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Received: 5 March 2026

Accepted: 11 May 2026

Published online: 16 May 2026

Cite this article as: Thai T.D., Ren J., Oh S.H. *et al.* Advances in biotechnological methods for genetic and metabolic engineering in *Methylobacter* sp. DH-1. *J Biol Eng* (2026). <https://doi.org/10.1186/s13036-026-00700-6>

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Advances in Biotechnological Methods for Genetic and Metabolic Engineering in *Methylobacter* sp. DH-1

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ABSTRACT

Methylobacter sp. DH-1 is a Gram-negative methanotrophic bacterium that utilizes methane as a carbon source and presents a potential chassis for sustainable production of value-added metabolites from this greenhouse gas. Realizing this potential requires the development of reliable and tailored genetic engineering methodologies. Recent progress has expanded the molecular toolbox for this strain, including optimized transformation methods to overcome host restriction barriers and plasmid-free genome engineering using cell-penetrating Cre recombinase. In addition, a tunable promoter

library for tunable transcriptional control and a synthetic small regulatory RNA platform for post-transcriptional modulation of gene expression have been established. Promising complementary engineering strategies, including adaptive laboratory evolution, synthetic consortia, and system biology approaches can be used to improve strain robustness and metabolic performance. The review highlights these advances, along with metabolic engineering efforts that have enabled the synthesis of diverse value-added chemicals in *Methylobacter* sp. DH-1. Collectively, these developments provide the foundation for metabolic engineering and synthetic biology in *Methylobacter* sp. DH-1 and may inform the design of genetic tools in other methanotrophs.

Keywords Methanotroph; *Methylobacter* sp. DH-1; Methane; Synthetic biology; Metabolic engineering

BACKGROUND

Methane (CH₄) is a critical climate pollutant since its effect on global warming is approximately 28 to 34 times stronger than that of carbon dioxide [1, 2]. Anthropogenic activities—such as fossil fuel extraction, agriculture, and waste disposal—have rapidly elevated atmospheric methane levels and amplified their climate impact [2]. Traditional methods for methane abatement include flaring, which involves high-temperature combustion to convert methane to carbon dioxide, and cryogenic distillation, which relies on low-temperature conditions for methane separation and recovery. While being effective for methane emission control, they typically require energy-

intensive operating conditions and do not transform methane into economic value [3]. In contrast, methanotrophic bacteria provide a biologically efficient route for converting methane into value-added chemicals under mild conditions [4-6].

Within the field of aerobic methanotrophy, *Methylococcus capsulatus* (Bath) and *Methylovulum burkatense* 5GB1C have emerged as the primary model organisms, extensively employed for metabolic engineering of high-value compounds (mevalonate, isoprene, taxadiene, organic acids and lipids) [7-11]. These Type I methanotrophs are Gammaproteobacteria characterized by rapid growth kinetics and the use of the ribulose monophosphate (RuMP) cycle—a pathway characterized by higher carbon efficiency compared with the serine cycle employed by Type II methanotrophs [12]. Versatile molecular toolkits, including conjugation and electroporation protocols, have been well-established for these strains [13, 14]. Furthermore, advanced genome editing is supported by allelic exchange vectors utilizing sucrose-based counterselection for unmarked mutations as well as broad-host-range replicative plasmid systems derived from pBBR1, RK2/RP4, and RSF1010 origins, which allow heterologous gene expression [12-14]. These foundational technologies have further enabled the implementation of clustered regularly interspaced short palindromic repeats (CRISPR)-based systems for efficient genome engineering in Type I methanotrophs [15-18].

Beyond established model methanotrophs, *Methylobacillus* sp. DH-1 has emerged as a promising chassis for metabolic engineering, offering distinct

physiological advantages over conventional Type I methanotrophs. Although *Methylomonas* sp. DH-1 is categorized as a Type I methanotroph, it is distinguished by the concurrent presence of both the RuMP cycle and serine cycle, thereby facilitating the enhanced carbon fixation and broader substrate flexibility across methane, methanol, and methylamine [19, 20]. This strain exhibits a methanol tolerance of up to 7% (v/v), surpassing the thresholds typically reported for other model Type I methanotrophs and supporting its application in high-titer methane-to-methanol bioconversion [4, 21, 22]. Additionally, *Methylomonas* sp. DH-1 possesses the endogenous carotenoid biosynthesis pathway, providing a native scaffold for the production of high-value pigments [19, 20]. While the availability of the complete genome and plasmid sequences, along with its transcriptomic profiles, offer a detailed roadmap for rational design, the genetic engineering of *Methylomonas* sp. DH-1 remains constrained by its a robust restriction-modification (R-M) system and a persistent high-copy endogenous plasmid, which continues to present challenges for efficient engineering efforts [19, 20, 23].

To extend the metabolic capacity of non-model bacteria, it is essential to redesign and optimize the existing methodologies to achieve reliable and functional bacterial engineering for various purposes. However, the absence of standardized molecular toolkits for the newly isolated bacterium presents a significant bottleneck to strain development. Recently, diverse molecular tools have been developed for the engineering of *Methylomonas* sp. DH-1

(**Fig. 1**). Firstly, optimized physicochemical transformation and electroporation protocols enabled the delivery of plasmids or DNA fragments for genetic engineering [24-26], and **the finding of a new methylation site** and corresponding methylase allowed for further improved DNA transfer efficiency [23]. In addition, a direct protein delivery system employing cell-penetrating peptides (CPPs) was established to enable efficient marker gene excision and expand the range of applicable genetic tools [27]. Beyond DNA and protein delivery strategies, the precise modulation of gene expression was refined at both the transcriptional and translational levels through the use of the well-characterized promoter library with varying strengths and the well-established synthetic small regulatory RNA (sRNA) platform, respectively [23, 28, 29].

In addition to the genetic engineering toolkits, the complementary engineering approaches such as adaptive laboratory evolution were also employed to improve the production yield in *Methylobacter* sp. DH-1, and several value-added bioproducts were successfully enhanced, including lactate [30] and succinate [31]. These non-rational approaches also offer interoperability with genetic engineering workflows by serving as preliminary tools for identifying promising targets and streamlining downstream genetic tool development.

This review provides an overview of genetic engineering toolkits specifically developed for *Methylobacter* sp. DH-1, and also discusses

relevant advances in other methanotrophs that may be adapted for future applications in *Methylomonas* sp. DH-1.

MAIN TEXT

Synthetic genetic tools

Methylomonas sp. DH-1 presents several challenges for genetic manipulation due to the intrinsic cellular barriers, yet the rapid advancement of synthetic biology has enabled the development of genetic engineering strategies tailored for this strain (**Fig. 1**). These strategies encompass DNA delivery methods, genome engineering approaches and gene expression control systems, which together support strain development and optimization. An overview of these strategies is provided in **Table 1**.

DNA delivery strategies: overcoming the host barriers

The introduction of exogenous DNA remains the bottleneck in engineering *Methylomonas* sp. DH-1 due to its strong R-M system. To overcome this challenge, several transformation strategies have been developed, each presenting the trade-offs between efficiency, reproducibility and experimental complexity (**Fig. 1**).

