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Research review paper

Type II methanotrophs: A promising microbial cell-factory platform for bioconversion of methane to chemicals

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ABSTRACT

Methane, the predominant element in natural gas and biogas, represents a promising alternative to carbon feedstocks in the biotechnological industry due to its low cost and high abundance. The bioconversion of methane to value-added products can enhance the value of gas and mitigate greenhouse gas emissions. Methanotrophs, methane-utilizing bacteria, can make a significant contribution to the production of various valuable biofuels and chemicals from methane. Type II methanotrophs in comparison with Type I methanotrophs have distinct advantages, including high acetyl-CoA flux and the co-incorporation of two important greenhouse gases (methane and CO_2), making it a potential microbial cell-factory platform for methane-derived biomanufacturing. Herein, we review the most recent advances in Type II methanotrophs related to multi-omics studies and metabolic engineering. Representative examples and prospects of metabolic engineering strategies for the production of suitable products are also discussed.

1. Introduction

Methane, which is the major constituent of biogas and natural gas, is a far stronger greenhouse gas than CO_2 (Lee et al., 2019b; Nguyen and Lee, 2020). Due to the low cost and abundance of methane in natural sources, it is considered a potential next-generation carbon feedstock for the biochemical industry, as well as a sustainable energy resource (Conrado and Gonzalez, 2014; Fei et al., 2014; Kalyuzhnaya et al., 2015; Strong et al., 2016a). The direct chemical conversion of methane is troublesome due to the high activation energy of C—H bonds (Haynes and Gonzalez, 2014). Hence, there has been a resurgence in interest in methanotrophs, which are able to use methane as a sole energy and carbon source under mild growth conditions (Kalyuzhnaya et al., 2015; Lee et al., 2016). These strains have great potential as a platform strain for the utilization of methane for chemical production, and also in the mitigation of climate change via removing methane as greenhouse gas (Kalyuzhnaya et al., 2015; Strong et al., 2016a).

There are different groups and types of methanotrophs depending on the three different pathways primarily employed by methanotrophs to assimilate formaldehyde into biomass: the Ribulose monophosphate (RuMP) pathway occurring in Group I methanotrophs (Gammaproteobacteria, Type I and Type X), the serine cycle of Group II methanotrophs (Alphaproteobacteria, Type II and Type III), and the Calvin–Benson–Bassham (CBB) cycle occurring in Group III methanotrophs (*Verrucomicrobia*, Type IV) (Kalyuzhnaya et al., 2015). To date, most advances in the metabolic engineering of methanotrophs for methane bioconversion have focused on Type I methanotrophs (Henard et al., 2016; Lee et al., 2019a; Nguyen et al., 2018, 2019a). Nonetheless, Type II methanotrophs provide distinctly attractive features that are beneficial for chemical production (Fig. 1). Recently, the successful strategies to engineer the Type II methanotrophic alphaproteobacterium for the biosynthesis of 3-hydroxypropionic acid (3-HP), cadaverine, and lysine were described (Nguyen et al., 2020a, 2020b). These studies are a proof-in-principle that Type II methanotrophs could be employed as a platform for the production of valuable chemicals and fuels via metabolic engineering. Since there has been no review focusing on Type II methanotrophs, this paper aimed to review the physiology and metabolism in these microbes to address appropriate engineering strategies for the production of value-added products. This review presents the recent progress on the development of engineered Type II methanotrophs as a microbial cell factory, including genetic tool development, multi-omics study, and suitable products suggestion, as well as strategies to enhance carbon pool for target product production (Fig. 1).

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2. Outstanding features of Type II methanotrophs

2.1. Why Type II methanotrophs?

Type II methanotrophic alphaproteobacteria possess many fascinating metabolic properties that are beneficial for biotechnological applications. First, this group has a comparably high flux of CoA-derivatives, such as acetyl-CoA, propionyl-CoA, and crotonyl-CoA, which could be used for biofuel production (Bordel et al., 2019b; Kalyuzhnaya et al., 2015; Yang et al., 2013). Second, a typical Type II methanotroph (e.g. *M. trichosporium* OB3b) can assimilate significant amount of CO_2 (Yang et al., 2013). Type II methanotrophs are appropriate biocatalyst for biogas conversion because of their ability to co-utilize methane and CO_2. Third, many Type II methanotrophs have broad-spectrum substrates that can assimilate from C_1 to C_2 and C_3 compounds, which is advantageous for methane-to-methanol bioconversion based on the methanol dehydrogenase (MDH) knock-out (Crombie and Murrell, 2014; Dedysh and Dunfield, 2011; Kalyuzhnaya et al., 2015; Semrau et al., 2011). The soluble methane monooxygenase (sMMO) (mostly present in Type II more than Type I methanotrophs) can catalyze both the oxidation of methane and the transformation of a wide spectrum of aliphatic and aromatic hydrocarbons and their derivatives (Trotsenko and Murrell, 2008). Thus, sMMO can play an important role in the bioremediation of polluted environments as well as biocatalyst for biotransformation (Dalton, 2005; Trotsenko and Murrell, 2008). Moreover, most of Type II methanotrophs have been shown to be capable of nitrogen fixation, while only some Type I methanotrophs have such capability (Auman et al., 2001; Stein, 2018; Trotsenko and Murrell, 2008). Their nitrogen-fixing capability is useful for trichlorethylene (TCE) oxidation, since methanotrophs require an adequate nitrogen source to effectively remove TCE in contaminated environments (Auman et al., 2001). Furthermore, Type II methanotrophs employ the glutamate cycle with glutamine synthetase and glutamine-oxoglutarate aminotransferase (GOGAT) system for NH_4 assimilation, whereas Type I methanotrophs assimilate NH_4 predominantly via reductive pyruvate or α-ketoglutarate amination (Trotsenko and Murrell, 2008). The GOGAT of Type I is peculiar to NADPH and is quite unsteady, while this enzyme is more stable in Type II methanotrophs and specific for NADH cofactor (Trotsenko and Murrell, 2008). Another advantage of methanotrophs is their ability to utilize either nitrate or ammonium as a nitrogen source, where the growth rate of methanotrophic bacteria is affected by the nitrogen source. Theoretically, the use of ammonium as a nitrogen source is more bioenergetically favorable than nitrate because it can be directly assimilated into biomass and ammonium is more reduced form as nitrogen source (Naizabekov and Lee, 2020). Additionally, some gammaproteobacteria have been traditionally described to favor high O_2 and low CH_4 concentrations for their growth, while alphaproteobacteria prefer low O_2 and high CH_4 concentrations (Strong et al., 2015), indicating that Type II methanotrophs can play better as a biocatalyst at higher methane concentration. Lastly, a Type II methanotroph, *M. silvestris,* with ability of n-alkanes utilization, can be a potential platform for the biological conversion of natural gas or petroleum gas containing C_2-C_4 alkanes together with C_1 as the main component (Etiope and Ciccioli, 2009; Kalyuzhnaya et al., 2015).

2.2. Classification and characteristics of Type II methanotrophs

The main characteristics of Type II methanotrophs are summarized in Table 1. Type II alphaproteobacteria possess two distinct forms of methane monooxygenase (MMO): pMMO and sMMO, or only one form of MMO. pMMO is a copper-dependent enzyme that is associated with the intracytoplasmic membrane. sMMO is a complex of non-heme iron enzymes located in the cytoplasm (Kalyuzhnaya et al., 2019). The expression of sMMO and pMMO is regulated by the concentration of copper. pMMO is expressed at a high copper concentration, whereas sMMO is produced only at low copper concentrations. Most Type II methanotrophs can grow on nitrate mineral salt (NMS) and ammonium mineral salt (AMS) medium, and have the capacity for nitrogen fixation (Auman et al., 2001; Stein, 2018; Trotsenko and Murrell, 2008).

