fold changes, indicating PPI-PFK of *M. alcaliphilum* 20Z might be related directly or indirectly to the regulation of *pmo* (Fig. 2a) with unknown mechanisms. Another downstream gene for methane oxidation *xoxF*, lanthanide-dependent methanol dehydrogenase,
contributes to the methanol oxidation process and it was down-regulated 1.8-fold changes as well. In contrast, the expression level of calcium-dependent methanol dehydrogenase, mxaFJGI, did not change significantly (Fig. 2a). Furthermore, formaldehyde-activating enzyme (fae) and formate dehydrogenase (fdh), which are the main enzymes for formaldehyde oxidation, were significantly down-regulated 2.7- and 7-fold, respectively (Additional file 3: Table S2).

The expression of genes in the C1 assimilation pathway changed significantly in M. alcaliphilum 20Z grown on methane when pfk was deleted. Metabolic enzymes in the ED pathway, such as glucose-6-phosphate isomerase (pgi), glucose-6-phosphate dehydrogenase (zwf) and keto-hydroxyglutarate aldolase (eda), were down-regulated 2.3-, 1.5-, and 1.5-fold, respectively, when pfk was knocked out. Interestingly, the significant downregulation was also observed for the genes in the EMP pathway and the serine cycle in Δpfk. In detail, the expression of glyceraldehyde-3-phosphate dehydrogenase (gapA) and phosphoglycerate kinase (pgk) in EMP pathway were decreased 2.9-, and 1.5-fold, respectively; the expression of ribulose-phosphate 3-epimerase (rpe) belonging to RuMP cycle were downregulated 1.7-fold, and the expression of phosphoketolase (xfp) in phosphoketolase pathway were downregulated 2.8-fold. Additionally, the expression of glycerate 2-kinase (gck2), methylenetetrahydrofolate dehydrogenase (mtdA), serine-glyoxylate aminotransferase (sga) which belong to serine cycle were downregulated 1.8-, 2-, and 1.8-fold, respectively (Fig. 2a). However, most of the genes in the TCA cycle did not change, except for slightly up-regulated fumA. These responses in the central metabolic pathway along with growth defect on Δpfk indicated that metabolic fluxes through the core carbon metabolic pathways were changed when pfk was deleted, suggesting its metabolic importance in methane assimilation in M. alcaliphilum 20Z.

**Different transcriptional response of PPI-PFK knockout on methanol**

Compared to methane, the growth rate of M. alcaliphilum 20Z on methanol is much higher [16]. Moreover, it was reported that *Methylotuvimicrobium* species could accumulate a large amount of glycogen up to 30% dry cell weight and formate when cells are grown on methanol [10, 17]. Interestingly, our current study revealed that C1 assimilation pathways and energy regulatory mechanisms were regulated in a different manner for different carbon sources including methane and methanol in M. alcaliphilum 20Z [10]. As noted above, pfk deletion decreased growth rate more severely on methanol, suggesting it might possess different regulatory roles in C1 metabolism on methanol compared to methane (Additional file 1: Figure S1B). In order to evaluate transcriptome change on different carbon sources and pfk deletion, RNA-seq was performed with M. alcaliphilum 20Z wild-type and Δpfk on methanol. As a result, 880 genes were identified with significant changes in transcription level of Δpfk compared to wild-type grown on methanol: 481 genes down-regulated and 399 genes up-regulated (Additional file 4: Table S3). Functional analysis with COG was performed and revealed that differentially expressed genes have enriched functions in signal transduction mechanisms, post-translational modification, and protein turnover/chaperone functions (Fig. 1c).

As expected, comparative transcriptomic analysis between Δpfk and wild-type on methanol showed differences in expression patterns of genes in central metabolism compared to methane. As for the C1 oxidation pathway, the expression of pmo did not change between Δpfk and wild-type on methanol (Fig. 2b). When substrate shifts from methane to methanol, pmo was extremely down-regulated in wild-type because methane is one of key enhancer factors for pmo expression [18]. Not similar to that of wild-type, this effect did not occur in Δpfk whereas similar expression of pmo was observed on methane and methanol (Additional file 1: Figure S3A, B). This observation supported our previous hypothesis that pfk might contribute to the regulation of the pmo operon. In addition, genes related to methanol oxidation also showed different expression patterns on methanol compared to methane when pfk was knocked out. While the expression of mxfA was not changed significantly on methane, the expression of mxaF was significantly down-regulated which was approximately 2.3-fold changes on methanol (Fig. 2b). Similar to that of mxaF, expression of genes related to pyrroloquinoline biosynthesis pqqB and pqqC, which provide cofactors for methanol oxidation, was also down-regulated (Additional file 4: Table S3). In contrast, xoxF was slightly up-regulated (1.9-fold change) on methanol (Fig. 2b). In the formaldehyde oxidation pathway, expression of fdh, which encodes an enzyme to convert formate to CO2 with the generation of NADH, was greatly decreased which was sevenfold changes.

