Fatty Acid Biosynthesis Pathways in *Methylomicrobium buryatense 5G(B1)*

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Methane utilization by methanotrophic bacteria is an attractive application for biotechnological conversion of natural or biogas into high-added-value products. Haloalcaliphilic methanotrophic bacteria belonging to the genus *Methylomicrobium* are among the most promising strains for methane-based biotechnology, providing easy and inexpensive cultivation, rapid growth, and the availability of established genetic tools. A number of methane bioconversions using these microbial cultures have been discussed, including the derivation of biodiesel, alkanes, and OMEGA-3 supplements. These compounds are derived from bacterial fatty acid pools. Here, we investigate fatty acid biosynthesis in *Methylomicrobium buryatense 5G(B1)*. Most of the genes homologous to typical Type II fatty acid biosynthesis pathways could be annotated by bioinformatics analyses, with the exception of fatty acid transport and regulatory elements. Different approaches for improving fatty acid accumulation were investigated. These studies indicated that both fatty acid degradation and acetyl- and malonyl-CoA levels are bottlenecks for higher level fatty acid production. The best strain generated in this study synthesizes 111 ± 2 mg/gDCW of extractable fatty acids, which is ~20% more than the original strain. A candidate gene for fatty acid biosynthesis regulation, *farE*, was identified and studied. Its deletion resulted in drastic changes to the fatty acid profile, leading to an increased pool of C18-fatty acid methyl ester. The FarE-regulon was further investigated by RNA-seq analysis of gene expression in *farE*-knockout mutants and *farE*-overexpressing strains. These gene profiles highlighted a novel set of enzymes and regulators involved in fatty acid biosynthesis. The gene expression and fatty acid profiles of the different *farE*-strains support the hypothesis that metabolic fluxes upstream of fatty acid biosynthesis restrict fatty acid production in the methanotroph.

**Keywords:** methane valorization, methanotrophs, fatty acid metabolism, *farE*, fatty acid elongation

**INTRODUCTION**

Methane bioconversion by methanotrophic bacteria is attracting attention from biotechnologists because of its inexpensiveness and the abundance of natural gas as its potential feedstock. However, economically effective examples of such bioconversions have been limited to just the production of single-cell protein and polyhydroxybutyrate products (Levett et al., 2016; Strong et al., 2016).
Advancement in this area is limited by the paucity of approaches to manipulate the methanotroph genome and the even more limited knowledge of the genomic targets to be manipulated. We and others are developing genomics and genetic tools for methanotroph genome manipulation (Ojala et al., 2011; Puri et al., 2015; Henard et al., 2016). In this work we focus on identifying novel genomic targets—specifically, we studied fatty acid (FA) biosynthesis/degradation pathways and regulation as a means to increase cellular lipid content. Total FAs extracted from bacteria have been shown to be valuable precursors for production of liquid transportation fuels (Lennen and Pfleger, 2012). Furthermore, the interest in methanotrophic microbes as methane-driven microbial factories for the production of a variety of FA-based products, ranging from industrial surfactants and detergents to cosmetic carotenoid-based products and OMEGA supplements, is on the rise. Methanotrophic bacteria produce high levels of FAs for the synthesis of intracytoplasmic membranes (ICM, Patt and Hanson, 1978; Jahne and Nicholst, 1986; Sessions et al., 2002). However, the metabolic pathways for FA biosynthesis have never been thoroughly investigated.

*Methylophilus* *buryatense* 5G(B1), a gammaproteobacterial methanotrophic bacterium, can use methane as a sole carbon and energy source. A set of unique properties of the strain, including its fast growth rate, its ability to grow over a broad range of salinity and pH, and the availability of a draft genome, a validated flux balance model, and a set of genetic tools, make it an ideal model for studying lipid biosynthesis (Kaluzhnaya et al., 2001; Khmelenina and Beck, 2013; De la Torre et al., 2015; Puri et al., 2015; Henard et al., 2016). The metabolic pathways for methane utilization in *M. buryatense* 5G(B1) have been recently refined (De la Torre et al., 2015; Gilman et al., 2015). Methane utilization starts with the oxidation of methane to methanol by a particulate or soluble methane monoxygenase enzymes (Kaluzhnaya et al., 2001); methanol is then converted to formaldehyde by PQQ-dependent methanol dehydrogenases, a two subunit Ca-containing enzyme or a one subunit La-induced enzyme (Chu and Lidstrom, 2016; Chu et al., 2016). Formaldehyde is either oxidized to CO2 to supply energy or assimilated into sugars through a ribulose monophosphate (RuMP) pathway, a fraction of which is then converted through pyruvate to acetyl-CoA (*Figure 1A*), a building block for FA synthesis (*Figure 1B*). Similar to other methanotrophic bacteria, *M. buryatense* 5G(B1) produces significant amounts of ICMs, which can occupy up to 60% of its cellular volume (Collins and Kalyuzhnaya, unpublished data). Methanotroph membranes are composed of phosphatidylethanolamine- (63–95%), phosphatidylglycerol- (2.5–18%), and phosphatidylserine- (2.2–7.6%) linked C16 or C16:1 FAs (Kaluzhnaya et al., 2001; Gilman et al., 2015). Here, we present a genome-based reconstruction of the FA biosynthesis pathways in *M. buryatense* 5G(B1). Assumptions from the analysis of genomic data were further supported by gene expression data and mutagenesis. A detailed search within the *M. buryatense* 5G(B1) genome revealed several candidate gene targets for strain engineering efforts to improve production of bulk FA-based chemicals.