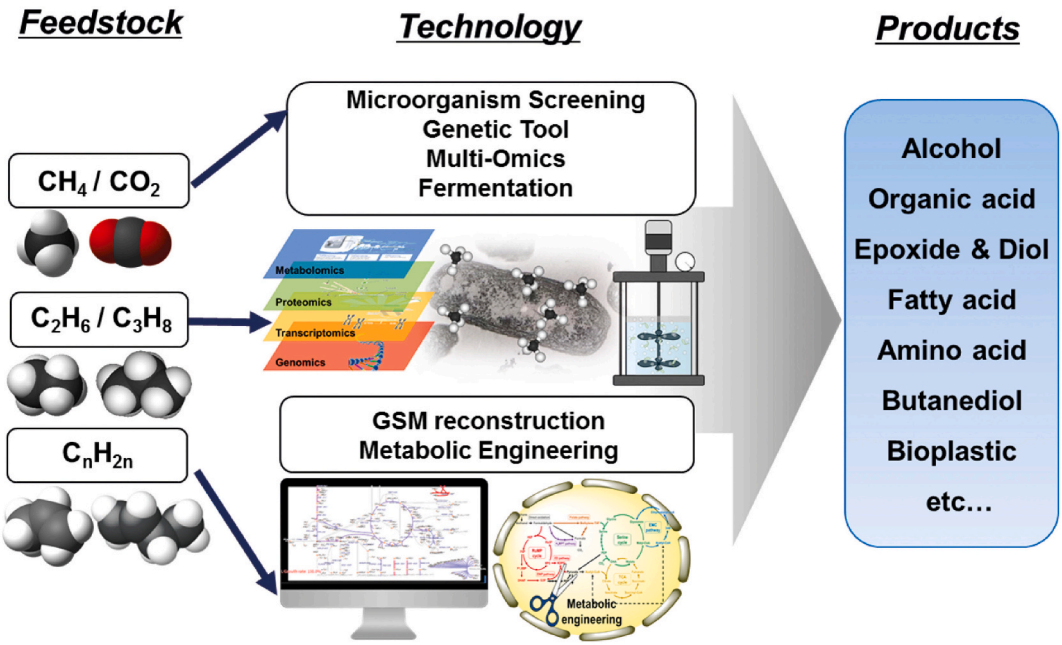

Fig. 1. Potential of Type II methanotrophs as a cell-factory platform for conversion of low-grade carbon feedstock into highly-valued products. (2020, July 26). Methane [Online]. Available: https://commons.wikimedia.org/wiki/File:Methane-3D-space-filling.svg#mediaviewer/File:Methane-3D-space-filling.svg (2020, July 26). Carbon dioxide [Online]. Available: https://simple.wikipedia.org/wiki/Carbon_dioxide (2020, July 26). Ethane [Online]. Available: https://marcellusdrilling.com/2017/10/wv-builder-tells-psc-power-plant-can-burn-methane-and-ethane/ (2020, July 26). Propane [Online]. Available: https://energyeducation.ca/encyclopedia/Propane (2020, July 26). Propene [Online]. Available: https://simple.wikipedia.org/wiki/Propene (2020, July 26). Butene [Online]. Available: https://www.jobilize.com/chemistry/test/isomers-of-alkenes-hydrocarbons-by-openstax

Table 1
Classification and characteristics of Type II methanotrophs.

Genus	Strain	Growth condition			Culture Medium	Characteristic	Reference
		Growth rate (h^{-1})	pH	T°C			
Methylocystis	*M. rosea* SV97	NA	5.5–9.0	5–37	NMS, AMS	pMMO only, no requirement of NaCl for growth, PHB production, capability of nitrogen fixation	Wartiainen et al. (2006)
	Methylocystis ATCC 49242 strain Rockwell	0.16	6.8	30	NMS, AMS	pMMO only, high tolerance of ammonium, capability of nitrogen fixation	Nyerges et al. (2010), Stein et al. (2011)
	M. hirsuta CSC1	0.05	7	30	NMS, AMS	pMMO and sMMO, capability of oxidizing aliphatic and aromatic compounds, ability of growing on methane and methanol, PHB production, capability of nitrogen fixation	Bordel et al. (2019a), Bordel et al. (2019b), Lindner et al. (2007)
	M. parvus OBBP	0.12	6–8	5–37	NMS, AMS	pMMO only, PHB production, capability of nitrogen fixation	del Cerro et al. (2012), Kalyuzhnaya et al. (2019), Pieja et al. (2011)
	Methylocystis sp. SC2	0.07	6–9	30	NMS, AMS	pMMO only, possession of genes for ammonia assimilation and nitrogen fixation.	Baani and Liesack (2008), Dam et al. (2012)
	Methylocystis sp. SB2	NA	6–9	10–30	NMS, AMS	pMMO only, facultative methanotroph, ability of growing on acetate or ethanol, diverse nitrogen metabolism capabilities	Dam et al. (2013), Kalyuzhnaya et al. (2019), Vorobev et al. (2014)
Methylosinus	*M. trichosporium* OB3b	0.12–0.14	6–8	5–37	NMS, AMS	pMMO and sMMO, well-established catalysis for PHB production, epoxidation, ability of degrading chlorinated aliphatic hydrocarbons, production of methanobactins, capability of nitrogen fixation	Oldenhuis et al. (1989), Stein et al. (2010), Whittenbury et al. (1970)
	Methylosinus sp. LW4	0.12–0.15	6–8	5–37	NMS, AMS	pMMO and sMMO, production of methanobactins and capability of nitrogen fixation.	Auman et al. (2001), Kenney et al. (2016)
Methylocella	*M. silvestris* BL2	0.014	4.5–7.0	4–30	NMS, AMS	sMMO only, facultative methanotroph, ability of growing on methane, and one-carbon and multi-carbon substrates such as acetate, pyruvate, propane, and succinate, capability of nitrogen fixation.	Chen et al. (2010), Dunfield et al. (2003)
	M. tundra sp. nov.	NA	4.2–7.5	5–30	NMS, AMS	sMMO only, growth inhibition by NaCl conc. above 0.8% (*w/v*), ability of growing on methane and methanol, best growth at methanol conc. of 0.5–1% (*v/v*), capability of nitrogen fixation.	Dedysh et al. (2004)
Methylocapsa	*M. acidiphila* B2	0.03	4.2–7.2	10–30	NMS	pMMO only, growth inhibition by NaCl conc. of 0.5% (*w/v*), ability of growing on methane and methanol, growth support at methanol conc. below 0.05% (*v/v*), capability of nitrogen fixation, PHB production.	Dedysh et al. (2002)
	M. aurea KYG	0.018	5.2–7.2	2–33	NMS, AMS	pMMO only, ability of growing on methane, methanol and acetate, growth inhibition with 0.3% (*w/v*) NaCl, growth support at methanol conc. below 0.1% (*v/v*); PHB production, capability of nitrogen fixation.	Dunfield et al. (2010)
Methyloferula	*M. stellata* AR4	0.005	3.5–7.2	4–33	NMS, AMS	sMMO only, growth reduction at NaCl conc. above 0.7% (*w/v*). Optimum methanol conc. between 0.1 and 1% (*v/v*), capability of nitrogen fixation.	Dam et al. (2013), Dedysh et al. (2015)

2.3. Central metabolic pathways and electron transfer of Type II methanotrophs compared to Type I methanotrophs

Methane oxidation is initiated by one of two MMOs, sMMO and pMMO, which oxidize methane into methanol in aerobic methanotrophic bacteria (Kalyuzhnaya et al., 2015; Lee et al., 2016; Semrau et al., 2010). Until now, most Type I methanotrophs possess pMMO, whereas Type II methanotrophs are able to express both sMMO and pMMO (Auman et al., 2001). MMO requires two electrons for methane oxidation. $NADH/H^+$ is the electron source for sMMO, while direct electron transfer by PQQ-MDH or ubiquinol (UQH2) is a possible way of electron transfer to pMMO (Nariya and Kalyuzhnaya, 2019; Semrau et al., 2010). For methane oxidation, type II methanotrophs are expected to employ the redox arm mechanism where pMMO takes electrons from ubiquinone/ubiquinol pool of the methanol oxidation process, resulting in lower biomass yields than Type I methanotrophs possessing the direct coupling system where electrons from methanol oxidation are directly transferred to pMMO (Akberdin et al., 2018; Bordel et al., 2019b).