The expression of the genes for the C1 assimilation pathway was also affected on pfk knockout when cells were grown on methanol. Not similar to growth on methane where gene expression for the ED pathway was repressed, expression of these ED pathway encoding genes did not change significantly on methanol. In addition, the down-regulation of some key genes in EMP pathway, RuMP cycle, and phosphoketolase pathway were observed on methanol. The expression of pgk and phosphoglycerate mutase (gpm) in EMP pathway were greatly decreased 1.8- and 1.8-fold, respectively. The expression of transaldolase (tal) belonging to RuMP
cycle and xfp from phosphoketolase pathway were also downregulated which were 1.9- and 4-fold, respectively (Additional file 4: Table S3). Furthermore, expression of sga, mtdA, hpr2 (D-glycerate dehydrogenase) and mclA1 (CoA ester lyase) which belong to the serine cycle also down-regulated which were 2.4-, 1.7-, 1.6- and 2-fold, respectively. Most of the genes in the TCA cycle did not change significantly except for slight down-regulation of succinate-semialdehyde dehydrogenase (gabD) (Fig. 2b).

In summary, knock-out of pfk resulted in down-regulation of many genes in the central metabolic pathways on methanol, whereas the overall transcriptional landscape on methanol were different compared to growth on methane, highlighting the differences of metabolic roles of PPI-PFK for methane and methanol metabolism in *M. alcaliphilum* 20Z.

**Recovery of growth fitness of Δpfk with ALE and identification of key mutations**

Deletion of pfk in *M. alcaliphilum* 20Z results in much slower growth than wild-type. Adaptive laboratory evolution (ALE) experiments with Δpfk resulted in evolved strains with significantly recovered growth rate (Fig. 3a, Additional file 1: Figure S4A). The evolved strains had similar growth rates as to the wild-type on both methane and methanol (Additional file 1: Figure S4B). To assess the genetic basis of the growth rate recovery, whole-genome resequencing was performed. Resequencing sequence reads were mapped onto the *M. alcaliphilum* 20Z reference genome to identify mutations in two ALE strains. Not similar to *E. coli* which can adapt to the loss of a major metabolic gene product such as PGI with only a handful of mutations [19], *M. alcaliphilum* 20Z overcame the loss of key regulator with a high number of mutations where 320 nonsynonymous substitution mutations were observed (Additional file 5: Table S4), including 219 mutations (68.4%) spanning in 40 intergenic regions and 101 mutations (31.6%) spanning in 56 ORFs. Interestingly, among these substitution mutations, 295 mutations (92.5%) were presented in both evolved strains. The similarity in mutation profiles between these two ALE strains suggested the unique adaptive responses to the loss of major metabolic enzyme, PPI-PFK, in *M. alcaliphilum* 20Z. Functional analysis with COG was also performed with the genes with mutations, and it was found that those genes have functions enriched in diverse categories including signal transduction mechanisms and lipid transport and metabolism (Additional file 1: Figure S5). Interestingly, among mutated points, we found a large region of 22 kb (from MEALZ_RS01220 to MEALZ_RS01310) spanning 17 mutated ORFs. In this region, there are three genes encoding sulfate transport via the ABC system (cysAW, sbp) and four genes related to fatty acid biosynthesis (fab) (Additional file 5: Table S4). Moreover, two endpoint strains have a single-nucleotide mutation in an RNA polymerase subunit beta (*rpoC*) (C→A; D170Y) (Table 1), which might implicate in the control of transcription pausing and elongation [20].