Physicochemical transformation

Conventional chemical transformation methods offer relatively high efficiency, whereas physical methods are simple and rapid; however, the efficiency of physical methods remains limited in bacterial transformation, motivating the development of alternative strategies [32-34]. To address this need, combined physical-chemical transformation methods have been

developed to expand the applicability of transformation methods to diverse Gram-positive and Gram-negative bacteria, including *Methylobacter* sp. DH-1 [24, 25]. Until this method, conventional chemical and physical methods were unable to transform *Methylobacter* sp. DH-1. This physicochemical approach leverages the actions of cationic agents and nanomaterials: cationic agents (Rb^+ , Mg^{2+} , Li^+ , Cs^+) to permeabilize bacterial membrane and nanomaterials (gold (III) chloride, multi-walled carbon nanotubes, and chitosan) to transiently disrupt cell membranes physically to enhance the DNA uptake [35-37]. This hybrid protocol **improves** transformation efficiency in recalcitrant bacterial species (*Cupriavidus necator*, *Bacillus* sp., *Enterococcus faecalis*, *Lactococcus lactis* subsp. *lactis*) as well as *Methylobacter* sp. DH-1. The highest transformation efficiency achieved using the hybrid method in *Methylobacter* sp. DH-1 was 8.78×10^4 CFU/ μg DNA when **1 M of RbCl** and 0.1 mg/mL of chitosan were used [25].

The physicochemical transformation strategy offers a versatile platform for engineering genetically recalcitrant bacteria by integrating chemical permeabilization with physical membrane disruption. By ensuring operational simplicity and eliminating the requirement for specialized equipment, this approach circumvents the technical constraints associated with conventional delivery methodologies, thereby enhancing broad accessibility. However, the efficiency of this strategy is often hindered by inherent variability; fluctuations in the characteristics of insoluble nanomaterials and preparation procedures frequently compromise inter-

batch reproducibility. While this methodology serves as a pivotal enabling technology for challenging strains, systematic optimization of both material properties and procedural parameters is essential to ensure robust reliability and broader applicability in bacterial biotechnology.

Electroporation

Electroporation introduces foreign DNA into bacterial cells by applying short, high-voltage pulses that transiently permeabilize the bacterial membrane for DNA entry before it reseals [38]. This method offers relatively high transformation efficiency and broad applicability, though its success depends on diverse parameters such as buffer composition, electric field strength, and DNA form and concentration. Recently, electroporation methods have been developed with enhanced transformation efficiency for type I methanotrophs, namely *M. buryatense* 5GB1C, *Methylocella silvestris* BL2 and *Methylomicrobium alcaliphilum* 20Z [14, 34, 39]. This method has been adapted for *Methylomonas* sp. DH-1 with several modifications [26]. Optimization efforts have focused on multiple parameters, including the growth phase of cells, electroporation buffers, competent cell volume, DNA concentration, electroporation conditions (electroporation cuvette width, electric field strength, capacitance and electrical resistance) and DNA forms. Specifically, 200 μ L of competent cells resuspended in sterile water and 10% glycerol with 500 ng of DNA in 2-mm cuvettes at 2.5 kV, 25 μ F, and 400 Ω achieved up to 3.2×10^3 CFU/ μ g DNA (linear DNA) and 2.5×10^3 CFU/ μ g

DNA (circular DNA), which demonstrates electroporation versatility for genetic manipulation of *Methylobacter* sp. DH-1 [26].

Electroporation is a robust DNA delivery platform, widely utilized for its reproducibility and broad applicability across diverse bacterial species. Its independence from permeabilizing chemical facilitates systematic optimization, as demonstrated by its successful application in various methanotrophic lineages. However, in *Methylobacter* sp. DH-1, the efficacy of this technique is constrained by an exceptionally narrow operational window. Transformation efficiency remains highly sensitive to physiological conditions, buffer compositions, and electrical parameters, which often compromises inter-laboratory reproducibility. Furthermore, electroporation cannot circumvent intrinsic biological barriers, such as R-M systems, leading to the rapid degradation of internalized DNA despite optimized pulse parameters. These observations suggest that physical membrane disruption alone is insufficient to overcome intracellular defense mechanisms. Consequently, while electroporation offers a versatile framework, its utility in *Methylobacter* sp. DH-1 remains fundamentally limited unless integrated with strategies designed to mitigate host-specific enzymatic barriers.

DNA pre-methylation for improved transformation efficiency

The most fundamental defense mechanism in bacteria is the R-M system that protects host cells from foreign DNA such as phage genome by restriction endonucleases while safeguarding the host genome from self-digestion by methyltransferases [40]. Since R-M system degrades foreign DNA including

plasmid DNA through restriction endonuclease activity, it is crucial to characterize R-M system for efficient transformation [23, 41]. Previous studies have reported that plasmids prepared from methanotrophic cells themselves are transformed into *M. alcaliphilum* 20Z and *M. buryatense* 5GB1C more efficiently than those obtained from *Escherichia coli* [14, 42]. This increased efficiency is attributed to the plasmids being modified by the donor methanotroph's R-M system, thereby protecting the incoming plasmid from restriction. Recently, a DNA methylation motif (TGGCCA) has been identified through whole-genome bisulfite sequencing in *Methylobacter* sp. DH-1 [23]. A cytosine-specific methyltransferase recognizes and methylates the fourth nucleotide within the sequence (TGGCCA). Although the TGGCCA motif has been experimentally validated in *Methylobacter* sp. DH-1, genomic databases such as REBASE have yet to identify this specific motif in other *Methylobacter* lineages [43]. Notably, related strains—including *Methylobacter koyamae* R-45283, *Methylobacter methanica* R-45371, and *Methylobacter* sp. 2B—possess a truncated GGCC motif that partially overlaps with the recognition sequence characterized in *Methylobacter* sp. DH-1 [23, 43]. These observations suggest a model of strain-specific divergence within R-M systems, wherein the core recognition machinery remains conserved while flanking sequence requirements evolve independently. However, a definitive resolution of these variations in the epigenetic landscape requires systematic, direct methylome characterization across the genus.

The introduction of plasmid DNA pre-methylated by the identified methyltransferase of *Methylobacter* sp. DH-1 led to a 124% increase in transformation efficiency (2.5×10^3 CFU/ μ g DNA) [23]. Pre-methylation of exogenous plasmids with an appropriate methyltransferase can assist in overcoming R-M systems, thereby improving transformation efficiency while shortening the recovery time (4 hours).

Pre-methylation of exogenous plasmids with an appropriate methyltransferase can provide a targeted strategy to bypass host R-M systems. However, its efficacy is often limited by the requirement for comprehensive methylome data, which are frequently unavailable for non-model organisms. Furthermore, in hosts harboring multiple redundant R-M systems, protection of a single motif is often insufficient to prevent DNA degradation. Consequently, while pre-methylation addresses a fundamental barrier to transformation, it remains a partial solution that must be integrated with complementary strategies to achieve robust and reliable DNA delivery.