Methanol is subsequently converted to formaldehyde by a PQQ-MDH (Lee et al., 2016). Formaldehyde assimilation is a characteristic that differentiates Type I methanotrophs from Type II. Type I methanotrophs use the RuMP pathway for formaldehyde utilization, while Type II methanotrophs mainly assimilate formaldehyde via the serine cycle and the ethylmalonyl-CoA (EMC) pathway (Kalyuzhnaya et al., 2015; Lee et al., 2016). In *M. trichosporium* OB3b, the typical Type II methanotroph, the serine cycle plays a role in glyoxylate regeneration and the refilling of phosphoenolpyruvate (PEP) for the generation of pyruvate by PEP conversion (Yang et al., 2013). It was also assumed that the serine cycle is split into two branches, which exert distinct metabolic controls. The first branch is involved in C1-fixation, which includes all steps from glycine to PEP, accounting for 53% of the biomass. Most of the C1-carbon assimilated at the first step of the branch is directly transferred to gluconeogenesis and amino acid biosynthesis. The second part of the serine cycle (considered as "glyoxylate regeneration") is interconnected with the EMC pathway which significantly contributes to resupplying the intermediates for assimilation (Yang et al., 2013).

2.4. Recent advances in genetic tools for Type II methanotrophs

To date, despite the fact that Type II methanotrophs are potential methane-based cell factories for chemical production, a limited number of genetic tools and engineering of Type II methanotrophs have been conducted (Table 2). Only the metabolic engineering of *M. trichosporium* OB3b has recently been applied for the accumulation of 3-hydroxypropionic acid, cadaverine, and lysine using pAWP89 as an expression vector and conjugation method for gene transfer (Nguyen et al., 2020a, 2020b). Conjugation is the most common method used for genetic manipulation in Type II methanotrophs (Lloyd et al., 1999; Martin and Murrell, 1995; Smith and Murrell, 2011). Electroporation has also been applied to some Type II methanotrophs, including *Methylocystis* sp. SC2,

Table 2
Genetic tools used in Type II methanotrophs.

Vector	Selectable marker	Strain	Reference
pMHA200 pMHA203	Km	*M. silvestris* BL2	Theisen et al. (2005)
pCM184 Linear DNA	Km		Crombie and Murrell (2011)
pGSS33	Km	*M. trichosporium*	Murrell (1992)
pVK100	Tet, Km	OB3b	Lloyd et al. (1999), Murrell (1992)
pBR325	Km		Martin and Murrell (1995)
pBR325	Spe, Str, Km		Smith and Murrell (2011), Smith et al. (2002a)
Linear DNA	Gm		Ro and Rosenzweig (2018)
pTJS141 pTJS142 pTJS175	Amp		Smith et al. (2002b)
pK18mob	Km		Stafford et al. (2003)
pBBR1MCS pMD2	Km		Borodina et al. (2007)
pk18mobsacB pAWP89	Gm, Km Km		Ro and Rosenzweig (2018)
pAWP89	Km		Nguyen et al. (2020a, 2020b)
pUC18	Km	*Methylocystis* sp. SC2	Baani and Liesack (2008)
pVK100 pVK104	Tet, Km	*M. parvus* OBBP	(Lloyd et al., 1999)

M. silvestris BL2, and *M. trichosporium* OB3b (Baani and Liesack, 2008; Crombie and Murrell, 2011; Ro and Rosenzweig, 2018). Electroporation was performed in *M. trichosporium* OB3b and *M. silvestris* BL2 using linear DNA fragments (Crombie and Murrell, 2011; Ro and Rosenzweig, 2018) and *Methylocystis* sp. SC2 using the suicide vector pUC18 (Baani and Liesack, 2008). Despite limited genetic manipulation in type II methanotrophs compared to other industrially relevant strains such as *E. coli*, on-going development of robust genetic tools will facilitate efficient genetic and metabolic engineering of Type II methanotrophs.

2.5. Understanding Type II methanotrophs via multi-omics approaches

A holistic vision of the cellular functions of Type II methanotrophs is known through genomics, transcriptomics, metabolomics, proteomics, and fluxomics (Lee et al., 2016, 2019b). Metabolic engineering via in silico modeling has potentially improved microbial biocatalysis, endowing it with desirable properties (Nguyen et al., 2018). Advanced investigations using omics technologies have provided key points from which to further develop methanotrophic strains. Recent systems biology approaches are listed in Table 3.

Transcriptomics became essential for gaining an overview of the central metabolic pathway from the utilization of methane in methanotrophs. *M. trichosporium* OB3b is a well-known model strain of Type II methanotrophs (Matsen et al., 2013). Studies combining transcriptomics with genomics have provided insights into the central metabolic pathways of C_1-metabolism in *M. trichosporium* OB3b (Matsen et al., 2013). The transcriptomic data of *M. trichosporium* OB3b indicates that methane oxidation involves many genes, including pMMO, PQQ-MDH, the genes of the H_4MPT-pathway, and NAD^+-FDH. The expression of genes in the serine cycle, the EMC pathway, and the TCA cycle was also proved (Matsen et al., 2013). The transcriptomic data of *Methylocystis* sp. strain SB2 provided information about carbon assimilation and the operation of the complete TCA and EMC pathways (Vorobev et al., 2014). The expression level of genes for the methane-to-methanol conversion is remarkably higher than those of downstream oxidative transformations, demonstrating the role of this step in cell growth in methane. Furthermore, transcriptomic analyses of this strain grown on ethanol compared to methane suggested that acetyl-CoA is generated from ethanol, which then either goes through the TCA cycle for energy generation or is incorporated into biomass through the EMC pathway (Vorobev et al., 2014). These results indicated that Type II methanotrophs have large metabolic flexibility, with a pathway operation that is highly controlled and interconnected.

Table 3
Summary of systems-level investigation in Type II methanotrophs.

Omics approach	Species	References
Transcriptomics	*M. trichosporium* OB3b	Matsen et al. (2013)
	Methylocystis sp. SB2	Vorobev et al. (2014)
Proteomics	*M. silvestris* BL2	Crombie and Murrell (2014)
Metabolomics	*M. trichosporium* OB3b	Yang et al. (2013)
^{13}C flux analysis	*M. trichosporium* OB3b	Matsen et al. (2013)
Kinetic models	*M. trichosporium* OB3b *M. parvus* OBBP	Rostkowski et al. (2013), Yoon and Semrau (2008)
Stoichiometry models	*M. trichosporium* OB3b *M. parvus* OBBP	Rostkowski et al. (2013)
Genome-scale models	*M. hirsuta* CSC1	Bordel et al. (2019b)
	Methylocystis sp. SC2	
	Methylocystis sp. SB2	
	M. parvus OBBP	Bordel et al. (2019c)
	M. trichosporium OB3b	Naizabekov and Lee (2020)

Metabolomics and ^{13}C-labeling were applied to the Type II methanotrophic bacterium, *M. trichosporium* OB3b, to evaluate the central metabolism for methane utilization (Yang et al., 2013). It has been reported that the serine, EMC, and TCA cycle are tightly linked. This strain uses the EMC pathway for carbon assimilation, and a significantly large portion of the cellular carbon (approximately 60%) is derived from CO_2. The data suggested that the serine cycle is divided into two functional branches, including the "C_1-fixation" branch and the "EMC pathway" branch (known as "glyoxylate regeneration"). The EMC pathway plays an important role in providing intermediates such as malate, acetyl-CoA, and glyoxylate, to cells (Yang et al., 2013).