In the carbon metabolism, single nucleotide mutation in the bifunctional methylenetetrahydrofolate dehydrogenase/methylenetetrahydrofolate cyclohydrolase encoded by *folD* (A→T; D87E) was discovered, and it may contribute to NADPH production and C1 assimilation via the folate pathway (Table 1). It was predicted that this pathway may carry more flux in the Δpfk ALE strain, suggesting the mutation in *fodD* might enable a specific function. Although *M. alcaliphilum* 20Z possesses both of methylenetetrahydrofolate cyclohydrolase (*fchA*) and methylenetetrahydrofolate dehydrogenase (*mtdA*) for methylene tetrahydrofolate pathway, it also contains *folD* which encoded a bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase. The function of these genes have not been characterized in type I methanotrophs. All of these three genes were expressed, with slightly higher expression of *mtdA* and *fchA* than *folD* (Additional file 6: Table S5). The dehydrogenase activity of *folD* catalyzes NADP+-dependent oxidation of 5,10-methylenetetrahydrofolate to 5,10-methenyl-tetrahydrofolate and subsequently to 10-formyltetrahydrofolate (10-CHO-THF) by the cyclohydrolase activity [21]. In order to confirm if the mutation point of *folD* might be led to change in its binding activity, multiple alignment was performed among *folD* from wild-type *M. alcaliphilum* 20Z, ALE strain and other well-studied *folD* with available crystal structures [21]. Even the 3D structures of FolD from different species are similar, they possess different specific activities and affinities for the inhibitors. The amino acid at position 87 is diverse among these species (D87, E87, and A87) so that substitution from D87 to E87 in the Δpfk ALE strain may provide valuable new candidates for rationally manipulating *folD* activity in metabolic engineering of methanotrophs. Additionally, seven mutations were also detected in the intergenic region upstream of *fodD* which might be related to the transcriptional regulation of this gene. Similar to *fodD*, sodium-translocating pyrophosphatase (*hpp*), which is involved in energy regulation mechanism, was also found to gain one mutation in the ORF (A→G; N30S) (Table 1). Notably, two more mutations were found in the upstream intergenic region of *hpp* where 1-bp deletion was identified. Transcriptome analysis between wild-type and one evolved strain was performed to further investigate how these mutations could enable the increasing growth rates from the initial state.
Transcriptome analysis for mutated genes of ALE strain

Integration of mutation analysis with the evolved strains and functional analysis for the genes with mutations narrowed down possible genes that could render different phenotypes after adaptive evolution. First of all, a mutation of RNA polymerase subunit, rpoC, could be a potent rescue solution for the pfk deletion. In the previous report with E. coli adaptive evolution after deletion of a major enzymatic gene of the EMP pathway, pgi, the catalytic subunit of RNA polymerase, rpoB, was mutated to change the genome-wide transcriptional landscape [22]. Similar to RpoB, a mutation of RpoC might lead

![Diagram](image-url)
to transcriptional change of multiple enzymatic genes. Besides, mutations in two transcriptional regulators (locus tag: MEALZ_RS18010 and MEALZ_RS01220) which encoding for an uncharacterized transcriptional regulator and HTH-type transcriptional regulator CysB were also identified in each of the fast-growing evolved strains. It can be hypothesized that these mutations might contribute to growth recovery by modulating transcription level of alternative pathways in the ALE strains (Fig. 3b).

Mutations in transcriptional regulatory machineries including RNA polymerase subunits and transcription factors necessitated investigation of genome-wide transcriptome to compare the wild-type and evolved mutant strain, hoping to characterize the rewiring of central metabolism to overcome the deletion of pfk. As a result, transcriptome analysis with RNA-seq approach was carried out when cells were grown on methane and revealed expression change of 1040 genes, where 514 and 526 genes were up- and down-regulated in the ALE strain compared to the parental strain (Additional file 2: Table S5). Functional analysis with COG was then performed to show differentially expressed genes had enriched functions in signal transduction mechanisms followed by inorganic ion transport and metabolism (Fig. 3c).

Comparative transcriptomic analysis showed the global expression changes in central metabolism which might be associated with growth recovery in the ALE strain (Fig. 3b). The expression of pmo did not change between the unevolved and ALE strain which still lower than the expression level of pmo in the wild-type. Interestingly, the up-regulation of methanol oxidation enzymes including lanthanide-dependent methanol dehydrogenase (xox) and calcium-dependent methanol dehydrogenase (mxa) was observed in the evolved strain which were 1.6- and 1.7-fold, respectively (Additional file 6: Table S5). Likewise, RT-qPCR also showed the upregulation of these metabolic genes in the evolved strain (Additional file 1: Figure S4C). In agreement with this observation, evolved strain had slightly higher methanol uptake rate compared to the wild-type (Additional file 1: Figure S4D). Furthermore, the formaldehyde oxidation pathway also was activated in the ALE strain, where genes in the H4MPT, H4F pathways were up-regulated (Fig. 3b).

Activation of the formaldehyde oxidation pathway in the evolved Δpfk strain brought more attention to investigate possible other changes in C1 assimilation pathway (Fig. 3a). First of all, activation of the ED pathway was observed with high up-regulation of key enzymatic genes such as pgi, edd, and eda (Fig. 3b, Additional file 1: Figure S4C). Without Ppi-PFK in the core carbon pathways, the ED pathway might need to be activated to accommodate carbon flux increase to produce sufficient G3P pool by making G3P from F6P (Fig. 3b). G3P, then, can subsequently enter the EMP pathway or the RuMP cycle. In agreement with this observation, in silico flux analysis with the GSM iLA407 and MCMC sampling also indicated an increasing flux thought the ED pathway when Ppi-PFK was removed from the model (Additional file 2: Table S1). At the G3P node, the RuMP cycle via the non-oxidative PP pathway—which mainly provided C5 unit for formaldehyde assimilation, was up-regulated with significant increase in the transcription level of transketolase.

