

Review

# Tools and Strategies for Engineering *Bacillus methanolicus*: A Versatile Thermophilic Platform for Sustainable Bioproduction from Methanol and Alternative Feedstocks

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**ABSTRACT:** *Bacillus methanolicus* MGA3 is a methylotrophic bacterium with a high potential as a production host in the bioeconomy, particularly with methanol as a feedstock. This review presents the recent acceleration in strain engineering technologies through advances in transformation efficiency, the development of CRISPR/Cas9-based genome editing, and the application of genome-scale models (GSMs) for strain design. The generation of novel genetic tools broadens the biotechnological potential of this thermophilic methylotroph. *B. methanolicus* is a facultative methylotroph and apart from methanol it can grow on mannitol, arabitol and glucose, and was engineered for starch and xylose utilisation. Here, the central carbon metabolism of *B. methanolicus* for various native and non-native carbon sources is described, with an emphasis on methanol metabolism. With its expanding product portfolio, *B. methanolicus* demonstrates its potential as a microbial cell factory for the production of tricarboxylic acid (TCA) cycle and ribulose monophosphate (RuMP) cycle intermediates and their derivatives. Beyond small chemicals, *B. methanolicus* is both a valuable source of novel thermostable proteins and a host for the production of heterologous proteins, enabled by advances in genetic tools and cultivation methods. Continued progress in understanding its physiology and refining its genetic toolbox will be decisive in transforming *B. methanolicus* from a promising candidate into a fully established industrial workhorse for sustainable methanol-based biomanufacturing.

**Keywords:** Methanol; Mannitol; Seaweed hydrolysate; Riboflavin; Amino acids; Genome-scale metabolic model; CRISPR/Cas9



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## 1. Introduction

The sustainable production of high-value biochemicals from methanol, a cost-effective and reduced C1 feedstock, has garnered increasing interest in the field of metabolic engineering. Among the currently explored microbial platforms, *Bacillus methanolicus* MGA3 (hereafter referred to as *B. methanolicus*) stands out as a thermophilic methylotroph that rapidly grows on methanol at 50–55 °C. At least thirteen *B. methanolicus* strains such as MGA3, NOA2 and PB1 have been isolated and tested for their methylotrophic growth so far [1–3]. However, the MGA3 strain attracted particular scientific interest thanks to its ability to overproduce L-glutamate to 59 g·L<sup>-1</sup> during methanol fed-batch fermentations [4]. Recent advances in strain engineering have opened the possibility of utilizing alternative sustainable feedstocks, including xylose and starch. While historically recognized for its ability to overproduce amino acids, notably L-glutamate and L-lysine, recent efforts have expanded its metabolic portfolio beyond TCA cycle derivatives. In this review, we discuss recent achievements and future challenges of a *B. methanolicus*-based bioeconomy.

## 2. Developments in Tools and Technologies for Strain Engineering

Here, we outline strategies used in *B. methanolicus* to uncover metabolic bottlenecks and improve production strains, focusing on chemical mutagenesis, adaptive laboratory evolution (ALE), plasmid-based gene dosage, and genome editing [2,5–7]. Early and recent random mutagenesis studies enhanced L-lysine and L-glutamate production, and genome sequencing of mutagenized strains in combination with reverse genetics identified causal mutations in biosynthetic enzymes [2,8]. ALE has been applied to select for robust phenotypes with improved tolerance to heterologous products, revealing adaptive changes that expand production capacity [6]. Plasmid-based systems and promoter characterization enable controlled overexpression of endogenous and heterologous genes, while recent developments in CRISPR technology and suicide vectors allow manipulation of essential genes and pathways [9].

### 2.1. Chemical Mutagenesis and ALE as Tools to Identify Bottlenecks of Biosynthetic Pathways

In initial engineering attempts in the early 1990s, random mutagenesis approaches were used to enhance the production of native metabolites in *B. methanolicus*. Cells were exposed to chemical mutagens, such as ethyl methanesulfonate or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and L-lysine titers of  $11 \cdot \text{g} \cdot \text{L}^{-1}$  were obtained in methanol fed-batch fermentations from mutants resistant to the L-lysine analogue *S*-(2-aminoethyl)-L-cysteine [2]. The resulting mutant, *B. methanolicus* M168-20, displayed deregulated L-lysine feedback inhibition, and whole-genome analysis revealed mutations in the homoserine dehydrogenase *hom-1* gene, which encodes a branch-point enzyme between the L-lysine and L-methionine biosynthetic pathways [8]. In recent efforts, classical random mutagenesis has been employed in combination with high-throughput screening to identify *B. methanolicus* clones characterized by enhanced L-glutamate production. Various mutagenic agents, including alkylating chemicals and ultraviolet irradiation, were applied. The resulting mutants exhibited ~50% higher L-glutamate titers compared to the wild type and subsequently served as chassis strains for pathway extension. Notably, the integration of recent random mutagenesis with a systems-level characterization of the mutant strain has provided a comprehensive understanding of L-glutamate biosynthesis in *B. methanolicus*, as described above [5].

