Metabolic Engineering 42 (2017) 43–51

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/meteng

The oxidative TCA cycle operates during methanotrophic growth of the Type I methanotroph *Methylomicrobium buryatense* 5GB1

Yanfen Fu[a], Yi Li[a], Mary Lidstrom[a,b,*]

[a] Department of Chemical Engineering, University of Washington, 616 NE, Northlake Place, Seattle, WA 98195, USA
[b] Department of Microbiology, University of Washington, 616 NE, Northlake Place, Seattle, WA 98195, USA

ARTICLE INFO

Keywords:
^{13}C tracer
Methanotroph
Methane
TCA cycle
Methylomicrobium buryatense

ABSTRACT

Methanotrophs are a group of bacteria that use methane as sole carbon and energy source. Type I methanotrophs are gamma-proteobacterial methanotrophs using the ribulose monophosphate cycle (RuMP) cycle for methane assimilation. In order to facilitate metabolic engineering in the industrially promising Type I methanotroph *Methylomicrobium buryatense* 5GB1, flux analysis of cellular metabolism is needed and ^{13}C tracer analysis is a foundational tool for such work. This biological system has a single-carbon input and a special network topology that together pose challenges to the current well-established methodology for ^{13}C tracer analysis using a multi-carbon input such as glucose, and to date, no ^{13}C tracer analysis of flux in a Type I methanotroph has been reported. In this study, we showed that by monitoring labeling patterns of several key intermediate metabolites in core metabolism, it is possible to quantitate the relative flux ratios for important branch points, such as the malate node. In addition, it is possible to assess the operation of the TCA cycle, which has been thought to be incomplete in Type I methanotrophs. Surprisingly, our analysis provides direct evidence of a complete, oxidative TCA cycle operating in *M. buryatense* 5GB1 using methane as sole carbon and energy substrate, contributing about 45% of the total flux for *de novo* malate production. Combined with mutant analysis, this method was able to identify *fumA* (METBUDRAFT_1453/MBURv2_60244) as the primary fumarase involved in the oxidative TCA cycle, among 2 predicted fumarases, supported by ^{13}C tracer analysis on both *fumA* and *fumC* single knockouts. Interrupting the oxidative TCA cycle leads to a severe growth defect, suggesting that the oxidative TCA cycle functions to not only provide precursors for *de novo* biomass synthesis, but also to provide reducing power to the system. This information provides new opportunities for metabolic engineering of *M. buryatense* for the production of industrially relevant products.

1. Introduction

Methane is the second most prevalent greenhouse gas, and is also a cheap, abundant and renewable carbon source (Fei et al., 2014; Haynes and Gonzalez, 2014). Converting methane to valuable products at an industrial scale using methanotrophs will require iterations of strain optimization for higher yield and efficiency. *M. buryatense* 5GB1 is a Type I (gamma-proteobacterial) methanotroph, which is highly resistant to contamination due to the high pH and salt in optimal culture conditions (Khmelenina et al., 1997). *M. buryatense* 5GB1 is a good candidate for industrial scale application of methane bioconversion to valuable chemicals (Kalyuzhnaya et al., 2015a), with a series of developed genetic manipulation tools (Puri et al., 2015; Yan et al., 2016) as well as bioreactor performance datasets (Gilman et al., 2015) available and a demonstrated growth rate (Gilman et al., 2015) of 0.23 h^{-1}. The whole genome of *M. buryatense* 5GB1 was also annotated and published (Khmelenina et al., 2013). However, in order to facilitate the process of metabolic engineering of *M. buryatense* 5GB1, which is a non-model organism, quantitative characterization of cellular metabolism using ^{13}C tracer analysis needs to be developed. Such approaches have facilitated applications of well-studied model organisms such as *E. coli* (He et al., 2014; Wada et al., 2017; Gonzalez et al., 2017). Stable isotope tracer experiments along with flux estimation and analysis is a key component of the metabolic engineering toolbox (Buescher et al., 2015; Klapa and Stephanopoulos, 2000; Toya and Shimizu, 2013). This approach has not yet been developed and used in Type I methanotrophs to quantitatively describe the metabolic phenotype of cells. Steady state ^{13}C flux analysis is well-established in other organisms with multi-carbon inputs (Wiechert, 2001; Wittmann, 2007; Zamboni et al., 2009). However, the details of the network topology and the single carbon input of methanotrophs results in only partial flux elucidation from steady state isotope tracer experiments. The predicted core metabolic