### Table 1 Key genetic changes in evolved strains

| Locus_Tag | Gene | Product | Reference_sequence_location | Mutation |
|-----------|------|---------|-----------------------------|----------|
| MEALZ_RS01065 | hppA | Sodium-translocating pyrophosphatase | 223,987 | N30S (AAC→AGC) |
| MEALZ_RS01220 | cysB | LysR family transcriptional regulator | 264,570 | E154D (GAA→GAC) |
| MEALZ_RS01235 | cysA | Sulfate ABC transporter ATP-binding protein | 268,089 | K19D (AAA→GAC) |
| MEALZ_RS01240 | cysW | Sulfate ABC transporter permease subunit CysW | 268,091 | K19D (AAA→GAC) |
| MEALZ_RS01250 | sbp | Sulfate ABC transporter substrate-binding protein | 268,121 | S9G (AGC→TGC) |
| MEALZ_RS01295 | fabG | 3-Oxoadipyl-ACP reductase FabG | 269,090 | S63A (TCT→TAC) |
| MEALZ_RS06915 | folD | Bifunctional methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase FolD | 269,198 | L27M (CTG→ATG) |
| MEALZ_RS16400 | rpoC | DNA-directed RNA polymerase subunit beta | 269,207 | K267N (AAG→AAT) |
| MEALZ_RS17985 | sbp | Outer membrane lipoprotein carrier protein LolA | 270,878 | K267N (AAG→AAT) |
| MEALZ_RS18010 | Transcriptional regulator | 271,623 | A19V (GCT→GTT) |

### Note
- The highlighted mutations are in the transcriptional regulators that were found to be associated with growth recovery in the ALE strain.
(tkt) and ribose 5-phosphate isomerase (rpi) which were 1.9- and 1.7-fold, respectively (Additional file 6: Table S5). In addition, the partial EMP pathway which further converts G3P to pyruvate was strongly activated with high up-regulation of glyceraldehyde-3-phosphate dehydrogenase (gapA) (3.6-fold), phosphoglycerate kinase (pgk) (2.6-fold), phosphopyruvate hydratase (eno) (1.8-fold) and pyruvate kinase (pyk) (2.1-fold) (Additional file 1: Figure S4C, Additional file 6: Table S5). Furthermore, the serine cycle, which interconnected with TCA and glycolysis pathway for acetyl-CoA formation, was also up-regulated with an increasing expression level of serine-glyoxylate aminotransferase (sga), serine hydroxymethyltransferase (glyA), bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase (folD) and formate-tetrahydrofolate ligase (ftfL) which were 3.2-, 1.6-, 1.9- and 1.5-fold (Additional file 6: Table S5). Consistent with the up-regulation of folD, some mutation points in the upstream region of folD was detected which might direct or indirect affect to transcription of this gene. It currently has been reported that the incomplete serine cycle only carries minor flux to acetyl-CoA production in M. alcaliphilum 20Z with a small amount of formate secretion [8]. However, higher secretion rate of formate from the ALE strain was observed when compared to wild-type (Additional file 1: Figure S4D), which explains the formaldehyde oxidation pathway and the serine cycle might carry more flux to contribute the formation of reducing equivalents as well as acetyl-coA production when pfk is deleted [10]. In addition, the expression level of genes in the TCA cycle did not change significantly except slight up-regulation of sucA, and sucB. In summary, transcriptome analysis with the evolved Δpfk strain illustrated possible activation of bypass pathway through the ED–EMP pathway. This result agreed with in silico flux analysis with the GSM and the mutation analysis with the ALE strain.

Differential regulation of PPI metabolism in the ALE strain
Inorganic pyrophosphate (PPI) is a by-product of several anabolic reactions such as biosynthesis of nucleic acids, proteins, coenzymes, isoprenoids, and oligo- and polysaccharides. Moreover, in M. alcaliphilum 20Z, the high accumulation of glycogen and sucrose suggested that the biosynthesis pathways of sucrose and glycogen might function as major sources to contribute to PPI generation (Fig. 4). It has been reported that intracellular PPI concentration in methanotrophs was 20 times higher than in E. coli while only low ATP concentration found which correlated with extremely low inorganic pyrophosphatase activity [12], suggested that energy-rich PPI molecules also play an important role in the energy metabolism in methanotrophs. The discovery of the activity of PPI-PFK along with a high intracellular concentration of PPI indicated that PPI-PFK could be one unique metabolic features of the energy generation mechanisms in
methanotroph [11]. With PPI-dependent glycolysis pathway, the energy of PPI can be re-utilized to generate ATP, which might significantly increase the efficiency of one-carbon assimilation.