### MATERIALS AND METHODS

#### Strains and Genetic Manipulations

Strains and genetic constructs used in this study are listed in Table 1. Strain AP18 was chosen as the base strain for most of the genetic modifications described here. The strain descends from the *M. buryatense* 5G(B1) strain, a lab-adapted variant of wild type *M. buryatense* 5G. The strain is resistant to rifampicin, has improved transformation efficiency, and lacks the 76 kB plasmid, the glycosyn synthase genes *gglA1* and *gglA2*, and the sucrose-phosphate synthase (sps) gene (Puri et al., 2015). These modifications resulted in a very modest increase in the FA pool as well as an inability to produce glycogen and/or sucrose. So far no significant impacts on cell growth and methane consumption have been observed. The strain AP18 and plasmid constructs used to create acetate kinase-deleted and acetyl-CoA carboxylase-overexpressing mutants were kindly provided by the Lidstrom Laboratory (University of Washington).

*M. buryatense* strains were grown on methane as described (Ojala et al., 2011), with some modification of the growth medium (Table S1). *Escherichia coli* strains were grown on Luria-Bertani media supplemented with kanamycin (100 μg/ml) and ampicillin (100 μg/ml). Genetic manipulations with *M. buryatense* were done as described (Puri et al., 2015). For unmarked gene deletions, the pCM433kanT plasmid carrying ~600-bp of sequences flanking the to-be-deleted genes was introduced. After conjugation, single-crossover kanamycin-resistant *M. buryatense* clones were plated on rifampicin to counter-select against *E. coli*. Then, to select for Kan-sensitive double crossover clones with deleted genes of interest, single-crossover clones were passaged on plates with 2.5% sucrose and the resulting colonies were PCR-genotyped for the absence of the gene of interest followed by sequencing. For overexpression of genes, the pAWP78 plasmid was used as a vector. All genes were cloned under the *tac*-promoter, which provides strong constitutive expression in *M. buryatense* (Puri et al., 2015). The nucleotide positions of the sequences cloned into plasmids are outlined in Table S2.

#### Induction of Acetyl-CoA Carboxylase

The acetyl-CoA carboxylase expression system (pAWP78:accABCD) was obtained from Lidstrom and Puri. The construct includes the native acetyl-CoA carboxylase genes (subunits A, B, C, and D) cloned into pAWP78 under the tetracycline-inducible pTet promoter (Puri, unpublished data). The plasmid was transformed into *E. coli* S17-1 and transferred into the 5GB1C, AP18, AP18 Δack, AP18ΔfadABEΔack, abd AP18ΔfadABE backgrounds via conjugation. Cells were grown until mid-exponential phase (at OD ~0.5). Each cell line was represented by biological (3) and technical (2 per biological replicate) replicates. Expression of acetyl-CoA carboxylase was induced by addition of anhydrotetracycline (1 μM final) followed by a 6-h incubation before collection of cells by centrifugation.

#### FA Methyl Ester (FAME) Analyses

Fifty milliliters of cell cultures grown at 30°C with 50 ml of methane to OD600 ∼1 were collected by centrifugation, washed...
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FIGURE 1 | Metabolic pathways for methane oxidation (A) and FA synthesis (B) in M. buryatense 5G(B1) based on a gene inventory study, transcriptomic data analysis, and genome-scale modeling (De la Torre et al., 2015). Numbers in blue represent that particular reaction’s fluxes according to the developed genome-scale model (De la Torre et al., 2015); numbers in black after gene names denote abundances of respective transcript normalized to an abundance of methanol dehydrogenase. Gene names coding enzymes of the methane assimilation pathways are extracted from BioCyc Database Collection (http://biocyc.org/). Enzyme EC numbers and corresponding gene IDs are listed in Table S5. Methane utilization starts from oxidation to methanol by methane monooxygenase enzyme in periplasm;

(Continued)
methanol is then metabolized to formaldehyde by a periplasmic pyroloquinolone quinone-linked methanol dehydrogenase. Formaldehyde is either oxidized to CO$_2$ or assimilated in cytoplasm through ribulose monophosphate (RuMP) and EMP/EDD (Embden-Meyerhof-Parnas/Entner-Doudoroff) pathways (A), fraction of which are then converted through pyruvate to acetyl-CoA, a precursor for FA synthesis (B). Designations: CH$_3$H, methane; CH$_3$O, formaldehyde; CH$_2$O$_2$H, formate; H$_4$MTP, tetrahydromethanopterin pathway; H$_4$F, methylene tetrahydrofolate pathway; CO$_2$, carbon dioxide; 6p, fructose 6-phosphate; acCoA, acetyl-CoA; NADH, nicotinamide adenine dinucleotide reduced; NADPH, dihydrornicotinamide adenine dinucleotide phosphate reduced; ATP, adenosine-triphosphate.