Genome engineering approaches

Beyond DNA delivery, precise genome engineering tools are required to program cellular metabolism. In *Methylobacter* sp. DH-1, several engineering strategies have been developed (**Fig. 1**).

*Integration of Cre recombinase gene into the genome of *Methylobacter* sp. DH-1*

A genome editing platform mediated by integrated Cre recombinase has recently been established in *Methylobacterium* sp. DH-1 [44]. In this system, the Cre recombinase (*cre*) gene from bacteriophage P1 was integrated into the chromosome under the control of the *trc* promoter and regulated by the *lacI^q* repressor, positioned between loci AYM39_00230 and AYM39_00235. The DNA construct also carried a kanamycin resistance gene (*km^r*) flanked by *lox66* and *lox71* sites at the same genomic locus. Upon induction with 1 mM IPTG, Cre recombinase specifically excised the *km^r* gene, leaving the integrated *cre* module intact. Notably, the engineered strain exhibited comparable growth to the wild-type strain, indicating that Cre recombinase integration imposed no apparent fitness cost. This system provides a practical framework for iterative genetic modifications in *Methylobacterium* sp. DH-1 without exhausting selectable markers, thereby facilitating more flexible and sustainable genome engineering strategies.

The Cre-lox system provides a robust framework for iterative genomic modifications in *Methylobacterium* sp. DH-1. However, its dependency on pre-installed *lox* sites necessitates substantial design overhead and significantly circumscribes targeting flexibility. Furthermore, successive editing is restricted by the limited availability of *lox* variant sites, and the resulting accumulation of residual *lox* scars may perturb the local genomic landscape, potentially compromising long-term transcriptional regulation or genomic integrity. Consequently, while Cre-lox remains an effective tool for marker excision and targeted rearrangements, it functions as a specialized auxiliary

rather than a primary platform in modern, high-efficiency genome engineering workflows.

Direct delivery of Cre recombinase using CPPs

For successful genome editing in bacteria, it is essential to develop a plasmid to express gene editing proteins (e.g., Cas or Cre) and necessary components (e.g., single guide RNA) inside the host bacterium or to integrate those genes into the host bacterium's genome [44-46]. However, for newly isolated and often uncharacterized bacteria, compatible plasmid vectors that can be stably maintained for gene editing are frequently unavailable. To overcome this limitation, a recent study has developed a plasmid-free delivery method for Cre recombinase directly into bacterial cells using CPPs [27]. To efficiently deliver Cre recombinase into *Methylobacter* sp. DH-1, a library of 98 CPPs was constructed through database mining and literature review. The CPPs were initially evaluated in *E. coli*, identifying several candidates that showed high protein cargo delivery efficiency and low cytotoxicity. Among these, Cre recombinase conjugated with CPP70 (GRKKRRQRRR) showed the highest efficiency (15%) in excising a *loxP*-flanked DNA sequence in the *Methylobacter* sp. DH-1 genome. This result implies that CPPs can be used to deliver other gene editing proteins, such as Cas, for genome modification. This plasmid-free strategy enables genome editing without the need for compatible vectors and expands the applicability to non-model bacteria. However, its relatively low efficiency and the need for protein purification and conjugation limit its scalability and practical implementation.

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system

The CRISPR/Cas9 system has emerged as a promising platform to streamline genetic modification in methanotrophs, offering faster and more precise results methods [17]. This technology has been successfully applied in several methanotrophic species for genetic modification to enhance metabolite production, including as *M. capsulatus* (Bath) and *M. buryatense* 5GB1C [15, 16]. However, a functional CRISPR/Cas9 system has not yet been established for *Methylobacter* sp. DH-1. The primary challenge lies in the bacterium's exceptionally robust R-M system, which significantly limits the transformation efficiency of large Cas9-carrying plasmids. Furthermore, the presence of a persistent, high-copy endogenous plasmid in *Methylobacter* sp. DH-1 may complicate the stable maintenance of heterologous plasmids required for CRISPR components. Due to these barriers, exogenous DNA encoding Cas9 and sgRNA is rapidly degraded or lost before effective genome editing can occur.

Despite the critical role of the R-M system in *Methylobacter* sp. DH-1, to date, the TGGCCA motif is the only methyltransferase recognition sequence that has been experimentally validated in *Methylobacter* sp. DH-1. It remains unclear which specific methyltransferase serves as the primary component of this defense system, and whether additional R-M systems exist in *Methylobacter* sp. DH-1 since it harbors several other methyltransferase genes including putative ones. Therefore, further characterization of the R-

M system in *Methylobacter* sp. DH-1 is essential for the development of efficient and reliable genetic engineering methodologies.

Although the implementation of CRISPR/Cas9 system is still challenging in *Methylobacter* sp. DH-1, existing synthetic toolkits developed for this bacterial species offer potential alternative methods to overcome these obstacles. For example, the use of CPPs for Cas9 delivery provides a non-traditional approach to facilitate extracellular protein delivery for genome editing, paralleling the successful plasmid-free gene excision achieved with CPP-fused Cre recombinase as done for Cre recombinase for gene excision [27]. Alternatively, pre-methylation of both the Cas9-expressing plasmid and the single guide RNA (sgRNA) expression plasmid may bypass host restriction and enhance editing efficiency. Furthermore, considering the large molecular size of Cas9, exploring smaller and more efficient Cas variants (Cas12a or Cas13a) may provide a more suitable and flexible genome editing platform for methanotrophs [47, 48]. These smaller systems may be particularly suitable for methanotrophic hosts because they offer a less burdensome alternative to the bulky Cas9 systems. For example, Cas12a is relatively compact, requires only a single CRISPR RNA (crRNA), and generates staggered DNA cuts, enabling efficient and precise genome editing. Additionally, its ability for multiplex guide RNA processing supports complex metabolic engineering [49, 50]. In contrast, Cas13a targets RNA rather than DNA, allowing reversible gene regulation without permanent genomic alterations, which is useful for studying essential genes and fine-

tuning metabolic pathways [51, 52]. Moreover, the smaller size of these Cas variants may facilitate their intracellular delivery via CPP-mediated systems.

The CRISPR/Cas9 system offers a genome engineering method in methanotrophs. However, a functional CRISPR platform has not yet been established for *Methylobacter* sp. DH-1. The primary technical hurdle lies in the strain's robust R-M system and persistent high-copy endogenous plasmids, which cause rapid degradation of Cas-carrying DNA and hinder the stable maintenance of editing components. To overcome these hurdles, compact Cas variants (e.g., Cas12a or Cas13a) and CPP-mediated delivery are being explored as potential strategies to bypass host barriers and establish host-specific delivery architectures.

Gene expression control systems: fine-tuning metabolic flux

Precise regulation of gene expression is essential for balancing the metabolic pathways, minimizing the cellular burden and preventing the accumulation of toxic intermediates. In *Methylobacter* sp. DH-1, both transcriptional and post-transcriptional regulatory strategies have been developed to enable fine-tuning of metabolic flux.