In the absence of PPI-PFK, PPI-dependent glycolysis pathway was repressed and could not re-utilized PPI efficiently, which might affect cell energetics, subsequently leading to decrease growth rate. So it was hypothesized that the PPI metabolism might change to adapt the absence of PPI-PFK in the ALE strain. To investigate the roles of PPI metabolism, we further confirmed the changes of others key PPI-linked enzymes in the ALE strain. Pyruvate phosphate dikinase (PPDK) catalyzes the reversible conversion of phosphoenolpyruvate (PEP) into pyruvate accompanied by ATP synthesis from AMP. With this enzyme, the PPI-dependent glycolysis pathway was completely reversible and energetically 2.5 times more efficient in comparison to the ATP-dependent glycolytic pathway [23]. Interestingly, the expression level of ppdk was increased by twofold in the ALE strain, suggesting that PPDK might take over the role of PPI-PFK to generate ATP via re-utilization of PPI (Fig. 3a, Additional file 6: Table S5). In addition, M. alcaliphilum 20Z possesses a membrane-bound proton-translocating H+–Ppase which might contribute to the regeneration of PPI from ATP. The predicted ratio of ATP hydrolysis: PPI formation for this enzyme is 1:3 and it was expected that these enzymes contribute to 25% of PPI pool upon active growth on methane in this strain [24]. Mutation analysis with resequencing showed that mutation points were found in the coding sequence of hpp as well as its upstream intergenic region, suggesting transcription regulation of hpp might change. In agreement with this, the transcription level of hpp in the ALE strain was significantly down-regulated, so that the mutations points in the intergenic region might directly affect transcriptional regulation of hpp. It is highly likely that the decreasing expression of hpp might contribute to ATP generation from PPI in the evolved strain.

Discussion
Loss of function strategy with gene knockout has been broadly used in biology to identify the gene functions and their roles in the biological networks. In particular, this approach has long been used in the study of microbial metabolism by observing responses to knockouts of metabolic enzymes and global regulators. The primary objective of this study was the elucidation the functional role of PPI-PFK, a key regulatory step of carbon metabolism in type I methanotrophs, in different culture conditions by using gene knockout and transcriptome analysis with RNA-seq. PPI-PFK has been found in all aerobic gammaproteobacterial methanotrophs and kinetic properties of this enzyme was characterized recently [24]. It also was tempting to speculate that PPI-PFK could be related to energy generation regulation in type I methanotrophs [24]. In this knockout studied, it was observed that growth rate of pfk mutant was extremely decreased on both methane and methanol possibly due to insufficient C1 metabolism. PPI-mediated EMP pathway is the main route for C1 assimilation which mainly contributed to pyruvate synthesis [6]. Due to knockout of pfk, the preferential route of carbon flow becomes the ED pathway, where the strain recruits the reducing powers as well as key intermediates G3P and pyruvate for the system. However, as noted above, genes in the ED pathway were significantly down-regulated. Thus, the decreasing growth rate may be due to the fact that the pfk knockout apparently disrupts the energy and reducing power balance. Type I methanotrophs possess sugar-phosphate dependent metabolic pathways which are similar to those in E. coli. The deletion of PGI to block the glycolytic pathway also led to decrease specific growth rate due to the imbalance of reducing power with overproduction of NADPH [14, 19].

When methanol is metabolized as a sole carbon source, the effect of pfk knockout is slightly different from that of the case used methane as a sole carbon source. The most striking difference is the decrease in growth rate as compared to methane, suggesting the important role of pfk during growth on methanol. Additionally, transcriptional responses of central metabolism showed different effects when pfk was inactivated on methanol. The C1 assimilation pathway via the RuMP cycle also was slightly different than that of methane, where the genes in the ED pathway did not change in Δpfk mutant was extremely decreased on methanol. The C1 assimilation pathway via the RuMP cycle also was slightly different than that of methane, where the genes in the ED pathway did not change in Δpfk mutant was extremely decreased on methanol. The C1 assimilation pathway via the RuMP cycle also was slightly different than that of methane, where the genes in the ED pathway did not change in Δpfk mutant was extremely decreased on methanol. The ED pathway contributes less to methane assimilation where 25% of intracellular pyruvate comes from this pathway and 75% comes from the EMP pathway. However, the ratio of carbon flux through EMP and ED pathways could alter under different carbon sources. In our recent study, the roles of EMP and ED pathway on methanol-grown cells were investigated to show the EMP pathway still dominates over the ED pathway for C1 assimilation and the ED pathway carries very low carbon flux [10]. In addition, the accumulation of a large amount of glycogen on methanol could lead to higher PPI generation on methanol (Fig. 4) and PPI-mediated EMP appears to re-utilize PPI to generate energy and reducing power for cell growth. It has been found that the expression of the serine cycle was more activated on methanol and deletion of PPI-PFK
showed more defects on this pathway, suggesting the operation of a functional serine cycle. In agreement with this observation, when shifting the substrate from methane to methanol, our current studies showed the activation of serine cycle on methanol-grown for energy regulation [10, 16, 18], while the serine cycle only carries negligible flux for growth on methane [8]. In summary, those results demonstrated the impact of pfk knockouts on the metabolic phenotype is more expressive and also showed to have a different influence on the metabolism of grown on methane and methanol.