TABLE 1 | List of strains and plasmids used in this study.

| Strain or Plasmid | Description | Reference source |
|-------------------|-------------|------------------|
| E. coli DH5α      | F–Φ80lacZ,ΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1 | Puri and Lidstrom, unpublished data |
| E. coli DH5α      | Strain with chromosomally integrated conjugal RP4 transfer functions for biparental conjugation | |
| AP18              | M. buryatense 5G(B1) ΔΔglgA1 ΔΔglgA2 ΔΔpsp (WP_017842105.1, WP_017842106.1, WP_017840914.1), cured of native plasmid | |
| AP18Δack          | AP18 ΔΔack (WP_017840011.1) | This study |
| AP18ΔfadABE       | AP18 ΔΔfadA (WP_017841934.1) ΔΔfadB (WP_017841933.1) ΔΔfadE (WP_017841932.1) | This study |
| AP18: ΔΔack:acc   | AP18 with pAWP145 containing accA (WP_017842193.1), accB (WP_017839152.1), accC (WP_017839151.1), and accD (WP_017839055.1) | This study |
| pCM184            | Plasmid for making kanamycin-resistant mutants | Marx, 2008 |
| pCM184Δsck        | Plasmid for making kanamycin-resistant mutants | Marx and Lidstrom, 2002 |
| pAWP78            | IncP-based broad host range plasmid for gene overexpressions | Puri et al., 2015 |
| pAWP145           | Tetracycline-inducible construct for overexpression of AccA (WP_017842193.1), AccB (WP_017839152.1), AccC (WP_017839151.1), and AccD (WP_017839055.1) | Puri and Lindstrom lab, UW |
| pCM433Δsck        | Variant of pCM433 containing flanks to knock out Sck | Lindstrom, UW |
| pCM433ΔfadABE     | Variant of pCM433 containing flanks to knock out FabA, FabB, and FabE | This study |
| pCM184ΔfarE       | Variant of pCM184 containing flanks to knock out FarE | This study |
| pAWP78::farE      | Variant of pAWP78 containing FarE | This study |
| pAWP78::fadB      | Variant of pAWP78 containing FabB | This study |
| pAWP78::acpP      | Variant of pAWP78 containing ScpP | This study |
| pAWP78::fadB::acpP| Variant of pAWP78 containing FabB and ScpP | This study |

Construction and Refinement of the Flux Balance Model for M. buryatense 5G(B1) in COBRA Toolbox

To convert the flux balance model for *M. buryatense 5G(B1)* into the COBRA Toolbox format (Schellenberger et al., 2011) we used the published genome-scale metabolic network of this strain (De la Torre et al., 2015) developed in Pathway Tools (Karp et al., 2010). Subsequently, all zero-flux reactions were removed from the model. The information about zero-flux reactions was extracted from the solution file of the Pathway Tools. We manually extended the COBRA model by adding exchange rates for both nutrients and secreted metabolites as well as by the biomass equation. Moreover, we used the Paint4Net tool (Kostromins and Stalidzans, 2012)
installed with the COBRA Toolbox to define dead-ends in the model network and to add corresponding exchange rates for them. To standardize reaction and metabolite IDs, we used abbreviations according to BiGG Models ID Specification and Guidelines (King et al., 2016) and KEGG abbreviations for enzymatic reactions for which we could not find corresponding BiGG reactions. To link genes with certain enzymatic reactions we turned to account NCBI Reference Sequence annotation: NZ_KB455575. Moreover, we added missing formulas for metabolites and checked out the model in COBRA Toolbox to ensure all reactions were mass balanced. The model was also extended by the addition of enzymatic reactions (EC 3.1.2.2; 1.3.8.-; 4.2.1.17; 1.1.1.35; 2.3.1.16) for FA degradation via the β-oxidation pathway and a reaction catalyzed by phosphoketolase (EC 4.1.2.22). Flux balance analysis (FBA) was performed by solving the Linear Programming problem for biomass optimization on the basis of GNU Linear Programming Kit (GLPK) (http://www.gnu.org/software/glpk/) solver in MATLAB. The converted model for *M. buryatense 5G(B1)* in COBRA format is available on the web-site: http://sci.sdsu.edu/kalyuzhlab/.

### Computational Analysis of the RNA-Seq Data

The genome sequence and annotation files of *M. buryatense 5G(B1)* were obtained from NCBI (RefSeq accession number GCF_000341735.1). Reads were aligned to the genome sequence using the Rockhopper 2 software system (Tjaden, 2015) with default settings. It is worth noting that the tool reports integral values for analyzed transcriptomic characteristics, even if the underlying value is a decimal. If a read aligns partially to a transcript, it will contribute a fractional amount to the raw count. Rockhopper 2 normalizes each RNA-seq data set using upper quartile normalization, because the normalization procedure of the mapped read counts is an essential preprocessing step for differential expression detection (Bullard et al., 2010). To quantify transcript abundance levels, Rockhopper 2 employs a modified RPKM value based on the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Appropriateness of the q-value threshold <0.05 was verified with a set of housekeeping genes and genes encoding key C1-enzymes, and those showed no differential expression [for example: 3-hexulose-6-phosphate synthase, q(AP18 vs. AP18ΔfarE) = 0.28, q(AP18 + pAWP78 vs. AP18:farE) = 0.53; pyruvate carboxylase, q(AP18 vs. AP18AfarE) = 0.41, q(AP18 + pAWP78 vs. AP18::farE) = 0.64; arginyl-tRNA-protein transferase, q(AP18 vs. AP18ΔfarE) = 1, q(AP18 + pAWP78 vs. AP18::farE) = 1].