Standardized promoter library

Uncontrolled expression of metabolic enzymes often results in resource depletion and accumulation of toxic intermediates, thereby constraining metabolic pathway efficiency and overall yield [43]. Promoters serve as central regulatory elements, making them indispensable tools for balancing gene expression [44]. The development of promoter libraries with a spectrum

of activities has emerged as a powerful strategy to fine-tune metabolic flux, alleviate cellular burden, and optimize production outcomes [53-55]. Recent studies have highlighted the utility of natural or synthetic promoters in methanotrophs, where such libraries enable nuanced control over heterologous pathways [56-58]. In this context, the identification and characterization of promoters with variable strengths is particularly important for expanding the genetic toolkit of newly explored hosts, including *Methylobacter* sp. DH-1.

A tunable promoter library was established to enable fine control of gene expression in *Methylobacter* sp. DH-1 [28]. In this study, 110 putative promoters were predicted from the 100-bp upstream regions of annotated genes using two computational tools, Softberry BPROM and the BDGP neural network promoter predictor. Complementing the *in silico* predictions, 2D-PAGE analysis of *Methylobacter* sp. DH-1 revealed five additional strong promoters that were not identified computationally. From these resources, a library of 38 promoters was constructed, comprising five experimentally identified promoters, four previously characterized promoters from *Methylobacter trichosporium* OB3b and *E. coli*, and 29 randomly selected candidates from the predicted set. Promoter activity was systematically assessed using green fluorescent protein (GFP) as a reporter, revealing strengths ranging from 0.24% to 410% relative to the *lac* promoter. This broad dynamic range highlights the library's utility for tuning metabolic flux in *Methylobacter* sp. DH-1. Specifically, the endogenous *mxoA* and *tal*

promoters exhibited the highest strengths, while the *lac* and *tac* promoters from *E. coli* supported medium-to-high expression levels in this host. However, since the activities of these library promoters were characterized under methane-supplemented conditions, their activity across distinct C1 substrates (e.g., methane versus methanol) remains to be further elucidated. Although promoter library offers precise and modular control of transcriptional strength as well as flexibility in pathway optimization, their regulatory output is largely static and may not adapt to the dynamic cellular or environmental conditions. In addition, the promoter activity can vary depending on the host physiology.

Gene knockdown with synthetic small regulatory RNAs (sRNAs)

sRNAs serve as versatile tools for fine-tuning gene expression and redirecting metabolic flux in diverse bacteria [29, 59]. Each synthetic sRNA contains a target-binding module that base-pairs with target mRNA and a scaffold region that recruits the Hfq chaperone for enhanced stability and knockdown efficiency. Synthetic sRNAs offer advantages over gene knockouts, including design simplicity, essential gene targeting, and broad host applicability, making them efficient and programmable regulators for metabolic engineering [59]. The feasibility of this approach has recently been demonstrated in *Methylobacter* sp. DH-1 [23]. In this study, a synthetic sRNA was designed to target the phytoene synthase (*psy*) gene involved in methylerythritol phosphate (MEP) pathway, a key node for carotenoid biosynthesis (**Fig. 2**). The target-binding sequence was rationally optimized

to achieve a binding energy of -30.7 kcal/mol, within the favorable range for effective sRNA-mRNA interactions [60]. Instead of relying on the canonical Hfq-dependent system, the design employed the RyhB-derived scaffold, which has been reported to function independently of *E. coli* Hfq in *Methylobacter* sp. DH-1, thereby offering broader applicability across bacterial species [23, 61, 62]. The anti-*psy* sRNA construct was expressed under the control of the *tac* promoter and integrated into the genome of *Methylobacter* sp. DH-1. Functional validation revealed a 40% reduction in carotenoid production compared with the wild-type strain, demonstrating that RyhB-based synthetic sRNAs can effectively downregulate target gene expression in *Methylobacter* sp. DH-1. This study demonstrates the early example of applying synthetic sRNA-mediated regulation in methanotrophs and highlights its potential as a genetic tool for metabolic engineering in emerging microbial species. Synthetic sRNA-based regulation provides a versatile method for tunable gene repression, particularly in modulating the expression of essential genes that are often inaccessible through traditional knockout strategies. While its relatively simple design allows for rapid implementation, variables such as mRNA secondary structure frequently decouple rational design from actual knockdown levels [60]. Furthermore, while Hfq-independent scaffolds offer broader applicability, reliance on host-specific RNA-processing machinery and potential off-target interactions remain significant hurdles to achieving the robust, quantitative control required for precise metabolic network engineering.

Adaptation of the genetic toolkits for *Methylobomonas* sp. DH-1 to other methanotrophs

The genetic toolkits and engineering strategies established for *Methylobomonas* sp. DH-1 provide a technical framework that can be adapted to other methanotrophic species, regardless of their specific physiological characteristics. A primary example of this broad applicability is the CPP-mediated protein delivery system developed for genome engineering of *Methylobomonas* sp. DH-1 [27]. Unlike traditional plasmid-based methods, CPP-based direct delivery of functional proteins into the cytoplasm bypasses the need for host-specific replicative vectors. For instance, a similar strategy has been successfully employed in *E. coli* to achieve plasmid curing by delivering CPP-fused restriction enzymes [27]. This plasmid-free approach is particularly valuable for newly isolated methanotrophs where compatible plasmids or stable expression systems have not yet been identified.

Furthermore, the physicochemical transformation methods developed for *Methylobomonas* sp. DH-1 have already demonstrated success across diverse bacterial species, including both Gram-positive and Gram-negative cells [25]. While these methods can be directly applied to other methanotrophs, the systematic optimization workflow used for *Methylobomonas* sp. DH-1 serves as a generalizable model, facilitating the development of highly efficient transformation protocols tailored to specific strains.

While promoter libraries are inherently host-specific, the integrated methodology used for *Methylobomonas* sp. DH-1—which combines

computational prediction with experimental validation through 2D-PAGE or transcriptomics—offers a robust blueprint for rapidly expanding the genetic toolbox of non-model methanotrophs [28].

Finally, the characterization of host-specific R-M motifs through methylome analysis provides a general strategy for improving DNA uptake in methanotrophs with strong defense systems [23]. By pre-methylating exogenous DNA based on these motifs, the host restriction barrier can be systematically bypassed, thereby enabling the implementation of advanced tools, such as CRISPR-based systems, across a broader range of methanotrophic species.

