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Spotlight

Insights into methanotroph carbon flux pave the way for methane biocatalysis

Calvin A. Henard [1,2,*]

Methanotrophic bacteria are used industrially as catalysts for the bioconversion of methane (CH_4) to valuable products. A landmark study by Kalyuzhnaya *et al*. identified the primary metabolic route for CH_4 flux to central metabolic intermediates and alternative fermentative products in an industrially promising methanotroph, leading to a systems-level understanding of methanotrophy.

CH_4-rich gas represents an abundant, high-energy substrate for the sustainable production of valuable chemicals and liquid fuels and is expected to have a significant role in the expanding circular bioeconomy. CH_4-rich gas sources include natural gas derived from hydraulic fracturing and biogas produced via anaerobic digestion of agricultural waste or municipal solid waste (MSW) at landfills and MSW treatment facilities. An estimated 8 quad BTU of CH_4-rich gas is available at these sites annually (equivalent to ~60% of the US electricity use) (see Biogas Opportunities Roadmap: https://www.epa.gov/sites/default/files/2015-12/documents/biogas-roadmap.pdf). However, gas leaks, flaring, and combustion for electricity generation squander the gas and significantly contribute to increasing atmospheric greenhouse gases. Thus, alternative technologies are needed to use these CH_4-rich carbon sources while concurrently mitigating further greenhouse gas production.

Bacteria that use CH_4 as a carbon and energy source (methanotrophs) are critical players in biogeochemical cycling in terrestrial and aquatic ecosystems, where they convert this single-carbon (C1) substrate into multicarbon molecules. Due to their unique CH_4 metabolism, methanotrophs also have significant biotechnology potential as biocatalysts for the conversion of CH_4-rich gases and greenhouse gas mitigation. Significant insights into methanotroph metabolism and physiology have been gained over the past 50 years through seminal research that elucidated the primary biochemical pathways used by methanotrophs for CH_4 assimilation and conversion [1]. Two generalizable CH_4 conversion pathways function in diverse methanotrophs: the serine cycle in alphaproteobacterial methanotrophs and the ribulose monophosphate (RuMP) cycle in gammaproteobacterial methanotrophs. Although much was learned about methanotrophs over the later part of the 20th century and early 2000s, significant knowledge gaps remained that limited the development of methanotroph-based bioprocesses compared with their heterotrophic microbe counterparts (i.e., *Escherichia coli* and *Saccharomyces cerevisiae*).

Several independent lines of research converged during the early 2010s to enable key advancements in methanotroph biology and genetic engineering. The relatively fast-growing gammaproteobacterial haloalkalitolerant *Methylotuvimicrobium alcaliphilum* 20Z (previously *Methylomicrobium alcaliphilum* 20Z) was isolated [2], its optimal cultivation conditions were characterized [2,3], and its genome was sequenced and annotated [4], enabling a landmark study by Kalyuzhnaya and colleagues in 2013 [5]. Before this landmark study, CH_4 flux to pyruvate in gammaproteobacterial methanotrophs was thought to occur through the Entner–Doudoroff (EDD) pathway (Figure 1, orange highlight). This conclusion was based on biochemical studies in which high activity of EDD pathway enzymes was detected but not of pyruvate kinase activity, a key enzyme of the Embden–Meyerhof–Parnas (EMP) glycolytic pathway (Figure 1, green highlight) in methanotroph cell lysates [6]. Interestingly, analysis of the *M. alcaliphilum* 20Z genome showed that this bacterium encodes a complete EMP pathway, including pyruvate kinase, as well as the EDD pathway [4], raising the question of what the primary route of CH_4-derived carbon flux in *M. alcaliphilum* 20Z was.

To address this question, Kalyuzhnaya and coworkers showed that a complete EMP pathway is transcribed at higher levels compared with EDD pathway genes in *M. alcaliphilum* 20Z during growth on CH_4, pointing toward the EMP pathway as the primary route of CH_4 flux in this bacterium. This conclusion was further supported by higher intracellular EMP metabolite levels compared with EDD metabolites under similar CH_4 cultivation conditions. $^{13}CH_4$-tracing experiments showed pyruvate isotopic labeling consistent with this central metabolite being derived via the EMP pathway rather than via the EDD pathway [5], providing definitive support that primary carbon flux is through the EMP pathway in *M. alcaliphilum* 20Z. The theoretical carbon conversion efficiency (CCE) using the EMP variant of the RuMP pathway agreed with measured CCE values (>60% mol/mol) in many gammaproteobacterial methanotrophs; thus, this finding resolved an outstanding question in the field. Notably, the EMP variant of the RuMP pathway is predicted to be the most energy efficient of the CH_4 assimilation pathways (Figure 1); thus, it is not surprising that many gammaproteobacterial methanotrophs exhibit the fastest growth rates under optimal cultivation conditions and represent promising microbes for CH_4 bioconversion.