Metabolic model simulations are necessary for the identification of potential targets for genetic modification and possible influences of genetic perturbation on cells, as well as in silico phenotype predictions (Akberdin et al., 2018; Bordel et al., 2019c; Kalyuzhnaya et al., 2015; Nguyen et al., 2018). The early Genome Scale Models (GSMs) of Type II methanotrophs, including *Methylocystis hirsuta* and two *Methylocystis* strains, have been established (Bordel et al., 2019b). The models estimated the oxygen consumption rates and biomass yields, and uncovered the mechanism for methane oxidation by pMMO. Type I methanotrophs exploit the "direct coupling" system, while Type II methanotrophs use the "redox branch" mechanism for methane oxidation, leading to lower biomass yields. Nonetheless, Type II methanotrophs have high carbon fluxes through acetoacetyl-CoA under nitrogen-insufficient conditions, which is the potential for the production of acetoacetyl-CoA-derived products through metabolic engineering. *Methylocystis* sp. strains are also able to slowly use C_2 as carbon sources (Bordel et al., 2019b). Another GSM of *M. parvus* OBBP was also reconstructed (Bordel et al., 2019c). The model was compared to GSMs of the closely related methanotrophs, *M. hirsuta,* and *Methylocystis* sp. SC2. The biomass yield of *M. parvus* was anticipated and suited with the experimental yield based on the "redox arm mechanism" presumption for methane oxidation. The model revealed an anaplerotic role of stored PHB, which is further utilized for replenishing glyoxylate for the serine pathway and succinyl-CoA for the TCA cycle. PHB storage serves as an energy source under anoxic circumstances coupled to denitrification. The yield of *M. parvus* was similar to that of *M. hirsuta* and *Methylocystis* sp. SC2 based on GSMs analysis, but *M. parvus* showed the highest specific growth rate and methane oxidation rate (Bordel et al., 2019c). Recently, the GSM for

M. trichosporium OB3b, a model organism of Type II methanotrophs, was reconstructed (Naizabekov and Lee, 2020). This model predicted oxygen: methane uptake rates, as well as specific growth rates on nitrate-supplied medium with methane as the sole carbon source. The "redox-arm" system was found to be the most fitting mode of electron transfer for methane oxidation, which is in agreement with the above findings. Methane metabolism in the ammonium-supplemented medium was validated, and the flux distribution in the central metabolism was investigated in silico by the model. This model can be employed as a specific knowledgebase to theoretically investigate the methane metabolism in alphaproteobacteria (Naizabekov and Lee, 2020).

3. Potential targets for the application of methane bioconversion in Type II methanotrophs

3.1. Existing technologies utilizing whole-cell methanotrophs as a biocatalyst

3.1.1. Alcohol production via alkane oxidation

The methane-to-methanol bioconversion using methanotrophs is one of the simplest processes of methane valorization (Fig. 2). Even though methane is converted to methanol by MMO, in order to accumulate methanol, MDH needs to be inhibited to prevent the conversion of methanol to formaldehyde (Hwang et al., 2014). Methane-to-methanol bioconversion has been widely investigated in *M. trichosporium* OB3b. A variety of MDH inhibitors consisting of metal chelating agents, such as cyclopropanol, NaCl, cyclopropane, EDTA, and $MgCl_2$, and high phosphate concentrations have been used to prevent the conversion of methanol (Hwang et al., 2014). Cyclopropanol has been employed for the MDH inhibition in *M. trichosporium* OB3b for methanol production with a relatively high efficiency (Furuto et al., 1999; Takeguchi et al., 1997). NaCl has also been successfully used for hindering MDH in *M. trichosporium* OB3b (Lee et al., 2004), but both pMMO and MDH activities were suppressed. Due to the greater extent of inhibition for MDH than pMMO enzyme, methanol still accumulated. A novel diffusion system for culturing *M. trichosporium* OB3b, using 100 mM NaCl and 1 mM EDTA as MDH inhibitors and 20 mM sodium formate as reducing power regeneration, produced methanol with high productivity (Kim et al., 2010). In another study, over 1.12 g/L methanol was generated with 60% conversion using *M. trichosporium* OB3b in 10 mM $MgCl_2$, 20 mM sodium formate, and 400 mM phosphate buffer (Duan et al., 2011). Also, the accumulation of methanol was attained in a membrane-aerated reactor using various MDH inhibitors including 100 mM potassium phosphate and 0.5 mM EDTA, and 40 mM sodium formate as a reducing power source with 73.8% conversion (mol methanol/mol methane) (Hwang et al., 2015). *M. sporium* was immobilized using encapsulation methods (employing sodium alginate and silica gel), and the high methanol production by the encapsulated cells was recorded. This suggested a suitable approach to enhance stable methanol production by the encapsulation of methanotrophs (Patel et al., 2016a). Moreover, several Type II methanotrophs were tested for their ability to produce methanol from methane, including *M. silvestris, M. bryophila,* and *M. stellata*. Among them, *M. bryophila* exhibited the highest methanol production. Optimization of parameters, 100 mM phosphate buffer and 50 mM $MgCl_2$ as an MDH inhibitor, and formate supplementation led to sharp improvements in methanol production by *M. bryophila* (Patel et al., 2016b).

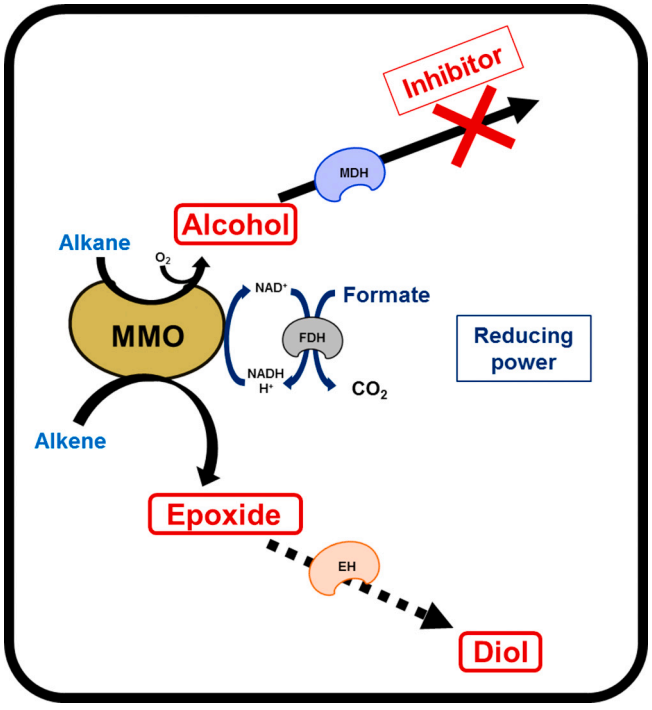

Fig. 2. Bioconversion of alkane to alcohol, and alkene to epoxide in Type II methanotrophs. The epoxide is converted into diol be heterologous expression of epoxide hydrolase (EH), indicated by the dotted arrows. Genes: MMO, methane monooxygenase; MDH, methanol dehydrogenase; FDH, formate dehydrogenase.