Potential complementary engineering strategies for *Methylomonas* sp. DH-1

The synthetic genetic engineering tools have been directly utilized in metabolic engineering to enhance the **production** of value-added compounds such as acetol [63], cadaverine [28], lactate [30, 64], succinate [26, 31], squalene [44], 5-aminolevulinic acid [65] and 2,3-butanediol [65] by precisely manipulating the genes of *Methylomonas* sp. DH-1 (**Table 2**). However, this rational approach requires prior knowledge regarding which genes benefit productivity enhancement. In contrast, adaptive laboratory evolution (ALE) offers a **less knowledge-dependent**, randomness-based engineering strategy [66]. Furthermore, when metabolic engineering for the final target product becomes overly complex, the synthetic consortia approach can be employed, where multiple microorganisms are co-cultured to allow one strain to convert

a metabolite produced efficiently by another [67]. In addition, the emergence of systems biology—encompassing genome-scale metabolic modeling and multi-omics analysis—now provides a predictive framework to understand and optimize methane metabolism [68, 69]. Systems biology approaches enable the prediction of carbon flux distribution and identification of metabolic bottlenecks, which may support more rational strain design and improved production of value-added compounds in *Methylobacterium* sp. DH-1. This section introduces these three complementary strategies.

Adaptive laboratory evolution (ALE)

ALE is a long-established experimental strategy used to study microbial evolution under controlled conditions [66]. Although this method relies on spontaneous mutation and selection rather than a rational engineering strategy, it has been successfully applied to isolate improved bacterial strains by cultivating them under appropriate selection pressure [66]. In ALE, bacterial cells are continuously cultivated in a selective environment, either deprived of specific nutrients or supplemented with stress-inducing compounds for extended periods (weeks to months or even years), enabling the gradual accumulation of beneficial mutations that result in adaptive phenotypes. Beyond improving specific traits, ALE provides valuable insights into metabolic flexibility, stress response mechanisms, and evolutionary trajectories, thereby informing subsequent rational metabolic engineering by analyzing accumulating beneficial mutations [70, 71].

Synthetic consortia

Synthetic microbial consortia offer a promising approach to enhance methane bioconversion by coupling *Methylobacter* sp. DH-1 with other heterotrophic bacteria [72-74]. As a type I methanotroph, *Methylobacter* sp. DH-1 efficiently oxidizes methane into intermediates such as methanol, formaldehyde, and organic acids, which can be further metabolized by co-cultured bacteria to synthesize complex biochemicals [21, 26, 64].

Such cooperative systems enable metabolic division of labor, improved carbon utilization, and detoxification of inhibitory intermediates, while maintaining redox balance [73, 74]. Similar strategies have been demonstrated in other methanotrophs, for example, consortia of *Methylobacter capsulatus* (Bath) and *E. coli* for producing mevalonate [72], and that of *Methylobacter* sp. OK1 and *E. coli* for yielding polyhydroxybutyrate (PHB) [75].

Though no study on synthetic consortia of *Methylobacter* sp. DH-1 with other bacterial species has been reported, it would broaden its biosynthetic capabilities and establish a modular, sustainable platform for methane-to-chemical conversion owing to its high metabolic capacity to convert methane to other intermediates [19-21].

Systems biology approaches

Systems biology serves as a fundamental bridge between high-throughput data and actionable engineering strategies. Genome-scale metabolic models (GEMs), typically analyzed using constraint-based methods, allow for the prediction of carbon flux distribution through *in silico* simulations and the

identification of competing pathways that limit methane-to-product conversion efficiency [76]. Concurrently, multi-omics datasets (e.g., transcriptomics and proteomics) provide insights into condition-specific gene expression and regulatory constraints [77]. When integrated with multi-omics data, the GEMs allow for the identification of regulatory constraints that are not apparent at the genomic level alone [78]. The iterative Design-Build-Test-Learn (DBTL) cycle employed in synthetic biology can enable to refine the GEMs by learning from high-throughput data and eventually can achieve a balance between energy conservation and biosynthetic demands to build more efficient biofactories [79]. Although a comprehensive GEM has not yet been established for *Methylobacter* sp. DH-1, insights from GEMs of related methanotrophs can support model construction in *Methylobacter* sp. DH-1 [80, 81]. Furthermore, the availability of its genome sequence and transcriptomic and metabolic data provides foundation for future development of *Methylobacter* sp. DH-1 GEM and finally enables model-driven metabolic engineering, promising a more streamlined path towards industrial-scale methane bioconversion [19, 20].

Metabolic production of value-added compounds in *Methylobacter* sp. DH-1

Methylobacter sp. DH-1 has been extensively engineered as a microbial cell factory for the production of diverse value-added compounds. Herein, we introduce a few key metabolites and engineering strategies employed to construct these effective biofactories (**Table 2**) (**Fig. 2**).

Acetol

Acetol, a compound containing both hydroxyl and carbonyl groups, is an important intermediate used in the production of various industrial materials, including textiles and cosmetics [82]. Growing interest has focused on its biological synthesis as a sustainable alternative to conventional chemical processes, since conventional chemical strategies require high temperature (220-550 °C) and expensive metal catalysts (cerium oxide, zirconium oxide, gallium oxide), which increases cost for acetol production and releases harmful byproducts [83]. A direct conversion pathway has been identified in methanotrophs, where acetone is oxidized to acetol either by cytochrome P450 oxidoreductases in the type II versatile methanotroph *M. silvestris* BL2 [84] or acetone monooxygenase (PmoCAB3) - a homolog of particulate methane monooxygenase (pMMO) - in the thermoacidophilic methane-oxidizing bacterium *Methylophilum* sp. IT6 [85].

Methylophilum sp. DH-1 was recently shown to be capable of producing acetol from acetone, albeit at low levels [63]. To enhance this production, the *pmoD* gene from *Methylophilum* sp. IT6 was introduced into *Methylophilum* sp. DH-1. PmoD is predicted to function as a transport protein that facilitates acetone delivery to the pMMO active site, improving acetone oxidation by pMMO (**Fig. 2**). Under an optimized condition, acetol production reached 18.3 mM (1.35 g/L) after 6 hours with 10 g/L acetone and 9.6 g of dry cell weight per liter (gDCW/L) biomass. This study also proposed a novel

acetone (or propane) assimilation pathway in *Methylobacter* sp. DH-1, with acetol serving as an intermediate.

Cadaverine

Cadaverine, a foul-smelling diamine, is an important precursor for polyamides such as PA54 and PA56 and has diverse applications in agriculture and medicine [86]. In microorganisms, it can be produced by lysine decarboxylase (*ldcC* or *cadA*) using lysine as a substrate [87]. Production of cadaverine in methanotrophic bacteria **has been demonstrated** in *M. trichosporium* OB3b by heterologous expression of the *E. coli*-derived *cadA* gene, **revealing** the potential of a methane-based platform for diamine biosynthesis [88].

Recently, *Methylobacter* sp. DH-1 has been engineered as a host for cadaverine production [28]. Since this strain lacks the native cadaverine biosynthetic pathway, the *cadA* gene from *E. coli* was expressed under a range of endogenous promoters with different strengths. Moderate promoters ($P_{\text{Intergrase}}$ and P_{rpmB}) supported both normal growth and higher cadaverine titer, reaching up to 11.6 mg/L and 8.32 mg/L, respectively. To further enhance yields, the *E. coli* antiporter gene (*cadB*) was co-expressed with *cadA* under fine-tuned promoters, achieving titers of 14.46 mg/L in the mutant strain harboring $P_{\text{rpmB}}\text{-}cadA/P_{\text{DnaA}}\text{-}cadB$. These findings demonstrate that transcriptional tuning, rather than strong overexpression, is crucial for balancing cell growth with efficient cadaverine production in *Methylobacter* sp. DH-1.