On methane, the expression level of pmn along with xoxF showed a similar expression pattern when pfk was removed. Our previous study suggested that xoxF could contribute to the methane oxidation process in type I methanotrophs [18] by formation of an alternative structure of MDH-pMMO association with the binding of XoxF monomer to pMMO [25]. So that, pfk likely serve as regulatory effects to control this structure. However, these effects might occur due to the imbalance of PPI, which potentially serves as an alternative phosphoryl donor instead of ATP. The pMMO located in the membrane of methanotrophs which also possesses an electron transport chain (ETC) and ATP synthesis through oxidative phosphorylation. These two processes, methane oxidation and ETC, might occur simultaneously, suggesting a complicated regulatory network including these processes. It was previously suggested that the source of PPI for PPI-PFK may be energy-dependent PPI synthesis on the cell membrane [11]. The disruption of PPI-PFK reaction might change the cell membrane integrity, subsequently affects to pMMO as well as aerobic ATP synthesis through ETC.

In the next step, we further investigated how the recovery of growth rate in Δpfk is enabled by genetic mutations and rewiring of the metabolic network. Comparison of genome, transcriptome, and phenotype of wild-type and Δpfk ALE strains could provide insight into how M. alcaliphilum 20Z may adapt to the loss of a key metabolic enzyme, PPI-PFK. The fitness recovery might be obtained by global transcriptional regulation such as RNA polymerase component rpoC which could contribute to a rebalancing of the proteome to promote growth [26]. This led to increased flux through oxidative pentose phosphate and ED pathways for generation of reducing powers and G3P which subsequently enters RuMP cycle for C5 unit regeneration as well as go to the EMP pathway for pyruvate synthesis. The increasing carbon flux through oxidative pentose phosphate pathway could increase NADPH availability which is a key factor for enhancing the biosynthesis of secondary metabolites from methane using methanotrophic bacteria as a biocatalyst. Furthermore, the upregulation of ED pathway is also an advantage for production of isoprenoids via methylerythritol phosphate (MEP) pathway since pyruvate and G3P were generated simultaneously via ED pathway, providing a more balanced precursor pool for the MEP pathway [27]. Moreover, carbon flux likely also enters \( \text{H}_4\text{MPT}, \text{H}_4\text{F} \), and serine cycle to generate more reducing powers as well as contribute to acetyl-CoA production which is advantageous to produce various products derived from intermediates from the serine cycle, such as amino acids and CoA derivatives. The availability of C1 assimilation pathway may enable flexibility when adapting to changing conditions. The mutation of this global transcription pattern has been found in E. coli through adaptive evolution reprogram in different conditions. For example, the mutation in rpoC improved metabolic efficiency to gain growth advantage from adaptation to glycerol as well as growth in minimal media [22, 28]. Besides, the adaptation of E. coli Δpgi also was accompanied with mutations in rpoC.

Notably, it was also found that the fitness recovery also relieve an alternative PPI mediated glycolysis. Inorganic pyrophosphate created during biosynthetic polymerization reactions in most organisms is hydrolyzed by inorganic pyrophosphatase in order to thermodynamically favor the anabolic processed [11]. However, with the negligible activity of inorganic pyrophosphatase found in methanotroph, the use of PPI instead of ATP as a phosphoryl donor in some key metabolic reactions such as PPI-PFK may provide a solution for economizing ATP synthesis, regulation of hexosephosphates and triosephosphates interconversion as well as PPI levels in cells [11, 12]. With regard to the previous studies, high flexibility at the key glycolytic steps could provide some advantages for methanotrophs, allowing metabolism to adapt to different energy and carbon sources. With the loss of PPI-PFK, energetics of cells was dramatically affected, and glycolytic steps turned into another strategy to adapt by using alternative PPI-dependent enzyme in glycolysis pathway PPDK for ATP synthesis and coupled with PPI recycling.