Due to the broad substrate specificity of sMMO, propanol can be produced from propane (Fig. 2). The 2-propanol is then transformed into acetone by NAD^+-alcohol dehydrogenase and PQQ-MDH. To accumulate propanol, propanol oxidation needs to be hindered. 2-propanol accumulation has been observed in *M. silvestris* BL2 grown on propane in the presence of putative propane monooxygenase (Crombie and Murrell, 2014). *M. trichosporium* OB3b has also been previously employed for 2-propanol production and showed higher productivity compared to Type I methanotrophs (Nguyen et al., 2019a). The optimized process using EDTA, sodium phosphate as inhibitors, and sodium formate for enhancing the conversion of propane to propanol was carried out to obtain a maximum titer of 0.610 g/L 2-propanol (Nguyen et al., 2019a).

Similarly, the conversion of ethane to ethanol has been examined using methanotrophs as a biocatalyst (Fig. 2). Ethane could be converted into ethanol relying on MDH inhibition and the addition of sodium formate in *M. trichosporium* OB3b, which theoretically demonstrates the biotransformation of ethane substrate (Shimoda et al., 1989). The production of ethanol was also investigated under ambient conditions using Type I and Type II methanotrophs, where the highest titer of 0.52 g/L ethanol was obtained under optimal batch reaction conditions using *M. trichosporium* OB3b (Oh et al., 2019).

3.1.2. Alkene epoxidation and diol production

MMO has been found to catalyze the epoxidation of gaseous alkenes to their corresponding epoxides (Fig. 2) (Hou, 1984; Xin et al., 2017; Zhang et al., 2008b). The accumulation of propylene oxide from propylene in a gas–solid bioreactor using *M. trichosporium* OB3b and *Methylosinus* sp. CRL 31 was performed with a 2.7% conversion rate for propylene (Hou, 1984). Among the 4 types of strains of methanotrophic bacteria (*M. trichosporium* OB3b, *M. capsulatus* HD6T, *M. trichosporium* IMV3011, *Methylomonas* sp. GYJ3), *M. trichosporium* OB3b showed the highest propene epoxidation activity (Zhang et al., 2008b). Xin et al. successfully synthesized epoxypropane from propene using methanol as the carbon source and an electron-donating substrate for NADH regeneration (Xin et al., 2010). In addition, 0.016% methanol was found to have the highest propene epoxidation capacity of *M. trichosporium* IMV3011. Exhaustion of the reductant and the suppression of toxic products are difficulties associated with producing epoxyethane from ethylene. The regeneration of NADH was achieved in batch experiments of *M. trichosporium* IMV 3011 using methane and methanol. The production of epoxyethane was enhanced in 10% gas-phase methane using methane as a reductant for epoxidation. In the case of methanol, the

maximum production of 6.6 nM epoxyethane occurs with 3 mM methanol. The removal of the product was proposed to overcome the inhibition of epoxyethane formation (Xin et al., 2017).

Epoxide hydrolases (EHs) catalyze the addition of water to the epoxides to generate the corresponding vicinal diols, which can be applied for the synthesis of value-added chiral pharmaceuticals (Lee et al., 2007). Methanotrophs with the ability to convert alkenes to epoxides could be a potential host for the production of diols from alkenes by the expression of suitable EH (Fig. 2). The reaction of alkene to diol confirmed that the alkene-to-diol reaction was successful using a wide range of substrates from C_3 to C_6 alkenes in methanotrophs expressing three types of EH enzymes (CcEH from *Caulobacter crescentus*, McEH from *Mugil cephalus*, and RgEH from *Rhodotorula glutinis*). Among the alkene substrates, the highest titer was 92.18 mg/L of 1,2-propanediol (1,2-PDO) for propylene conversion in a CcEH-expressed Type II methanotroph. The reaction conditions were optimized using the *M. trichosporium* OB3b strain containing CcEH, and the final concentration was 263.5 mg/L 1,2-PDO under the optimal reaction conditions (Lee et al., 2020a).

3.2. Production of native products in Type II methanotrophs

The most attractive native-product in Type II methanotrophs is polyhydroxybutyrate (PHB), due to its inherent biodegradability, biocompatibility, water resistance, optical purity, and piezoelectric properties (Fig. 3) (Laycock et al., 2013; Strong et al., 2016b). PHB is generally synthesized in nutrient-deficient conditions and is utilized as a reducing equivalent source in nutrient-sufficient conditions (Khosravi-Darani et al., 2013). Nitrogen limitation is the most common way to accelerate PHB accumulation (Strong et al., 2016b). The route for PHB generation begins with acetyl-CoA molecules and passes through three enzyme-mediated reactions as follows: (1) condensation of two molecules of acetyl-CoA to yield acetoacetyl-CoA by β-ketothiolase (*phaA*); (2) conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA by NADPH-acetoacetyl-CoA reductase (*phaB*); (3) polymerization of 3-hydroxybutyrate to produce PHB by PHA synthase (*phaC*) (Khosravi-Darani et al., 2013; Strong et al., 2016b). Most studies on quantitative PHB accumulation are well-documented in Type II genera, including *Methylocystis*, *Methylococcus*, *Methylosinus*, and *Methylomonas* sp. (Asenjo and Suk, 1986; Dedysh et al., 2004; Helm et al., 2008; Helm et al., 2006; Pieja et al., 2011; Shah et al., 1996; Vecherskaya et al., 2001; Wendlandt et al., 2005; Wendlandt et al., 2001; Xin et al., 2007; Zhang et al.,