### RESULTS

#### Lipid Biosynthesis and Degradation: *M. buryatense 5G(B1)* Genome Mining

Methane metabolism in the *M. buryatense 5G(B1)* strain is outlined in Figure 1A. The automatic annotation of the genome predicts a typical Type II FA biosynthesis pathway. The FA-biosynthesis genes form several clusters: (1) a plsXfabHDGacpfabF-cluster, which includes genes for a fatty acid/phospholipid synthesis protein, 3-oxoacyl-ACP synthase I, malonyl-CoA-ACP transacylase, 3-oxoacyl-ACP reductase, acyl carrier protein (ACP), and 3-oxoacyl-ACP synthase II. The cluster also includes a hypothetical protein and a S05 ribosomal protein L32 gene upstream of plsX. The organization of this cluster is strongly conserved among all methanotrophic bacteria; (2) a fabAB-cluster encoding 3-oxoacyl-ACP synthase I and beta-hydroxydecanoyl thioester dehydrase; (3) a fabG/zfzA cluster, encoding 3-oxoacyl-ACP synthase II, 3-oxoacyl-ACP reductase, and beta-hydroxyacyl-ACP dehydratase; and (4) an accBb-cluster gene encoding biotin carboxylase and biotin carboxy carrier protein (BCCP). Genes encoding the acetyl-CoA carboxylase carboxyltransferase alpha subunit (*accA*) and beta subunit (*accD*), a second copy of the acetyl-CoA carboxylase biotin carboxylase subunit (*accC*), an additional (3R)-hydroxymyrirstol ACP dehydratase (*fabZ*), and two genes for ACP are located at sites distant from each other and other FA-biosynthesis genes. 

Synthesis of phospholipids in *M. buryatense 5G(B1)* appears to follow the E. coli paradigm where a phosphatidic acid precursor is synthesized from glycerol 3-phosphate and FA precursors (Yao and Rock, 2012). However, no *plbB* (Glycerol-3-phosphate acyltransferase) homologs were identified within the *M. buryatense 5G(B1)* genome. Instead, homologs of *plsX* and * plsY* were found. *PlsXT* has been shown to preferentially utilize acyl-acetyl carrier protein and not acyl-CoA as an acyl donor (Lu et al., 2006). A set of phospholipid-modifying enzymes whose homologs have been shown to act on phospholipids post-synthetically were found in the genome. The first of these enzymes is a membrane-bound desaturase, *DesC*, which shows about 60% identity to other well-characterized desaturases that catalyze the oxygen-dependent introduction of a double bond at the Δ9 position of saturated FAs, particularly stearic acid (Zhu et al., 2006; Parsons and Rock, 2013). Additionally, *M. buryatense 5G(B1)* contains two copies of *cfa*, a cyclopropane FA synthase, which uses S-adenosyl methionine to generate a cyclopropyl group at a double bond position of a FA (Grogan and Cronan, 1997). Cyclopropane FAs have been previously observed in other methanotrophic bacteria (*Methylococcus capsulatus*, *Methyllobacterium organophilum*) during growth on methane and during oxygen limitation (Patt and Hanson, 1978; Jahne and Nicholst, 1986). An intriguing aspect of these lipid-modifying enzymes in *M. buryatense 5G(B1)* is that both copies of *cfa* and *desC* are clustered together as part of a larger operon. Additionally the operon appears to encode a histidine sensor kinase, a lipase/esterase, and a sterol reductase gene. The physiological significance of this is unclear; however, it is tempting to speculate that these various activities are genetically
biosynthesis in methanotrophs is not regulated as formation of an as-yet-to-be discovered mechanism. It seems unlikely that FA biosynthesis in M. buryatense 5G(B1) cannot transport fatty acids. However, the rest of genes, fadA, fadB, and fadE, responsible for the degradation and recycling of FAs via β-oxidation, were identified. The presence of the PlsXY pathway for phospholipids biosynthesis and the lack of a FadD homolog suggest that M. buryatense 5G(B1) is not capable of incorporating exogenous fatty acids into its phospholipid membranes.

Neither fabR/desT nor fabR (a known transcriptional regulators of fatty acid biosynthesis/degradation in E.coli) homologs were detected. The lack of any recognizable transcriptional regulatory factors is unique and could imply that FA biosynthesis in M. buryatense 5G(B1) is being regulated by an as-yet-to-be discovered mechanism. It seems unlikely that FA biosynthesis in methanotrophs is not regulated as formation of ICMs is commensurate with an increase of FA content per cell (Gilman et al., 2015).