Lactate

Lactate serves as a valuable platform molecule with applications in pharmaceuticals, food additives, and particularly in the production of polylactic acid bioplastics [89]. While *Methylobacter* sp. DH-1 is intolerant of the low pH caused by lactate accumulation; subsequent engineering strategies have enabled the lactate production.

To overcome this limitation, ALE has been effectively applied to *Methylobacter* sp. DH-1 to enhance its tolerance to lactate toxicity (low pH) for enhanced lactate production [30]. The bacterial cells were continuously cultivated in media with progressively higher lactate concentrations (0.5 - 8 g/L), leading to the selection of a mutant tolerant (JHM80) to 8 g/L of lactate. Genomic analysis of JHM80 revealed that a 2-bp (TT) deletion in the *watR* promoter region upregulated the WatR protein (LysR-type regulator) expression and in turn WatR protein activated the WatABO efflux pump genes, thereby enhancing organic acids tolerance [30, 90] (**Fig. 2**).

For D-lactate biosynthesis in the lactate-tolerant *Methylobacter* sp. DH-1 strain (JHM80), the D-lactate dehydrogenase gene from *Leuconostoc mesenteroides* subsp. *mesenteroides* (*Lm.ldh*) was introduced into the JHM80 genome [30]. In addition, the native glycogen synthase gene (*glgA*) was deleted to redirect flux towards pyruvate for enhanced lactate production (**Fig. 2**). As a result, the engineered strain produced 1.19 g/L of lactate with a yield of 0.245 g/g methane, demonstrating that tolerance engineering is a critical step for efficient lactate production.

In a following scale-up study, the *glgA*-deleted strain showed growth inhibition, resulting from the accumulation of ADP-glucose (toxic at high concentration) under carbon-excess conditions coming from the enhanced gas-to-liquid transfer in the bioreactor [64]. This limitation was overcome by additional disruption of glucose-1-phosphate adenylyltransferase (*glgC*), the enzyme responsible for ADP-glucose biosynthesis (**Fig. 2**). The resulting strain achieved 6.17 g/L of D-lactate at 50 μ M IPTG induction in a 5-liter bioreactor.

Succinate

Succinate is a high-value platform chemical with wide industrial applications [91, 92]. In methanotrophs, succinate biosynthesis is mainly channeled through the oxidative TCA branch and the glyoxylate pathway since methane assimilation requires oxygen [93] (**Fig. 2**).

Methylobacter sp. DH-1 was known to secrete succinate and exhibit high tolerance up to 7.2 g/L of succinate, which highlights its potential as a platform for succinate production [19, 26]. Inactivation of succinate dehydrogenase (*sdh*) in the TCA cycle resulted in a 10-fold increase in succinate production (95 mg/L) compared with the wild-type strain (9.54 mg/L) [26]. Furthermore, the glyoxylate shunt pathway of *E. coli*, comprising of isocitrate lyase (*il*) and malate synthase (*ms*), was additionally introduced into the succinate-producing *Methylobacter* sp. DH-1 strain to compensate for the incomplete TCA cycle and thus enhance the serine cycle to supply more oxaloacetate and acetyl-CoA precursors for succinate synthesis [26]

(**Fig. 2**). As a result, the engineered strain produced 195 mg/L of succinate in fed-batch culture.

In another study, *Methylobacter* sp. DH-1 was engineered for succinate production by integrating *E. coli* glyoxylate shunt genes and deleting the genomic *sdh* gene [31]. ALE was subsequently employed to further enhance succinate production, which resulted in a titer of 323 mg/L. This improvement was attributed to enhanced methane and oxygen consumption, extended substrate uptake, and a more than two-fold increase in growth rate relative to the parental strain. Genomic analysis identified an 87-kilobase genomic duplication containing 68 genes, including the key enzymes for succinate production: malyl-CoA lyase (*mcl*), malate thiokinase subunit alpha (*mtkA*), and succinate-CoA ligase subunit beta (*sucC*). Furthermore, the up-regulation of transcriptional regulators (*creB*, *gcvR* and a TetR family transcriptional regulator, AYM39_08625) likely modulated genes associated with pMMO complex, thereby enhancing methane assimilation. Further rational engineering—including the overexpression of endogenous citrate synthase (*gltA*), pyruvate carboxylase (*pc*) and phosphoenolpyruvate carboxylase (*ppc*) genes combined with succinate semialdehyde dehydrogenase (*ssadh*) gene disruption—elevated the succinate titer to 702 mg/L of succinate, representing a nearly two-fold increase over the ALE-evolved strain. These results highlight the effectiveness of combining ALE with targeted genetic modifications to improve substrate utilization, metabolic activity, and production performance in *Methylobacter* sp. DH-1.

Squalene

Squalene, a lipophilic triterpene with significant market demand in cosmetics, nutraceuticals, and vaccine adjuvants, is traditionally obtained from shark liver oil or plant sources [94]. Interestingly, *Methylomonas* sp. DH-1 has been identified as a natural producer of squalene, offering potential as a microbial production platform [44].

In a recent study aimed at enhancing squalene production in *Methylomonas* sp. DH-1, several genes that consume the squalene precursor (presqualene diphosphate, PSPP) were deleted: phytoene desaturase (*pds*), diaplycopenene oxygenase (*crtN2*), and aldehyde dehydrogenase (*ald*) [44] (**Fig. 2**). Additionally, squalene-hopene cyclase (*shc*), that converts squalene to hopene, was partially inhibited using its inhibitor (ferulenol, 1.5 µg/mL). This partial inhibition reduced squalene consumption while avoiding the growth defects caused by the complete loss of cyclase activity. These modifications enabled squalene production of 7.4 mg/L in flask culture and 10.6 mg/L in fed-batch fermentation. Further improvement was achieved by integrating an extra copy of the native squalene synthase (*sqs*) under the strong *mxoF* promoter, yielding 8.4 mg/L in flask culture and 18.2 mg/L in fed-batch condition. Thus, this highlights the potential of *Methylomonas* sp. DH-1 as a robust microbial platform for squalene production.

5-Aminolevulinic acid

5-Aminolevulinic acid (ALA) serves as a starting point for heme, chlorophyll, and vitamin B₁₂ (cobalamin) formation and finds applications in medicine,

agriculture, and as a bio-stimulant [95]. In many bacteria, including methanotrophs, ALA is synthesized by the C5 pathway, in which glutamate is converted through a series of enzymatic steps to ALA (**Fig. 2**). *Methylomonas* sp. DH-1 has been reported to naturally produce ALA through this pathway, achieving 2.19 mg/L after 120 hours of cultivation [65]. To explore the potential for ALA production by the C4 pathway, in which glycine and succinyl-CoA are condensed by 5-ALA synthase (ALAS), two ALAS genes from *M. trichosporium* OB3b (*OB-alas*) and *Rhodobacter* sp. (*Rs-alas*) were individually expressed in *Methylomonas* sp. DH-1. The two engineered strains exhibited increased ALA production, reaching approximately 3.78 mg/L for *OB-alas* and 3.94 mg/L for *Rs-alas*, representing a 1.8-fold improvement over the wild-type strain. These findings highlight the metabolic potential of *Methylomonas* sp. DH-1, benefiting from the glycine from the serine pathway and succinyl-CoA from TCA cycle, a combination absent in many other Type I methanotrophs [96] (**Fig.2**).