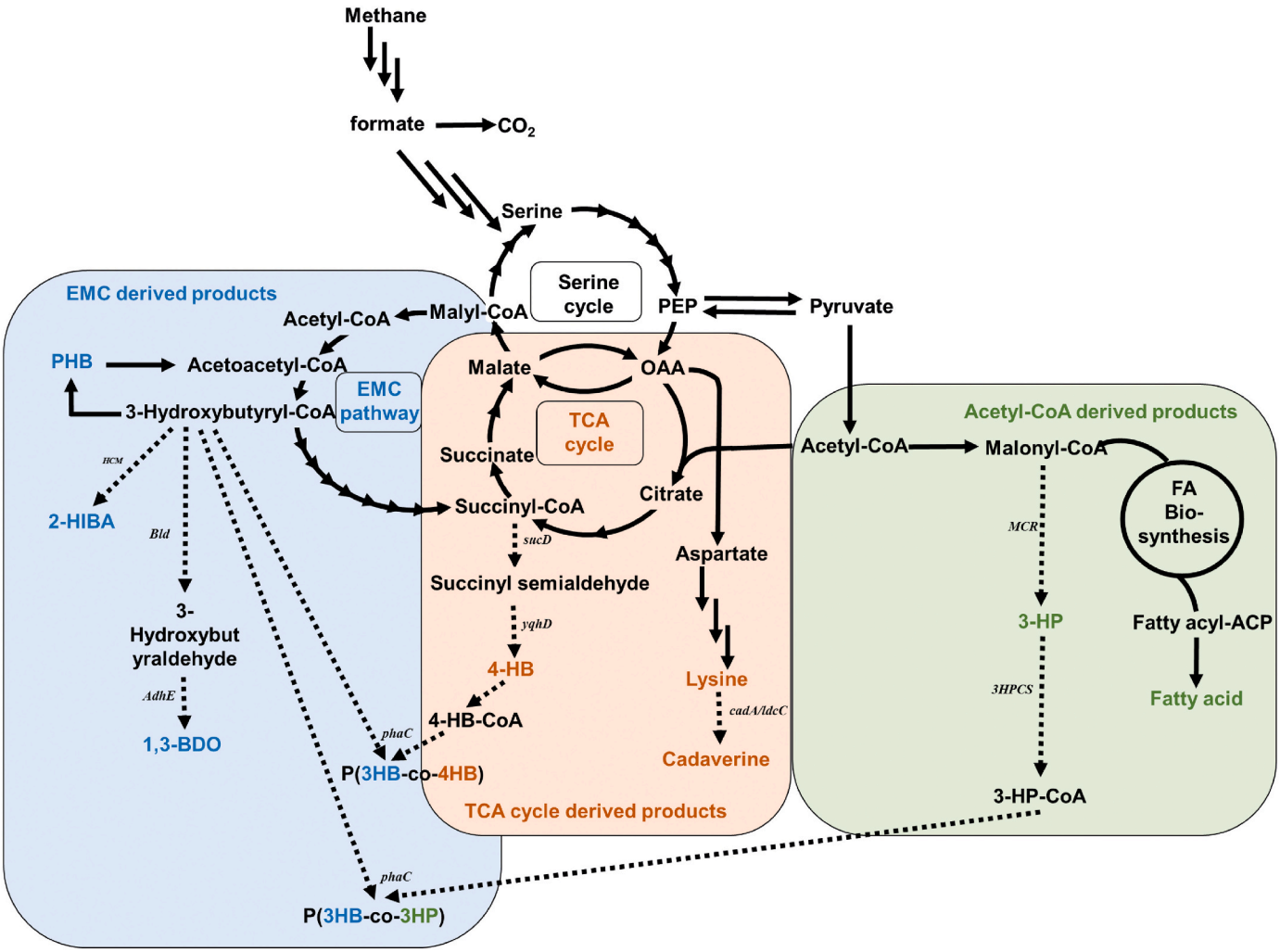

Fig. 3. Simplified metabolic pathway for production of chemicals and fuels from methane in Type II methanotrophs. The dotted arrows represent the non-native pathway, and the interactions mediated by the heterologous enzyme are marked in red. Enzymes (or encoding genes): *MCR*, malonyl-CoA reductase; *3HPCS*, 3-HP-CoA synthetase; *sucD*, succinate semialdehyde dehydrogenase; *yqhD*, succinate semialdehyde reductase; *cadA/ldcC*, L-lysine decarboxylase; *phaC*, PHA synthase; *HCM*, 2-hydroxyisobutyryl-CoA mutase; *Bld*, butyryl-CoA dehydrogenase; *AdhE*, alcohol/aldehyde dehydrogenase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2008a).

It has been reported that only a small amount of PHB (around 10%) was generated by *M. trichosporium* OB3b when methane and air were used as substrate mixtures. However, the PHB content rose to 45% when the air was switched to pure oxygen, indicating the role of nitrogen-limited conditions in PHB production because Type II generally can utilize molecular nitrogen (Shah et al., 1996). The Type II methanotrophic strain, *Methylocystis* sp. GB 25 can accumulate PHB in a brief non-sterile process with two-stage cultivation: a continuous growth phase and a PHB production phase under limiting conditions. The PHB content was 51% with a maximum PHB yield of 0.55 g/g with very high molecular mass of PHB produced (up to 2.5×10^6 Da) (Wendlandt et al., 2001). Furthermore, the biosynthesis of PHB with an ultra-high average-weight molecular weight can be induced by potassium-limited conditions with levels of 33.6% and a yield of 0.45 g/g in *Methylocystis* sp. GB 25 (Helm et al., 2008). The *M. trichosporium* IMV3011 can accumulate PHB up to 0.6 g/L using shake flasks via two-stage cultivation by adding methanol (0.1%) to improve methane oxidization. Then, to increase PHB composition, citric acid was supplied as an inhibitor of the TCA cycle, and the PHB yield increased from 12% to 40% (*w/w*). Generally, those resulting PHB possess high molecular weight up to 1.5×10^6 Da (Zhang et al., 2008a). Recently, nitrogen-free NMS was employed to examine the production of PHB in the presence or absence of N_2 by *M. trichosporium* OB3b (Zhang et al., 2019). In the absence of N_2, the highest PHB content (52.9% with 814.3 mg/L of PHB) was achieved at a ratio of CH_4:O_2 = 2:1, while in the presence of N_2, the highest PHB content (55.5% with 901.8 mg/L of PHB) was obtained at 0.2 atm O_2, and PHB accumulation was reduced at >0.3 atm O_2. These results show that the responses of PHB production in methanotrophs to oxygen in the absence and presence of N_2 were different (Zhang et al., 2019).

PHB production can be enhanced by optimizing the culture conditions. Flux balance analysis simulations showed that the *Methylocystis* strains can redirect high metabolic fluxes towards acetoacetyl-CoA precursor and then to PHB under nitrogen limitation, demonstrating that these operating conditions are particularly suitable for PHB production (Bordel et al., 2019b). Acetyl-CoA, which can be utilized by the TCA cycle and fatty acid pathway, is the precursor of acetoacetyl-CoA; therefore, the downregulation of the TCA cycle and fatty acid pathway helps to enhance the metabolic flux towards PHB. On the contrary, fatty acid production could be enhanced by the downregulation of the TCA and PHB cycles. Although fatty acids are attractive products of Type II methanotrophs (Fig. 3), as valuable precursors for the production of transportation fuels, there have been few reports on fatty acid production in Type I methanotrophs (Demidenko et al., 2017; Henard et al., 2016). In addition to the downregulated expression of the TCA and PHB cycles, acetyl-CoA carboxylase expression and the deletion of acetate kinase (block in acetyl-CoA to acetate conversion) are suitable strategies for improving carbon flux towards fatty acid building blocks (acetyl-CoA and malonyl-CoA) for an increased fatty acid accumulation (Demidenko et al., 2017).

3.3. Production of non-natural products via the introduction of the non-native pathway

The metabolic engineering of Type I methanotrophs has been extensively developed for the bioproduction of chemicals and biofuels (Henard et al., 2016; Lee et al., 2019a; Nguyen et al., 2018, 2019b). On the contrary, Type II methanotrophs have been recently applied for the production of non-natural products such as 3-HP and cadaverine (Nguyen et al., 2020a, 2020b) via metabolic engineering, indicating that Type II methanotrophs still have much room for further engineering for the production of potential products based on their metabolic characteristics. Type II methanotrophic alphaproteobacteria have a relatively high flux of acetyl-CoA, a key metabolic intermediate connected to TCA and EMC pathways, thus showing potential for applications in the biofuel production of target products originating from acetyl-CoA, as well as the TCA and EMC cycles (Fig. 3) (Kalyuzhnaya et al., 2015; Lee et al., 2019b).

3.3.1. Acetyl-CoA-derived molecules

3-HP is employed as a platform for the creation of many commercial compounds. It can be used for the production of industrial chemicals, such as acrylic acid, propiolactone, acrylamide, and poly(3HP), a favorable alternative to petrochemical plastics (Andreeßen and Steinbüchel, 2010; Kumar et al., 2013; Valdehuesa et al., 2013; Wang et al., 2013). The production of 3-HP from methane was performed using a Type II methanotroph (Fig. 3) (Nguyen et al., 2020a). To establish 3-HP production in *M. trichosporium* OB3b, the gene encoding *C. aurantiacus* bifunctional malonyl-CoA reductase (MCR) was overexpressed. Then, two strategies were carried out to increase the malonyl-CoA precursor and 3-HP production. First, the malonyl-CoA pool was increased by overexpressing native acetyl-CoA carboxylase (ACC), thus enhancing the production of 3-HP. The 3-HP titer was further increased in this strain by overexpression of biotin protein ligase and $NADP^+$-malic enzyme. Second, the pathway for acetyl-CoA carboxylation bypass was constructed to improve 3-HP production by overexpressing methylmalonyl-CoA carboxyltransferase (MMC). The co-expression of MMC and phosphoenolpyruvate carboxylase (PEPC) further enhanced the 3-HP titer. The highest 3-HP production was obtained in the OB3b-MCRMP strain, which expressed MCR, MMC, and PEPC. The strain accumulated 60.59 mg/L of 3-HP in bioreactor operation, increasing by 6.36-fold in volumetric productivity.