Conclusion

In summary, this study highlighted the importance of PPI-PFK on C1 metabolism of M. alcaliphilum 20Z, which is considered as an industrially promising candidate for bioconversion of methane and methanol into value-added products. Additionally, we first demonstrated the combination of adaptive evolution experiment and -omic analysis to study the regulatory mechanism in the Δpfk ALE strain. While whole-genome resequencing enabled the mutation discovery, and transcriptomic analysis coupled with genome-scale modeling revealed the metabolic pathways underlying the evolved phenotypes.
This knowledge could provide novel strategies for evolution-based metabolic engineering for methanotrophs.

**Methods**

**Bacterial growth conditions**

The strains used in this study were grown in a nitrate mineral salt (NMS) medium [29]. Liquid pre-cultures of *M. alcaliphilum* 20Z were grown in 180 mL baffled-flask with 10 mL NMS with a supplement of 50% methane (v/v) as a sole carbon source at 30 °C and 230 rpm agitation. Fresh NMS medium of 50 mL was then inoculated with the pre-culture in a 500 mL baffled flask sealed with a screw cap with methane supplied to a final concentration of 50% (v/v) by gas substitution using a gas-tight syringe. For methanol, *M. alcaliphilum* 20Z was cultured in the same medium containing 1% methanol instead of methane for a carbon source. The initial optical density of each culture was set to OD₆₀₀ = 0.01 for growth rate experiments. All culture experiments were performed with at least two biological duplicates. For strain construction, gentamicin was used at final concentrations of 10 μg/mL.

**Total RNA isolation and sequencing**

For RNA-seq experiments, all cultures were grown in biological duplicates for subsequent RNA extraction and sequencing steps. For sequencing library preparation, 5 mL of microbial culture broth containing either methane- or methanol-grown culture, grown as described above, in mid-exponential phase were harvested (OD~ 0.6–0.7 for methane growth and OD~ 1.2 for methanol growth) and immediately mixed with 10 mL of bacterial reagent RNAprotect (Qiagen, Hilden, Germany) for ensuring reliable gene expression and gene-profiling data. Total RNA was then extracted using RNeasy Mini Kit along with On-Column DNase Digestion using RNase-Free DNase Set (Qiagen, Hilden, Germany) following the manufacturers’ protocols. Total RNA quality and quantity were measured using an RNA 6000 Nano kit with the Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA) with an RNA Integrity Number (RIN) value greater than or equal to 7. In addition, total RNA samples were checked on 1.3% agarose gel to validate no DNA contamination (Additional file 1: Figure S7).

Next, for mRNA enrichment, rRNA was removed using the Ribo-Zero rRNA removal kit for gram-negative bacteria (Epicentre, Madison, WI, USA), and the remaining RNA was used for creating the sequencing library using the TruSeq Stranded Total RNA Sample Prep Kit (Illumina, USA) according to the manufacturer’s instructions. Transcriptome sequencing was conducted using the Illumina/Hiseq-2000 RNA sequencing platform with ~30 million/sample PE101 reads by Macrogen (Korea) (Additional file 7).

**Quantification of differentially expressed genes**

The quality of the raw sequence data was examined with FastQC ([http://www.bioinformatics.babraham.ac.uk/projects/fastqc/](http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)) for further quantitative analysis as previously described [30]. Sequencing reads were mapped onto the genome sequence of *M. alcaliphilum* 20Z (NC_016112 for the chromosome and NC_016108 for the plasmid). Bowtie toolbox [31] was used with a maximum insert size of 1000 bp and with two maximum mismatches after trimming 3 bp at the 3′ ends with other default option. The sequence alignment map (SAM) files were post-processed with SAMTools ([http://samtools.sourceforge.net/](http://samtools.sourceforge.net/)) to be converted to the binary alignment/map (BAM) files, and then were sorted and indexed. Indexed and sorted BAM files were fed to Cuffdiff [32] to calculate differential expression (with default options including geometric normalization method and a library type of dUTP RNA-seq). Genes with differential expression of log2 fold change ≥ 1.0 and false discovery rates (FDR) value ≤ 0.01 were considered as differentially expressed genes in this study.

**DNA sequencing**

Genomic DNA was isolated using a Promega Wizard DNA purification kit (Promega, Madison, WI, USA) following the manufacturers’ protocols. DNA was quantified by using a Qubit dsDNA high-sensitivity assay. Paired-end sequencing libraries were generated and sequenced with Illumina Hiseq-2000 sequencing platform (Macrogen, Korea). The breseq pipeline [33] was used to map sequencing reads and identify mutations relative to the genome sequence of *M. alcaliphilum* 20Z (NCBI accession NC_016112 for the chromosome and NC_016108 for the plasmid).