2,3-Butanediol

2,3-Butanediol (2,3-BDO) is an important platform chemical used in synthetic rubber, solvents, fuels, and pharmaceuticals [97]. In bacteria, it is typically synthesized from pyruvate by the sequential reactions of acetolactate synthase, acetolactate decarboxylase, and acetoin reductase [42].

Interestingly, *Methylomonas* sp. DH-1 naturally produces acetoin (2,3-BDO precursor) when cultured with a methane (20%)-ethane (10%) mixture [65]. Methane primarily supports biomass formation while ethane is oxidized

by pMMO, methanol dehydrogenase (*mdh*), and pyruvate decarboxylase (*pcd*) to yield acetoin (1.61 mg/L) [65]. Therefore, the replacement of endogenous glycogen synthase (*glgA*) with the acetoin reductase (*budC*) from *Klebsiella pneumoniae* enabled the conversion of acetoin to 2,3-BDO (21.4 mg/L) without significant carbon loss as carbon dioxide. This approach highlights *Methylobacter* sp. DH-1 as a promising host for 2,3-BDO synthesis from ethane. This strain may also offer advantages over conventional methanotrophs and heterotrophic microorganisms, with reduced carbon loss and relying on minimal genetic modification.

Conclusion and Future Perspectives

Methylobacter sp. DH-1 has transitioned from a promising isolate to a significant contender of methane-based biomanufacturing. To fully realize its potential as an industrial chassis, the research focus must now shift from foundational tool development toward an integrated engineering paradigm that harmonizes molecular biology with computational and process engineering.

The next frontier for *Methylobacter* sp. DH-1 lies in the establishment of high-throughput synthetic genetic platforms for rapid and multiplex genome editing or modulation. This evolution will allow researchers to move beyond single-target modifications toward the rapid construction and screening of expansive genetic libraries. In parallel, achieving system-level insights through the development of specialized metabolic models is paramount. A functional computational model is essential to navigate and

optimize the unique dual-pathway architecture of *Methylobacter* sp. DH-1. Such insights will enable the rational orchestration of carbon flux between the concurrent RuMP and serine cycles, ensuring that metabolic resources are precisely diverted toward high-value products without compromising cellular growth or redox balance.

Ultimately, the convergence of these experimental and computational advancements will facilitate a robust DBTL cycle for methanotroph engineering. Within this framework, high-throughput genetic interventions will be rationally guided by system-level predictions and iteratively refined through process-scale feedback. This holistic approach will not only accelerate the optimization of the metabolic pathways discussed in this review but also solidify the competitiveness of *Methylobacter* sp. DH-1 as a cornerstone of the global C1-based bioeconomy.

Abbreviations

als, 5-aminolevulinic acid synthase; *ald*, aldehyde dehydrogenase; *budC*, acetoin reductase; *cadA*, lysine decarboxylase; *crtN2*, diaplycopen oxygenase; *glgA*, glycogen synthase; *glgB*, 1,4- α -glucan branching enzyme; *glgC*, glucose-1-phosphate adenylyltransferase; *gltX*, glutamyl-tRNA synthetase; *hemA*, glutamyl-tRNA reductase, *hemL*, glutamate-1-semialdehyde aminotransferase; *il*, isocitrate lyase; *ldh*, lactate dehydrogenase; *mdh*, methanol dehydrogenase; *ms*, malate synthase; *pcd*, pyruvate decarboxylase; *pds*, phytoene desaturase; *pmoCAB*, particulate methane monooxygenase; *psy*, phytoene synthase; *sdh*, succinate

dehydrogenase; *shc*, squalene-hopene cyclase; *sqs*, squalene synthase; CadB, cadaverine/lysine antiporter; PmoD, putative acetone transporter; WatABO, Resistance-Nodulation-Division (RND)-type efflux pump; 2,3-BDO, 2,3-Butanediol; 6PG, 6-phosphogluconate; ALA, 5-Aminolevulinic acid; DMAPP, dimethylallyl pyrophosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; F1,6P, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; FPP, farnesyl pyrophosphate; G1P, glucose 1-phosphate; G3P, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl diphosphate; H6P, hexulose 6-phosphate; IPP, isopentenyl pyrophosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; PSPP, presqualene diphosphate; Ru5P, ribulose 5-phosphate; ALE, Adaptive laboratory evolution; Au³⁺, gold cation; CPP, Cell-penetrating-peptide; CRISPR, Clustered regularly interspaced short palindromic repeats; CS, chitosan; Cs⁺, cesium cation; ED, Entner-Doudoroff; EMP, Embden-Meyerhof-Parnas; GEM, genome-scale metabolic model; GFP, green fluorescent protein; Li⁺, lithium cation; loxP, Cre-recognition DNA site; Mg²⁺, magnesium cation; pMMO, Particulate methane monooxygenase; PPP, pentose phosphate pathway; Rb⁺, rubidium cation; R-M, Restriction-modification; RuMP, ribulose monophosphate; Sep, Sepiolite; sRNA, synthetic small regulatory RNA; TCA, tricarboxylic acid.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors have reviewed and approved the final version of the manuscript and are fully informed of its submission.

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (RS-2025-02303886) and the Chung-Ang University Research Grants in 2025.

Authors contributions

TDT contributed to conceptualization, writing, editing, and visualization. RJ contributed to conceptualization, writing, and editing. SHO contributed to the writing of the original draft. DN contributed to writing and editing of the review, as well as supervision and funding acquisition.

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Figure Legends

Fig. 1 Expanded synthetic genetic tools and complementary engineering strategies enabling methane-based biomanufacturing in *Methylobacter* sp. DH-1. The full names are listed in **Abbreviations**.

Fig. 2 Schematic of methane, ethane and acetone oxidation integrated with engineered biosynthetic pathways for high-value bioproducts in *Methylobacter* sp. DH-1. Red-labeled genes are heterologous genes and blue-labeled genes are native genes modulated for enhanced metabolites production. Red crosses denote gene deletions. Orange arrows show the oxidation process of carbon sources, and triple arrows represent sequential enzymatic reactions. Dashed lines indicate non-native or synthetic metabolic routes. Purple circles highlight target metabolites produced by genetic and non-genetic modifications. The full names are listed in **Abbreviations**.