**Determining Control Points in Fatty Biosynthesis**

The overreaching goal of the study was to identify targets to manipulate fatty acid biosynthesis in methanotrophs. The FBA suggests that about 7.5% of methane carbon enters the fatty acid biosynthesis pathway. According to genomic data, M. buryatense 5G(B1) possesses three different metabolic routes for production of acetyl-CoA from single carbon compounds with the main reaction contributing to acetyl-CoA formation predicted to be pyruvate dehydrogenase (Figure 1A). The FBA predictions are consistent with published enzymatic evidence and transcriptomic data (Figure 1; Kalyuzhnaya et al., 1999; De la Torre et al., 2015), as well as with transcriptomic data generated in this study.

ACP is one of the key proteins in FA biosynthesis. In the M. buryatense 5G(B1) genome there are three genes encoding ACP of which only one is highly expressed (Table 4). The differential regulation of ACP expression raised a question about whether M. buryatense 5G(B1) FA metabolism is saturated with ACP or whether overproduction of ACP would stimulate FA synthesis. If ACP is a limiting factor, its overexpression would lead to an overall increase of the FA production. Alternatively, adverse effects on cell physiology might be observed if cells do not tolerate higher ACP levels. To distinguish between these hypotheses, we chose to overexpress the highly expressed acp gene (WP_014148504.1). An ACP-overexpressing plasmid was constructed and introduced into the AP18 strain. Unlike most other strains tested in this study, the ACP-upregulated strain showed growth defects (Figure 2) and no signs of increased FA production (Table 2, Figure 3). Addition of FabB to the overexpressing ACP strain did not enhance its FAME content. Thus, higher levels of ACP production seem to be toxic for the methanotrophic culture, as seen for E. coli (Keating et al., 1995).

To determine whether FA biosynthesis in methanotrophs is limited by the elongation step, ketoacyl-ACP synthases (fabB) overexpression was examined. No changes in either the amount of FAME or composition of FAME were observed (Table 2, Figure 3). In order to define the role of β-oxidation in methanotrophic FA metabolism, the entire cluster of fadA, fadB, and fadE genes was deleted. The Δfad(ABE) mutant did not show significant changes in growth characteristics (Figure 2); furthermore, the Δfad(ABE)-mutant cells had higher levels of FAs (10% increase compared to original AP18 strain; Table 2, Figure 3).

**Improving Carbon Flux into Fatty Acid Building Blocks: Acetyl- and Malonyl-CoA**

Having established that neither ACP nor elongation of FA synthesis are limiting steps in FA accumulation, we then tested the possibility that directing carbon flux into acetyl-CoA and malonyl-CoA pools would have a positive impact on total FA accumulation. M. buryatense 5G(B1) cells express acetate (∼100 μmol gCDW−1) and formate (∼500 μmol gCDW−1) during growth on methane (Gilman et al., 2015).
concentrations of excreted organic acids are not high; however, the leak of C2-compounds might indicate an additional loss of carbon needed for FA biosynthesis. In order to increase carbon flow into FA biosynthesis two additional modifications were made: deletion of acetate kinase (Δack) and overexpression of acetyl-CoA carboxylase (acc). It should be mentioned that the overexpression of acetyl-CoA carboxylase (accABCD) in the M. buryatense 5G(B1) strain did not show a statistically significant increase (Puri and Lidstrom, unpublished data). Here, we tested acc-overexpression in different genetic backgrounds. The AP18Δack and overproducing Acc strains were found to produce 99 ± 7 and 108 ± 2 mg/gDCW of FAME, respectively (Table 2, Figure 3). Moreover, when combined together in the same strain, the effects of both mutations were additive resulting in the highest FA levels (111 ± 2 mg/gDCW, ∼20% more than in the original AP18) achieved. Deletion of fabABE genes in AP18Δack::acc background did not increase FAME levels.

### Regulation of Fatty Acid Biosynthesis in M. buryatense 5G(B1)

The M. buryatense 5G(B1) genome does not contain proteins homologous to typical bacterial regulators of FA metabolism, such as fabR, fadR, or desT. However, an orf (WP_017839568) with a weak homology to the nucleotide-binding Maf family proteins was identified upstream of the plsXfabHDGacpfabF-cluster. Homologous genes were identified in eight methanotrophic genomes (M. buryatense, Methylocrobium alcaliphilum, Methylobacterium marinus, Methylocrobium luteus, M. sp MK WGS, M. sp.11b, Methylocrobium methanica, and M. capsulatus), and in each case the gene located upstream of the FA biosynthesis genes. The conserved proximity of the fab-gene suggested it could be linked to FA metabolism. A WP_017839568 deletion mutant was generated and shown to have a severe growth defect (Figure 2). Cellular lipid content was also impaired: not only was the FAME content decreased but also there was an unusually high amount of C18:1 complemented by a decrease in C16:1 (Table 3). Reciprocal up-regulation (overexpression) of the WP_017839568 gene showed no growth defects and approximately the same FA profile as the initial strain (Table 3).