**Calculation of in silico metabolic flux with FBA analysis and MCMC sampling**

To calculate in silico metabolic flux, FBA analysis, and MCMC sampling was performed with *M. alcaliphilum* genome-scale model iLA407 [8] and COBRApy [34] as previously described [35]. The iLA407 model is available on the following web-site: [http://sci.sdsu.edu/kalyuzhlab/](http://sci.sdsu.edu/kalyuzhlab/). The flux distribution for each reaction in the model was determined using Markov chain Monte Carlo (MCMC) sampling [36]. The uptake rates for all of the carbon sources, methane and methanol, were constrained to the model as 11.7 mmol/DW/h. The biomass objective function (BOF) was set at a lower bound of 95% of the optimal growth rate as calculated by FBA. Thus, the
sample flux distributions by MCMC sampling method could be represented sub-optimal flux distributions. MCMC sampling was performed by optGpSampler sampling algorithm with 100,000 of feasible fluxes. After normalization of average flux value against BOF, flux value each reaction was used for further analysis.

Analytical analysis of culture supernatants
The culture supernatant was separated from cells by centrifugation. Formate and methanol concentrations were detected and measured using HPLC (Jasco Co., Easton, USA) with an RI detector and an Aminex HPX-87H organic acid column (Bio-Rad, Hercules, USA). Sulfuric acid (0.005 M) was used as the mobile phase at 60 °C with a flow rate of 0.7 mL/min. All solutions were filtered through a 0.2 µm membrane before use. The concentrations of formate and methanol were calculated by regression analysis compared to known standards.

Adaptive laboratory evolution
Evolution of *M. alcaliphilum* 20Z ∆pfk was conducted in 50 mL of NMS medium with a supplement of 1% methanol in baffled flasks as the batch cultures. At the start of evolution, ∆pfk was grown in 50 mL NMS medium in 500 mL baffled flasks at 30 °C and 230 rpm in 48 h and the cultures were then transferred to fresh medium for adaptive evolution at an initial OD₆₀₀ of 0.01. Cells were grown reach to exponential phase, then diluted by serial transfer into fresh medium. Each transfer into the fresh medium was started at an OD₆₆₀ of 0.01. This process, in batch growth and serial dilution, was conducted for 40 days for a total of 20 transfers to fresh medium. The strain from the last transfer was streaked out on NMS medium and incubated for 2 days. Then, 10 endpoint clones were selected to further confirm the growth rate.

COG functional enrichment
DE genes were categorized according to their annotated clusters of orthologous groups (COG) category. Functional enrichment of COG categories DE genes was determined by performing a hypergeometric test, and P-value < 0.05 was considered significant.

RT-qPCR
Total RNA samples were directly used for RT-qPCR experiments. The primers used for RT-qPCR provided in Additional file 8: Table S6. cDNA was synthesized from 500 ng total RNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA) and used as a template for RT-qPCR. RT-qPCR was performed by using GoTaq qPCR Master Mix (Promega, Madison, USA) in CFX Connect Real-Time PCR Detection System (Biorad, Hercules, USA). The cycle threshold (Ct) value for each gene was determined and normalized to the *rpoD* housekeeping gene. The relative expression between two samples was calculated by using the ΔΔCt method [37].

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12934-020-01382-3.

Additional file 1. Additional figures and legends supporting the results described in text.
Additional file 2. Nominalized flux distribution of wild-type and ∆pfk by MCMC sampling.
Additional file 3. Differentially expressed genes of *Methylotuvimicrobium alcaliphilum* 20Z wild-type and ∆pfk grown on methane.
Additional file 4. Differentially expressed genes of *Methylotuvimicrobium alcaliphilum* 20Z wild-type and ∆pfk grown on methanol.
Additional file 5. Mapped genetics changes in ALE strains.
Additional file 6. Differentially expressed genes of *Methylotuvimicrobium alcaliphilum* 20Z ∆pfk and ALE strains grown on methane.
Additional file 7. The Illumina NGS workflow for RNA-seq experiment.
Additional file 8. Primers used for RT-qPCR experiment.

Abbreviations
PPi-PFK: Pyrophosphate-dependent 6-phosphofructokinase; PPI: Inorganic pyrophosphate; EMP: Embden–Meyerhof–Parnas; ED: Entner–Doudoroff; ATP-PFK: ATP-dependent 6-phosphofructokinase; ALE: Adaptive laboratory evolution; GSM: Genome-scale metabolic model; MCMC: Markov Chain Monte Carlo; COG: Clusters of Orthologous Groups; NMS: Nitrate mineral salt; SAM: Sequence alignment map; BAM: Binary alignment map; PFKM: Fragments per kilobase of exon per million fragments; FDR: False discovery rate.

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Authors’ contributions
ADN and GN carried out the experimental procedures, performed a complete analysis, and prepared a draft of the manuscript. EYL and DK coordinated the study and finalized the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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