In addition to the malonyl-CoA pathway for 3-HP production, other metabolic pathways from glycerol and β-alanine precursors could be used for the biosynthesis of 3-HP (Borodina et al., 2015; Raj et al., 2008; Song et al., 2016). To produce 3-HP from glycerol, the glycerol metabolism in *K. pneumoniae* and *E. coli* could be applied by expressing glycerol dehydratase, which catalyzes the conversion of glycerol to 3-hydroxypropionaldehyde, and aldehyde dehydrogenase, which subsequently converts 3-hydroxypropionaldehyde to 3-HP (Raj et al., 2008). In another route of consecutive reactions converting β-alanine to 3-HP, β-alanine is converted into malonic semialdehyde by β-alanine pyruvate aminotransferase or γ-aminobutyrate transaminase, and malonic semialdehyde is further converted into 3-HP by 3-hydroxypropionate dehydrogenase or 3-hydroxyisobutyrate dehydrogenase (Borodina et al., 2015; Song et al., 2016). These pathways represent promising routes for the production of 3-HP in Type II methanotrophs.

3.3.2. TCA cycle-derived molecules

Type II methanotrophs are good candidates for the bioconversion of methane into TCA-derived products because of the complete TCA cycle and high carbon flux resulting in this cycle compared to Type I methanotrophs (Matsen et al., 2013). In the metabolism of Type II methanotrophs, succinyl-CoA generated from methylmalonyl-CoA via the EMC pathway and from alpha-ketoglutarate via the TCA cycle is thought to be higher than succinyl-CoA of gammaproteobacteria, which was only formed from alpha-ketoglutarate in the TCA cycle (Akberdin et al., 2018; Fu et al., 2017; Matsen et al., 2013; Yang et al., 2013). Type II methanotrophic bacteria are expected to possess a large pool of oxaloacetate (OAA) owing to the existence and expression of phosphoenolpyruvate carboxylase (*pepc*) and pyruvate carboxylase (*pc*) in its genome (Matsen et al., 2013; Stein et al., 2010). Moreover, flux balance analysis simulations revealed that Type II methanotrophs have significant potential for the production of chemicals from succinate and oxaloacetate derivatives (Bordel et al., 2019b). These features, combined with the fact that a large acetyl-CoA pool, provide advantages for the production of 4-hydroxybutyric acid derived from succinyl-CoA or succinate, lysine, and cadaverine derived from OAA (Fig. 3).

4-hydroxybutyric acid (4-HB), also known as gamma-hydroxybutyric acid, is an important precursor of chemicals and polymers, such as γ-butyrolactone, 1,4-butanediol, and various polyhydroxyalkanoates (Choi et al., 2013; Choi et al., 2016). 4-HB can be formed through two

metabolic routes: a reductive pathway (succinate route) and an oxidative pathway (α-ketoglutarate route) (Choi et al., 2013; Valentin et al., 2000; Yim et al., 2011). The redox balance in the 4-HB biosynthesis pathway should be considered since 4-HB is a reduced chemical, and therefore, optimizing the intracellular redox balance could enhance 4-HB production (Choi et al., 2013; Choi et al., 2016). Among these routes, a reductive pathway may be the most potent way for 4-HB production due to succinate and succinyl-CoA derivatives. Therefore, the 4-HB biosynthetic pathway was constructed in a Type II methanotroph, *M. trichosporium* OB3b, which expresses CoA-succinate semialdehyde dehydrogenase (*sucD*) and NADPH-succinate semialdehyde reductase (*yqhD*) genes. The engineered *M. trichosporium* OB3b accumulated 6.92 mg/L of 4-HB titer after shake flask culture. The 4-HB titer was increased up to 10.5 mg/L by further expressing the phosphoenolpyruvate carboxylase (*pepc*), isocitrate dehydrogenase (*icd*), and 2-oxoglutarate dehydrogenase (*sucAB*) genes in the engineered strain (Lee et al., 2020b).

L-lysine, one of the most essential amino acids, can be utilized as a precursor for the synthesis of several valuable products, such as glutaric acid, cadaverine, and 5-aminovaleric acid (Kim et al., 2015; Xu et al., 2014). L-lysine is natively produced in small amounts by methanotrophs. To enhance L-lysine production, aspartokinase III (*lysC*) and diaminopimelate decarboxylase (*lysA*) were expressed in *M. trichosporium* OB3b. The L-lysine level was improved by 1.37-fold and 2.78-fold in OB3b/lysC and OB3b/lysA, respectively. The expression of *lysC* or *lysA* led to a sharp increase in L-lysine accumulation (7.33 mg/L and 14.87 mg/L, respectively). This result indicated the role of *lysC* or *lysA* in the regulation of metabolic flux in the L-lysine biosynthetic pathway of *M. trichosporium* OB3b (Nguyen et al., 2020b).

Cadaverine (1,5-diaminopentane) is an industrial platform chemical with many applications for the production of polyamides or polyurethanes, chelating agents, or additives (Kind and Wittmann, 2011). The biosynthetic pathway of cadaverine encompasses the L-lysine production pathway. Cadaverine is formed through the decarboxylation of L-lysine, and thus its biosynthesis depends on L-lysine. Considering the capability of methanotrophs to natively produce L-lysine, cadaverine production could be achieved by using the L-lysine biosynthetic pathway and the expression of L-lysine decarboxylase (Akberdin et al., 2018; Chung et al., 2015; Stein et al., 2010). *M. trichosporium* OB3b was engineered to produce cadaverine by the expression of L-lysine decarboxylase (*cadA* and *ldcC* from *E. coli*) converting L-lysine directly to cadaverine. Compared to *cadA*, *ldcC* exhibited higher enzyme activity. L-lysine biosynthetic pool was increased by overexpressing native *lysC* and *lysA* and *Methylomonas* sp. DH-1 pyruvate carboxylase (*pyc*), resulting in a ~ 10-fold increase in the cadaverine titer (corresponding to 30.99 mg/L). Furthermore, cadaverine was further improved to 33.18 mg/L by introducing an *E. coli* cadaverine-lysine antiporter (*CadB*), which encourages the secretion of cadaverine. In that study, higher concentration of cadaverine was produced by using AMS medium with ammonium as a nitrogen source compared to using NMS medium with nitrate as a nitrogen source due to direct assimilation of ammonium into the lysine synthesis pathway. Finally, 283.63 mg/L of cadaverine with a volumetric productivity of 6.52 mg/g DCW/d was produced using a gas bioreactor system with AMS medium (Nguyen et al., 2020b).

3.3.3. EMC-derived molecules

Type II methanotrophs have the ability to alter the significant carbon fluxes into acetoacetyl-CoA under nitrogen-deficient conditions, combined with high metabolic fluxes that go through the EMC pathway (Bordel et al., 2019b; Yang et al., 2013), making these organisms attractive platforms for metabolic engineering for the production of EMC-derived molecules (Fig. 3).

1,3-Butanediol (1,3-BDO) is an important material for generating a variety of compounds, such as azetidinone derivatives and intermediate materials for pheromones, antibiotics, fragrances, and insecticides (Matsuyama et al., 2001). In the presence of *phaA* and *phaB* in Type II methanotrophs (Dam et al., 2012; Stein et al., 2011; Stein et al., 2010), 1,3-BDO could be accumulated by the expression of butyryl-CoA dehydrogenase (*Bld*), which catalyzes the conversion of 3-hydroxybutyryl-CoA into 3-hydroxybutyraldehyde, followed by the conversion of 3-hydroxybutyraldehyde into 1,3-BDO by alcohol/aldehyde dehydrogenase (*AdhE*) (Kataoka et al., 2013; Kataoka et al., 2014). Thus, Type II methanotrophs can be promising hosts for 1,3-BDO production.