The authors hypothesized that a functional EMP pathway may enable fermentative metabolism in *M. alcaliphilum* 20Z. Since fermentation typically occurs under anaerobic or oxygen-limited conditions and CH_4

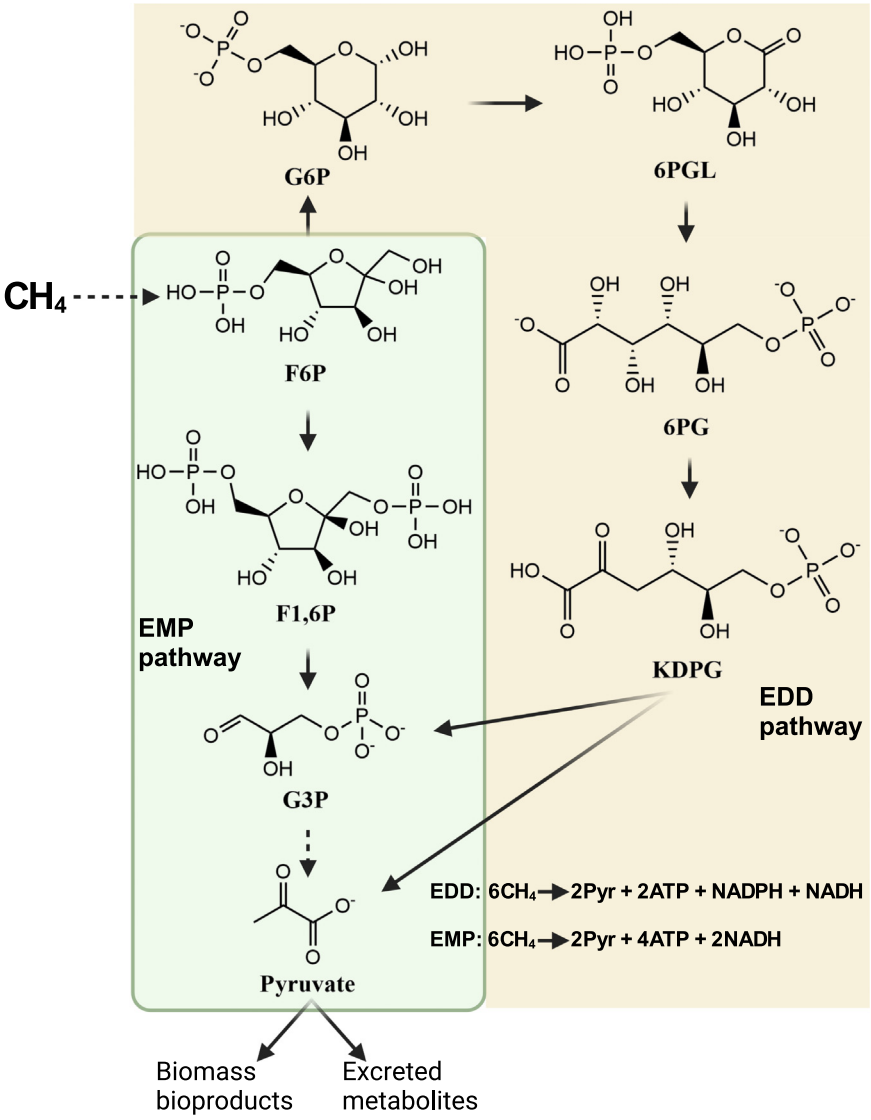

Figure 1. Methane (CH_4) flux to pyruvate in gammaproteobacterial methanotrophs primarily occurs through the Embden–Meyerhof–Parnas (EMP) pathway (green box) rather than the Entner–Doudoroff (EDD) pathway (orange box), paving the way for energy- and carbon-efficient CH_4 biocatalysis. Abbreviations: 6PG, 6-phosphogluconate; 6PGL, 6-phosphogluconolactone; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; F1,6P, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; Pyr, pyruvate.

oxidation requires oxygen, the authors assessed whether *M. alcaliphilum* 20Z produces fermentative products under hypoxia. Although the bacterium consumed limited CH_4 under low-oxygen conditions, 40–50% was converted to mixed, excreted fermentative products, including formate, acetate, succinate, lactate, and hydroxybutyrate [5]. Notably, all of these metabolites can serve as synthons to produce renewable polymers and materials.

With an active EMP pathway and the synthesis of valuable products from CH_4, it was readily apparent that metabolic engineering approaches used in other microbial hosts could be applied to methanotrophs. Following this landmark study, metabolic engineering of methanotrophic bacteria experienced a surge in breakthroughs, including the development of a suite of genetic tools and the first-in-class demonstration of CH_4 conversion to lactate in a genetically engineered methanotroph [7,8]. Kalyuzhnaya and colleagues applied several systems approaches to methanotrophs for the first time, including RNA-sequencing, targeted metabolomics, and ^{13}C tracing, which led to the development of genome-scale metabolic models that have since guided the rational engineering of these microbes [9–12]. Kalyuzhnaya and colleagues were the first to use the term 'methane biocatalysis' and its use in publications has increased steadily since 2013. These data underscore that this study significantly inspired and impacted downstream research in basic and applied methanotrophy, paving the way for significant progress in methane biocatalysis over the past 10 years. Continued research focusing on methanotroph metabolism and physiology, as well as their role in environmental nutrient cycles, will ensure that methane biocatalysis can be leveraged to meet international goals of reducing CH_4 emissions.

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Declaration of interests

No competing interests are declared.

[1]Department of Biological Sciences and BioDiscovery Institute, University of North Texas, Denton, TX, USA
[2]Laboratory website: https://bdi.unt.edu/calvin-henard

*Correspondence:
Calvin.Henard@unt.edu (C.A. Henard).

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