* Correspondence to: Department of Chemical Engineering, University of Washington, 616 NE Northlake Place, Benjamin Hall Room 440, Seattle, WA 98105, USA.
E-mail addresses: fyf@uw.edu (Y. Fu), liyi396679338@gmail.com (Y. Li), lidstrom@uw.edu (M. Lidstrom).

http://dx.doi.org/10.1016/j.ymben.2017.05.003
Received 7 December 2016; Received in revised form 19 May 2017; Accepted 20 May 2017
Available online 25 May 2017

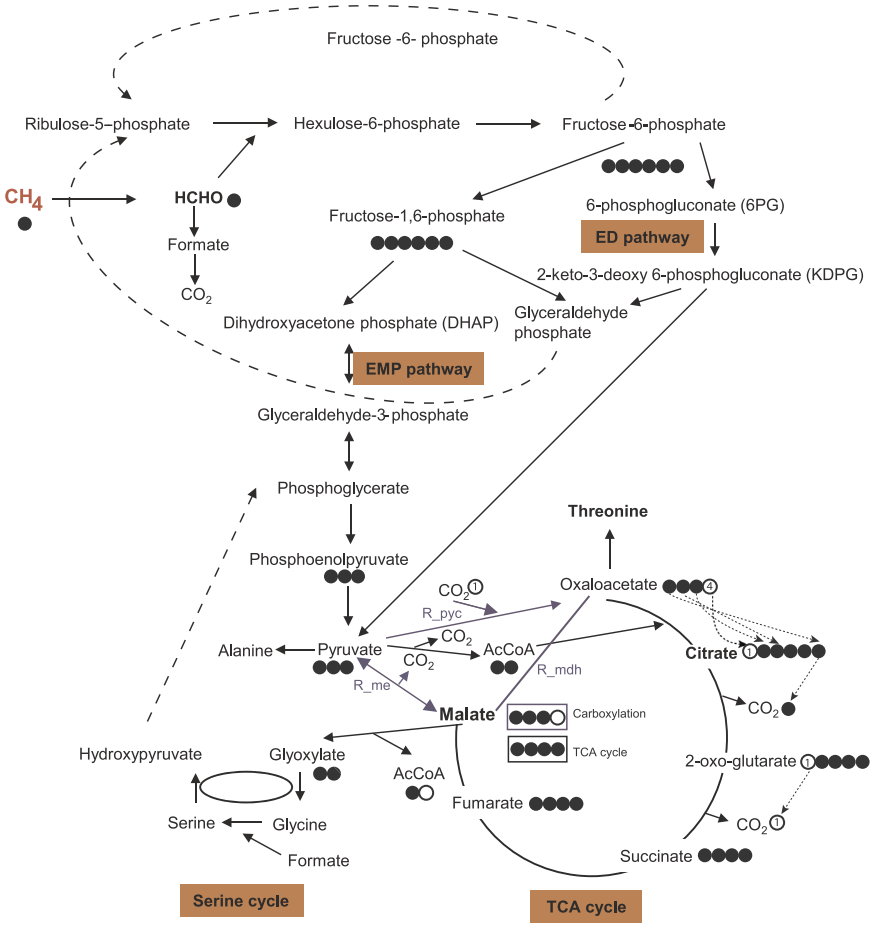

Fig. 1. Central carbon metabolism pathways of *M. buryatense* 5GB1 during growth on methane and atom transition mapping for the sub-network. The black circles with numbers are carbons with mixed labeling pattern, the numbers indicating the atom position in the carbon skeleton for specific metabolites. Methane (red) is the ^{13}C tracer substrate. The solid black circles indicate ^{13}C labeled carbon atoms. Based on the network topology as well as atom transition, two forms of malate could be synthesized through carboxylation pathways in blue (R_pyc, R_mdh, R_me) and TCA cycle. The C3 compounds as well as metabolites in RuMP cycle will be fully labeled are also selectively represented.