This striking phenotype of the WP_017839568-deleted strain suggested the possibility that this gene might be involved in the regulation FA synthesis, especially elongation of growing FA chains past 16 carbon atoms. In this regard, we proposed the name farE (for Fatty Acid Regulator of Elongation) for the WP_017839568 gene. In order to investigate the mechanism of such regulation, total RNA sequencing was performed (Table S3) for the following strains: (i) original AP18 strain (ii) AP18ΔfarE, (iii) AP18::farE, and (iv) AP18::pAWP78 (empty plasmid). By comparison of AP18 to AP18ΔfarE, ∼1600 differentially expressed transcripts were identified. The majority of the differentially expressed genes are related to general metabolism and cellular functions (not shown), which could be explained by significant differences in growth rates between wild type and the farE-mutant. A subset of differentially regulated genes upon farE deletion is involved in FA biosynthesis or maintaining the acyl-CoA pool (Table 4). They include all three copies of the acpP gene present in M. buryatense 5G(B1) genome, two copies of the fabF and fabA genes, and the fabG and fabD genes. Two more insufficiently annotated genes included another copy of 3-ketoacyl-ACP synthase (WP_017841167.1) and a new thioesterase (WP_040575583.1, acyl-CoA thioester hydrolase). Considering their function in elongation of FA biosynthesis, upregulation of either 3-ketoacyl-ACP synthase (two copies of fabF and WP_017841167.1) could be a logical explanation for the elevated levels of the normally uncommon C18 FA in M. buryatense 5G(B1). Upon deletion of farE, the Acp expression pattern changes so that all three acpP genes are slightly upregulated (1.32x, 1.75x, and 2.13x).

In order to further narrow down the list of potential candidates for explaining changes in the ΔfarE mutant, we compiled a table (Table 5) including only those FA

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**TABLE 2** | FA methyl ester (FAME) content of the strains used in this study (percent = FAME/dry cell weight).

| Strain           | FAME %   | Increase, folds over AP18 |
|------------------|----------|---------------------------|
| AP18             | 9.17 ± 0.80 | 1.00                     |
| AP18Δack         | 9.88 ± 0.72 | 1.08                     |
| AP18ΔfadABE      | 10.12 ± 0.89 | 1.10                     |
| AP18::acc        | 10.77 ± 0.18 | 1.17                     |
| AP18::ack::acc   | 11.07 ± 0.22 | 1.21                     |
| AP18ΔfadABEΔack::acc | 10.63 ± 0.45 | 1.16                     |
| AP18::farE       | 6.71 ± 0.51  | 0.73                     |
| AP18::fare       | 9.14 ± 0.15  | 1.00                     |
| AP18::fabB       | 9.04 ± 0.19  | 0.99                     |
| AP18::acp        | 8.76 ± 0.43  | 0.96                     |
| AP18::fabB::acp  | 8.54 ± 0.25  | 0.93                     |

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**FIGURE 3** | Graphic presentation of FAME levels in the same strains as in Table 2 (percent = FAME/dry cell weight).
TABLE 3 | FA profile of original AP18 and strains with up- and down-regulated farE.

| Strain                          | Fatty acid, mg/gDCW |
|---------------------------------|---------------------|
|                                 | C14:0 | C15:0 | C16:1n7 | C16:1n9 | C16:0 | C18:1 | C18:0 |
| AP18, replicate 1               | 3.23  | 0.48  | 55.38   | 12.07   | 14.95 | 0.25  | 0.30  |
| AP18, replicate 2               | 3.32  | 0.40  | 50.09   | 9.17    | 13.39 | 0.27  | 0.20  |
| AP18::faeR, replicate 1         | 0.00  | 0.00  | 23.49   | 6.24    | 8.70  | 0.97  | 0.97  |
| AP18::faeR, replicate 2         | 0.00  | 0.23  | 23.84   | 7.73    | 9.05  | 28.09 | 1.72  |
| AP18::faeR, replicate 1         | 4.53  | 0.62  | 54.34   | 15.36   | 16.89 | 0.24  | 0.49  |
| AP18::faeR, replicate 2         | 4.25  | 0.59  | 52.65   | 14.93   | 17.31 | 0.22  | 0.54  |

TABLE 4 | Differentially regulated genes of FA biosynthesis with down- and up-regulated farE compared to original AP18 (q < 0.05).

| Gene ID          | Gene function                                      | Expression |
|------------------|----------------------------------------------------|------------|
| fabA             | 3-Hydroxyacyl-ACP dehydratase                      | 89         |
| fabA             | 3-Hydroxydecanoyl-ACP dehydratase; 3-Hydroxyacyl-ACP dehydratase | 121        |
| fabG             | 3-Ketoacyl-ACP reductase                           | 39         |
| fabG             | 3-Ketoacyl-ACP synthase                            | 166        |
| fabF             | 3-Oxoacyl-ACP synthase                             | 40         |
| fabF             | 3-Oxoacyl-ACP synthase; 3-Ketoacyl-ACP synthase    | 73         |
| acpP             | Acetyl-CoA carboxylase subunit alpha                | 1336       |
| acpP             | Acyl carrier protein                               | 29         |
| acpP             | Acyl carrier protein                               | 1133       |
| fabD             | Malonyl CoA-ACP transacylase                       | 391        |
| farE             | Fatty acid regulator of elongation                 | 29         |