Table 1 Synthetic genetic engineering tools developed for *Methylobacter* sp. DH-1

Strategy	Method	Key features	Optimized conditions	Outcome	Advantages	Disadvantages	References
Delivery	Physicochemical transformation	Cationic agents (Rb ⁺ , Mg ²⁺ , Li ⁺ , Cs ⁺) and DMSO combined with nanomaterials (sepiolite, gold(III) chloride, multi-wall carbon nanotubes, chitosan).	1 M RbCl and 0.1 mg/mL chitosan.	8.78×10^4 CFU/ μ g DNA.	Simple method. No specialized equipment needed.	High chemical complexity. Potential cytotoxicity from DMSO or nanomaterials. Non-standardized process.	[25]
	Electroporation	DNA delivery by high-voltage pulse to create transient pores.	Cells at exponential phase or agar-plate grown (30% methane, 3-7	3.2×10^3 CFU/ μ g DNA (linear DNA). 2.5×10^3 CFU/ μ g DNA	Standardized protocol. Compatibility with different DNA types.	Experimental parameters sensitivity (cell preparation,	[26, 27]

			days), suspended in sterile water. Electro-program of 2.5 kV, 25 μ F, 400 Ω (or Ec2 setting for BioRad MicroPulser electroporator). Overnight recovery after electroporation.	(circular DNA).	Good reproducibility and broad adaptability.	electrical conditions). Lengthy overnight recovery culture period.	
	Plasmid pre-methylation	Methylation of plasmid DNA by cytosine methyltransferase (AYM39_01025) from <i>Methylobacterium</i> sp. DH-1. Electroporation of methylated plasmid into <i>Methylobacterium</i> sp. DH-1.	Plasmid pre-methylation using <i>Methylobacterium</i> sp. DH-1-derived cytosine methyltransferase gene (AYM39_01025) introduced inside <i>E. coli</i> . Four-hour recovery after electroporation.	The transformation efficiency of 2.5×10^3 CFU/ μ g DNA after 4-hour recovery, markedly shorter than the overnight recovery of conventional electroporation.	Host restriction-modification conquering. Short recovery culture duration.	Additional intermediate steps for methylated plasmid DNA preparation in a specific host.	[23]
Engineering	CPP-mediated direct protein delivery	Cre recombinase delivered directly into bacterial cells.	CPP70 (GRKKRRQRRR) conjugated with Cre showed a moderate gene excision efficiency from genome.	The 15% efficiency of plasmid-free gene excision flanked by <i>loxP</i> sites from genome.	The transient, non-genome integrative method. Host restriction-modification overcoming.	Moderate efficiency. Protein purification and conjugation requirement.	[27]
	Integrated Cre recombinase system	Genomic integration of <i>cre</i> under <i>trc</i> promoter and regulated by <i>lacI^q</i> .	Kanamycin resistance gene flanked by <i>lox66</i> and <i>lox71</i> excised upon 1 mM IPTG induction.	Stable strain (DH1-Cre) with no apparent growth defect under reported conditions for engineering established.	High stability for iterative engineering. Simplified workflow via chemical induction.	Permanent modification of host genome.	[44]
Expression	Promoter library	Library of 38 promoters identified from computational predictions and	Strength range: 0.24% - 410% relative to <i>lac</i> promoter (closely 1708-fold).	Tunable expression for fine metabolic flux control.	Graded tuning of metabolic flux. Dynamic promoter	Static expression. The strength may vary depending on growth	[28]

	2D-PAGE experiment.			range for pathway optimization.	substrates (methane or methanol).	
Synthetic sRNA-mediated knockdown	Programmable repression tool independent of Hfq.	Anti- <i>psy</i> sRNA targeting carotenoid biosynthesis (MEP pathway). Optimized binding energy (30.7 kcal/mol) and expressed under <i>tac</i> promoter.	40% reduction in carotenoid production. Early example of sRNA application in methanotrophs..	Simple design. Non-lethal and reversible gene silencing. Possibly targeting essential genes.	Partial knockdown of gene expression. mRNA accessibility-dependent regulatory efficacy.	[23]

Table 2 Value-added bioproducts synthesized by *Methylobacter* sp. DH-1 using metabolic engineering

Metabolite	Engineering strategy	Key modifications	Titer	Headspace air (% v/v)
Acetol	Genetic	Integration of <i>pmoD</i> gene (putative acetone transporter) from <i>Methylobacterium</i> sp. IT6 to enhance acetone oxidation by pMMO	1.36 g/L	30% Methane
Cadaverine	Genetic	Expression of <i>Escherichia coli</i> -derived <i>cadA</i> (for cadaverine production) and <i>cadB</i> (for cadaverine export) under moderate native promoters (P_{rpmB} - <i>cadA</i> / P_{DnaA} - <i>cadB</i>)	14.46 mg/L	30% Methane
Lactate	Genetic + ALE + Nitrate supplement	Elevated lactate tolerance strain (JHM80) established by ALE. <i>Lm.ldh</i> gene (for lactate production) expressed by native promoter of the <i>glgBA</i> operon. Disruption of the glycogen-synthesizing <i>glgA</i> gene	1.19 g/L	20% Methane
	Genetic + Nitrate supplement	KNO_3 optimization (2 g/L). <i>Lm.ldh</i> gene (for lactate production) expressed by IPTG-inducible <i>tac</i> promoter in JHM80. Deletion of <i>glgA</i> , <i>glgC</i> for glycogen synthesis disruption.	6.17 g/L	20% Methane
Succinate	Genetic	KNO_3 optimization (6 g/L). Deactivation of <i>sdh</i> gene. Integration of <i>E.coli</i> glyoxylate pathway (<i>il</i> and <i>ms</i> gene) under <i>mxnF</i> promoter.	195 mg/L	30% Methane
	Genetic + ALE	High-level succinate production strain (CS40) established by ALE.	702 mg/L	30% Methane

Squalene	Genetic + Nitrate supplement	Overexpression of <i>pc</i> and <i>ppc</i> gene by cold-shock protein promoter. Disruption of <i>ssadh</i> gene (succinate-semialdehyde dehydrogenase). Overexpression of <i>glcA</i> (citrate synthase) by <i>mxoF</i> promoter. Deletion of <i>pds</i> , <i>crtN2</i> , <i>ald</i> gene (C30 carotenoid synthesis). Repression of <i>shc</i> (squalene-hopene cyclase) expression by ferulenol (1.5 µg/mL) Overexpression of another copy of <i>sqs</i> (squalene/phytoene synthase) under <i>mxoF</i> promoter. KNO ₃ optimization (2 g/L).	31.3 mg/L	30% Methane
5-Aminolevulinic acid	Genetic	Heterologous expression of <i>alas</i> (for aminolevulinic acid production) genes from <i>Methylosinus trichosporium</i> OB3b and <i>Rhodobacter</i> sp.	3.78 mg/L (<i>M. trichosporium alas</i>) 3.94 mg/L (<i>Rhodobacter</i> sp. <i>alas</i>)	30% Methane
2,3-Butanediol	Genetic	Replacement of endogenous <i>glgA</i> gene (glycogen synthase) with <i>budC</i> (acetoin reductase) from <i>Klebsiella pneumoniae</i> .	21.4 mg/L	20% Methane 10% Ethane