2-Hydroxyisobutyric acid (2-HIBA), a hydroxylated carboxylic acid, has emerged as a fascinating building block for the production of acrylic glass, long-lasting coating, and inks (Rohwerder and Müller, 2010). The production of 2-HIBA is based on the prevalent overflow PHB pathway of bacteria; therefore, Type II methanotrophs as PHB native hosts could be employed for 2-HIBA production (Kalyuzhnaya et al., 2015; Rohde et al., 2017). In this route, acetyl-CoA is transformed into 3-hydroxybutyryl-CoA by *phaA* and *phaB*. Then, replacing the polymerization step catalyzed by *phaC*, the expression of 2-hydroxyisobutyryl-CoA mutase (HCM) can generate 2-hydroxyisobutyryl-CoA, which is consequently cleaved by intracellular thioesterases to excrete 2-HIBA (Kurteva-Yaneva et al., 2015; Rohde et al., 2017; Yaneva et al., 2012). As a proof-of-concept, the production of 2-HIBA in a non-methane utilizing methylotroph, *Methylobacterium extorquens* AM1, with a similar serine pathway for carbon utilization expressing HCM genes has already been demonstrated, and 2.1 g/L 2-HIBA was produced in the recombinant *M. extorquens* AM1 using methanol as the substrate (Rohde et al., 2017). Therefore, exploiting the PHB overflow metabolism in Type II methanotrophs can represent a potential strategy for 2-HIBA biosynthesis.

Many Type II methanotrophic bacteria can synthesize PHB under nitrogen-limited conditions, but the application of PHB is limited because of its stiffness and brittleness (Strong et al., 2016b). The *co*-polymerization of 3-hydroxybutyrate (3HB) with another hydroxyalkanoic acid (HA) monomer is an alternative to produce biopolymers with desirable properties, such as the polymers P(3HB-co-3HV) and P(3HB-co-4HB), which are comparatively tougher and more elastic than PHB. There are many strategies for the conversion of methane into more versatile polyhydroxyalkanoates (PHAs). The culture media can be supplied with a co-substrate that can be directly metabolized to produce fatty acid-CoA, followed by condensing with acetyl-CoA to generate the starting materials for copolymer synthesis. For example, by adding propionate or propionyl-CoA, 3-ketovaleryl-CoA is created and subsequently condensed with 3-hydroxybuturyl-CoA to generate the P(3HB-co-3HV) biopolymer. P(3HB-co-3HV) was produced by supplying valerate using a culture predominated by a *Methylocystis* spp., in which the molar % of 3HV rose with an increase in the amount of added valerate (Myung et al., 2015). Supplying different HA resulted in the accumulation of PHA copolymers in the culture of many methanotrophic strains, as well as two strains, including *M. parvus* OBBP and *M. trichosporium* OB3b (Myung, 2018). Only PHB was synthesized when methane was given as the only carbon source. Adding propionate enhanced PHBV production, while adding valerate further improved the PHBV content due to the formation of 3HV in the resulting copolymer (Myung, 2018). Even though these strategies have proven successful, the addition of precursors is an appended cost. Other combinations could be used to generate desirable PHA co-polymers from cheaper substrates. For example, poly(3HB-co-3HP) or poly(3HB-co-4HB) could be synthesized in Type II methanotrophs by combining the native PHB pathway and the 3-HP or 4-HB production pathway, together with the expression of 3-HP-CoA synthetase or 4-HB-CoA synthetase and native *phaC* or heterologous *phaC*, using methane as the sole carbon substrate.

4. Concluding remarks and outlook

Compared to Type I methanotrophs, Type II methanotrophic bacteria can be a more efficient biocatalyst for the production of important chemicals such as methanol, propanol, ethanol, epoxide, diol and so on which is directly related to methane oxidation pathway (Hwang et al., 2015; Hwang et al., 2014; Lee et al., 2020a; Nguyen et al., 2019a; Oh

et al., 2019; Patel et al., 2016b). Especially, due to a social issue from plastic pollution in the recent years, the capability to natively accumulate biodegradable plastics, PHB of Type II methanotrophs is an attractive characteristic (Laycock et al., 2013; Strong et al., 2016b). The physicochemical properties of PHB could be further improved by blending with another kind of polymer, P(3HP), or P(4HB), which can be used as a biodegradable polymer. Thus, metabolic engineering of Type II methanotrophs promises to bring a feasible environmentally-friendly solution for the production of green plastics with mitigation of greenhouse gas for acclimation of climate change.

Despite the large number of benefits provided by Type II methanotrophs for methane bioconversion, their applications are still limited due to a lack of fluent and efficient genetic engineering tools, as well as gaps in our understanding of their physiology and metabolism. In order to use Type II methanotrophs as an efficient industrial platform, an improved system-level understanding of cells, such as gene expression regulation, regulating mechanism of C1 metabolism, and metabolic flux distribution, is necessary for the suitable design of engineering strategies in Type II methanotrophs. Type II methanotrophs have lower biomass yields on methane in comparison with Type I methanotrophs due to the redox arm mechanism for methane oxidation (Bordel et al., 2019b; Bordel et al., 2019c; Naizabekov and Lee, 2020); this mechanism should be revisited to find a suitable way to improve cell growth. Additionally, the native serine cycle in Type II methanotrophs could be re-established to enhance methane utilization as well as the productivity of products from Type II methanotrophic strains (Nguyen and Lee, 2020). Type II methanotrophs showed potential for the production of acetoacetyl-CoA- as well as EMC-derived products (Bordel et al., 2019b), but the production of EMC derivatives could affect to the cell growth by disrupting the EMC pathway. Therefore, the construction of glyoxylate shunt as the replacement for the EMC pathway might be necessary for accumulation of EMC-derived products (Nguyen and Lee, 2020; Schada von Borzyskowski et al., 2018).

The establishment of a synthetic pathway in non-native host from the natural cycle existing in methanotrophs has emerged recently and brings another sustainable way for C1 bioconversion. By applying the suitable genes from natural serine cycle present in Type II methanotrophs, the reductive glycine pathway was constructed in the non-native hosts and considered a potential route to establish formatotrophic microbes such as *E. coli, S. cerevisiae,* and *C. necator*, opening the door for the C1-derived formate bioeconomy in a microbial platform (Claassens et al., 2020; Doring et al., 2018; Gonzalez de la Cruz et al., 2019; Yishai et al., 2018). Furthermore, the modified serine cycle built in *E. coli* allows the strain to assimilate methanol (or formate) together with CO_2 (Bang and Lee, 2018; Yu and Liao, 2018). Based on those concepts, the evolved synthetic formatotrophic and methylotrophic *E. coli* can grow on methanol and CO_2 using the reductive glycine pathway (Kim et al., 2020). Thus, synthetic methylotrophy could play a certain role in C1 gas biorefineries.

In this review, the latest advances in metabolic engineering of Type II methanotrophs are discussed, including progress in genetic tool development and multi-omics studies, metabolic divergence, and the capacity for employment of Type II methanotrophic bacteria as catalysts for the bioproduction of chemicals and fuels. This information can give strategies for metabolic engineering of Type II methanotrophs, including the selection of broad-host-range plasmid systems and novel strains with properties well-suited for metabolic engineering. Growing studies of methanotrophic metabolism will present opportunities for implementation of a variety of suitable metabolic engineering strategies. Such strategies will pave the way to employ the full potential of Type II methanotrophic biocatalysis. This review is expected to encourage researchers to take advantage of the methanotrophic machinery, as well as provide a driving direction for industrial implementation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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