Calculated normalized transcript abundance levels are show.

biosynthesis-related genes that are regulated in opposite directions in ΔfaeR and farE overexpression strains. In addition, genes differentially regulated in AP18 and AP18::pAWP78 (empty plasmid) were also removed. That allowed us to narrow the list to only three entries (FabF, WP_026130034.1; 3-hydroxydecanoyl-ACP dehydratase, WP_017839698.1; 2-isopropylmalate synthase, WP_017840721.1). Effects of up- and down-regulation of these genes will be tested in future studies.

DISCUSSION

Microbial fatty acid biosynthesis pathways are among the most attractive targets for the production of fuels and oleochemicals from renewable feedstocks (Lennen and Pfleger, 2013; Janßen and Steinbuchel, 2014). Different microbial systems as well as numerous metabolic engineering strategies have been explored for improving biofuel production (Lennen and Pfleger, 2013). The most common approaches aim to reduce the catabolism of fatty acids (e.g., deletion of fabBAE or fabR genes), to eliminate feedback inhibition in the biosynthetic pathway (e.g., overexpression of accABC, fabD with/without thioesterase I (tesA) genes and/or deletion of fabR), as well as incorporating heterologous enzymes or metabolic pathways for converting the FA intermediates into free fatty acids, fatty acid methyl esters, and fatty acid ethyl esters, olefins, etc. (Clomburg and Gonzales, 2010; Lennen and Pfleger, 2013; Janßen and Steinbuchel, 2014).

Core metabolic functions of many methanotrophic bacteria are interlinked with the development of intracytoplasmic membranes. As a result, methanotrophic bacteria have relatively high lipid/biomass content (9–10% of dry weight, mostly as phospholipids). Here, we investigated fatty acid biosynthesis in M. buryatense 5G(B1). Overall, the metabolic pathways for fatty acid production and degradation in M. buryatense 5G(B1) resemble the canonical bacterial pathways with only a few exceptions, such as the lack of recognizable fadD/fadL and regulators. Absence of the fadL gene is not surprising since M. buryatense 5G(B1) is known for not being able to utilize long-chain FAs as a carbon or energy source. On the other hand, the absence of fadD, the mechanism by which free FAs becomes activated (i.e., ligation to CoA) for beta oxidation is puzzling. The phenotype of fadABE mutants indicates that beta-oxidation pathway is active in M. buryatense 5G(B1). A set of
TABLE 5 | Opposite directions* of differentially expressed genes in two datasets: original AP18 vs. down-regulated farE and AP18+pAWP78 compared to up-regulated farE.

| FATTY ACID METABOLISM                      | Fold change |            |
|-------------------------------------------|-------------|------------|
|                                            | AP18 vs. AP18ΔfarE | AP18+pAWP78 vs. AP18::farE |
| WP_026130034.1                            | 0.9         | 2.5        |
| 3-Oxoacyl-ACP synthase (tabF)              | 1.3         | 1.9        |
| WP_014148504.1**                          | 0.8         | 1.1        |
| ACP                                       | 0.3         | 2.2        |
| WP_017839673.1***                         | 0.9         | 4.6        |
| Beta-ketoacyl-ACP reductase (tabG)         | 2.2         | 0.8        |
| WP_017839672.1 ***                        | 1.3         | 1.9        |
| Malonyl CoA-ACP transacylase               | 0.6         | 2.7        |
| WP_017839671.1 ***                        | 0.4         | 4.6        |
| 3-Oxacyl-ACP synthase                     | 0.9         | 1.8        |
| WP_017839570.1 ***                        | 0.9         | 1.8        |
| Phosphate acyltransferase                 | 0.9         | 1.8        |
| WP_017839698.1                           | 0.9         | 1.8        |
| 3-Hydroxydecanoyl-ACP dehydratase         | 0.8         | 1.1        |
| WP_017839697.1**                          | 0.9         | 2.5        |
| Beta-ketoacyl-ACP synthase I              | 1.3         | 1.9        |

| Acetyl-CoA POOL                           |            |
|-------------------------------------------|------------|
| WP_017840721.1                           | 0.9         | 1.2        |
| 2-Isopropylmalate synthase                | 1.4         | 0.6        |
| WP_026130168.1**                         |            |
| CDP-diacylglycerol-serine                 |            |
| 0-phosphatidyl transferase                |            |

*Down-regulated in AP18 vs. AP18ΔfarE and up-regulated in AP18+pAWP78 vs. AP18::farE;
**Neighboring gene; ***Neighboring genes from one gene cluster.

genes annotated as Acyl-CoA synthetases were identified in the genome, each of them might potentially fulfill the fabD function.