pathways operating in *M. buryatense* 5GB1 summarized in Fig. 1 have been identified through both genome sequence as well as transcriptome analysis (de la Torre et al., 2015). Methane is first oxidized to methanol through pMMO (particulate methane monooxygenase), and then converted into formaldehyde via methanol dehydrogenase. Formaldehyde is the first branch point, where it can either enter assimilation using the ribulose monophosphate cycle (RuMP cycle) or be further oxidized to formate and CO_2 to produce NADH. There are two variants of the RuMP cycle in *M. buryatense* 5GB1, the Embden-Meyerhof-Parnas (EMP) variant as well as the Entner–Doudoroff (ED) variant, which convert formaldehyde into C3 intermediate metabolites, such as pyruvate and phosphoenolpyruvate (PEP). Downstream of the pyruvate node, a series of multi-carbon compound inter-conversions lead to carbon precursors for biosynthesis, such as oxaloacetate (OAA), 2-oxoglutarate and acetyl-CoA (AcCoA).

One essential foundation of steady state ^{13}C tracer analysis is differentiated carbon rearrangements resulting from different metabolic pathways. However, this condition is not met in the *M. buryatense* 5GB1 metabolic network topology. The net outcome of the RuMP cycle is condensing 3 molecules of formaldehyde into 1 molecule of C3 compounds such as pyruvate. In this case, all intermediate metabolites will become fully labeled with the ^{13}C input. The two pathway variants both lead to fully labeled product, which makes the flux partition between EMP and ED variants unresolvable by steady state ^{13}C tracer analysis. However, carboxylation and decarboxylation steps downstream of the pyruvate and PEP nodes result in pathways that lead to differentiated labeling patterns. Several carboxylation reactions and decarboxylation reactions are involved in anaplerotic reactions as well as the TCA cycle. The carbon skeleton is also rearranged through malyl-CoA lyase reactions. Table 1 summarizes all the carbon atom transitions for the sub-network downstream of the PEP node. These transitions create the opportunity to determine fluxes through the steady state isotopomer labeling patterns. However, several parallel reactions (R_pyc, R_me, and R_mdh) with identical atom transitions (highlighted in blue, Fig. 1) exist in the network, which places more challenges on resolving the flux between those reactions. Here we use mutant analysis coupled with ^{13}C tracer analysis to solve this challenge and quantitate the flux ratio in a sub-network downstream of the PEP node.

Table 1
Carbon atom transition in sub-networks of core metabolism in M. buryatense 5GB1.

Reaction name	Stoichiometry	Atom transition
R_pk	PEP - > PYR	ABC - > ABC
R_pyc	PYR + CO2 - > OAA	ABC + D - > ABCD
R_me	PYR + CO2 - > MAL	ABC + D - > ABCD
R_mdh	OAA - > MAL	ABCD - > ABCD
R_mcl	MAL - > GOX + ACCOA	ABCD - > AB + DC
R_pdh	PYR - > ACCOA + CO2	ABC - > BC + A
R_sga	GOX + SER - > HPR + GLY	AB + CDE - > CDE + AB
R_TCA1	ACCOA + OAA - > CIT	AB + CDEF - > FEDBAC
R_TCA2	CIT - > AKG + CO2	ABCDEF - > ABCDE + F
R_TCA3	AKG - > SUCC + CO2	ABCDE - > BCDE + A
R_TCA4	SUCC - > FUM	ABCD - > ABCD
R_fum	FUM - > MAL	ABCD - > ABCD

One important question this approach can address is whether *M. buryatense* 5GB1 operates an incomplete TCA cycle when growing on methane. An incomplete TCA cycle has been proposed for Type I methanotrophs including *M. buryatense*, based on the inability to detect 2-oxoglutarate dehydrogenase enzyme activity as well as radioactive tracer studies (Davey et al., 1972; Hazeu et al., 1980; Kaluzhnaya et al., 2001; Patel et al., 1975). From these results, it has been assumed that in