Given that intra-cytoplasmic membranes are primarily produced during growth on methane and that a membrane-bound methane monoxygenase, pMMO, is associated with these membranes, a regulatory mechanism must exist to repress FA biosynthesis during growth on non-methane carbon sources (i.e., methanol). The initial hypothesis posed that farE could encode a FA metabolism regulator. Our results support this hypothesis. farE deletion changes expression of many metabolic genes including several involved in FA biosynthesis; the FA content of the mutant is also drastically changed. The phenotypic feature of AP18ΔfarE, i.e., conversion of a significant portion of C16 FA into C18 FA, could be potentially explained by overproduction of 3 FA synthesis elongation enzymes, two fabF genes and one less characterized 3-ketoacyl-ACP synthase gene (WP_017841167.1). Another option for accumulation of C18 FAs could be overregulated acpP genes. Only one of the 3 acpP genes is highly transcribed (WP_014148504.1); the two others are transcribed in low amounts. However, these low-expressed copies of acyl carrier proteins could have different properties from the main one resulting in synthesis of longer FAs. It has been recently demonstrated that the size of the Acp hydrophobic pocket influences the length of FA synthesized, with smaller pockets leading to cellular accumulation of shorter FAs (Liu et al., 2016). In our case we would expect that one of the Acp copies would have a bigger hydrophobic pocket resulting in synthesis of longer lipids. In addition, FarE contributed to upregulation of a number FA biosynthesis genes, including fabA (hydroxacyl-ACP dehydratase), fabG (3-ketoacyl-ACP reductase), and thioesterase. Overall, it could be suggested that FarE acts as a regulator of fatty acid metabolism, although there is a possibility that the observed AP18ΔfarE phenotype could be achieved through indirect action of unidentified regulatory mechanisms.

A set of different strategies was tested for improving fatty acid production in M. buryatense 5G(B1). While the overexpression of the fabB did not alter the FA composition significantly, the modulation of FA synthesis elongation seems to be a viable strategy to manipulate the FA content in methanotrophs. Analysis of transcriptomics data of the AP18ΔfarE strain suggested three potential candidates for synthesis of longer-than-usual C18 FA in M. buryatense 5G(B1). These proteins with 3-ketoacyl-ACP synthase (KAS) activities comprise 2 fabF genes and one previously unidentified KAS (WP_017841167.1) gene. Previously, fabF was identified as an activator of FA biosynthesis in E. coli (Lee et al., 2013), which makes it attractive target for biotechnological manipulation. Another difference is the ability of fabB but not fabB to elongate palmitoleic acid (C16:1) for synthesis of a C18:1 version (Edwards et al., 1997). However, whether the increased activity of fabF would be sufficient for switching of FA biosynthesis from predominantly C16 FA to C18 remains a question. In this regard, the third KAS enzyme (WP_017841167.1), which is still uncharacterized, could also be considered. Whether overexpression of any of them would result in changes in FA profile will be accessed in future studies.

It has been previously observed that overexpression of acpP from a multi-copy plasmid leads to inhibition of cell growth in E. coli (Keating et al., 1995). That effect was attributed to an unmodified (Apo) form of acyl carrier protein since overexpressed ACP was incompletely post-translationally modified and accumulation of apo-Acp resulted in an inability to transfer the completed FA to glycerol 3-phosphate. However, lower levels of Acp, including AcpS from other organisms, could be produced in E. coli without toxicity (Guerra et al., 1988). Since the overexpression plasmid used in the current study (pAWP78, Puri et al., 2015) is a medium- to low-copy number plasmid, we did not expect inhibitory effects of Acp overproduction in M. buryatense 5G(B1). However, adverse effects resulting in inhibition of growth upon overproduction of Acp were observed indicating that the mechanism of post-translation modification of Acp by holo-ACP synthase is adjusted to regulate Acp levels and might be inefficient when Acp is overproduced. Further attempts to upregulate FA accumulation by Acp overproduction should be supported by simultaneous overproduction of holo-ACP synthase.
The genetic alterations of fatty acid turnover as well as key elements of fatty acid biosynthesis resulted in very minor changes in overall lipid content, suggesting the capacity of the system is restricted by the overall influx of carbon into the pathway. However, strains with ack deleted (block in acetyl-CoA to acetate conversion) showed an increased FA content. Moreover, a strain with both \( \Delta \text{ack} \) and overexpression of \( \text{acc} \) had the highest FAME content among all tested in this study. The inconsistencies in acc-overexpression outcomes could be explained by insufficient biotinylatation of the protein.

**SUMMARY**

Metabolic pathways for fatty acid biosynthesis are reconstructed and a set of novel mechanisms contributing to FA-biosynthesis regulation and metabolic precursor formation are proposed. *M. buryatense 5G(B1)* naturally accumulates more FA than many other bacteria in order to maintain high amounts ICM structures. The existence of a yet-to-be discovered mechanism of regulation of FA levels.

**AUTHOR CONTRIBUTIONS**

AD, EA, and MK designed the experiments and coordinated the study. AD carried out all genetic, growth, and transcriptomic experiments and analyzed data. AD and MA performed genome mining and wrote the first draft of the manuscript. IA contributed to model construction and RNA-data analyses. MK and EA helped to analyze the data and write the manuscript. All authors reviewed and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.02167/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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