ALE is a method in strain engineering that addresses unforeseen challenges such as energy imbalances or occurrence of toxic intermediates by selecting for robust and optimized phenotypes under defined cultivation conditions. For instance, *B. methanolicus* underwent ALE to enhance tolerance to the heterologous product 5-aminovalerate (5AVA), to which it is naturally susceptible. Gradual supplementation with increasing concentrations of 5AVA led to selection for mutants with alterations in iron homeostasis genes, including one encoding an iron siderophore-binding protein. Overexpression of this mutant allele in the wild-type *B. methanolicus* markedly improved 5AVA tolerance under high  $\text{Fe}^{2+}$  conditions [6]. The ALE strategy to increase 5AVA tolerance directly expanded the production capacity for methanol-based added-value chemicals in *B. methanolicus*. ALE was also used to increase the growth rate of the *B. methanolicus* strain with pBM19 plasmid integrated into the chromosome [9].

### 2.2. Gene Dosage Effect of Plasmid-Based Gene (Over)Expression

Plasmids are commonly used for heterologous gene expression or for the overexpression of endogenous genes. Early strain engineering efforts in *B. methanolicus* also relied on gene expression using the plasmid pHP13, developed as an expression vector for *Bacillus subtilis* [10,11]. Later, the available vector repertoire was expanded by incorporating additional replicons, including the *Staphylococcus aureus* plasmids pNW33N and pUB110, as well as the *B. subtilis* plasmid pHCMC04. Plasmids pUB110, pHP13, and pNW33N are rolling-circle replicating vectors belonging to the same incompatibility group and display approximate copy numbers of 30–50, 5–6, and ~15 per genome, respectively, in *B. methanolicus*. By contrast, pHCMC04 is a theta-replicating plasmid with an estimated copy number of ~3 per genome; importantly, its replication mechanism is compatible with that of rolling-circle replicating plasmids, enabling stable co-expression from two different vectors within the same host [7]. The diversification of plasmid systems for *B. methanolicus* represented a milestone that henceforth provided a flexible and reliable genetic toolbox, laying the foundation for metabolic engineering and multi-gene pathway optimization in this organism [12,13].

Genome-wide transcriptomic analyses in *B. methanolicus* have enabled the characterization of native promoter architecture and transcriptional regulation. Promoter elements were identified, including a conserved -10 motif sequence (TATAaTaa) and a less conserved -35 consensus sequence (ttgaaa). RNA-seq further mapped genome-wide native promoter transcript abundances and transcription start sites, revealing that promoters of central metabolic genes, e.g., *hps* and *phi*, drive the highest transcript abundances, making them attractive targets for gene expression [7]. Further *in vivo* validation confirmed strong promoters upstream of the genes for glutamate synthase large and small subunits (*gltA*,

*glbB*) and dihydroxy-acid dehydratase (*ilvD*) [14]. Recently, a library of synthetic promoters was constructed and characterized, leading to creation of a promoter exhibiting over double activity in comparison to commonly used *mdh* promoter [15]. Nevertheless, the native methanol dehydrogenase promoter (*mdh*) remains the standard for constitutive gene expression in *B. methanolicus*. Regulated gene expression has also been established, for example xylose-inducible promoters derived from *Bacillus megaterium* or *Parageobacillus thermoglucosidasius* allow controlled expression from plasmids in *B. methanolicus* [7,12,15]. Additionally, inducible expression can be achieved via a *B. subtilis*-derived *pbuE* riboswitch, which responds to 2-aminopurine in the culture medium [14]. Both the regulator- and riboswitch-based systems are gratuitous and titratable, providing precise control over gene expression for sensitive metabolic engineering applications. Moreover, a previously mentioned native mannitol-inducible promoter controlled by a transcriptional activator MtlR can also be used as a genetic tool, with a major limitation of mannitol being a carbon and energy source for *B. methanolicus* [16].

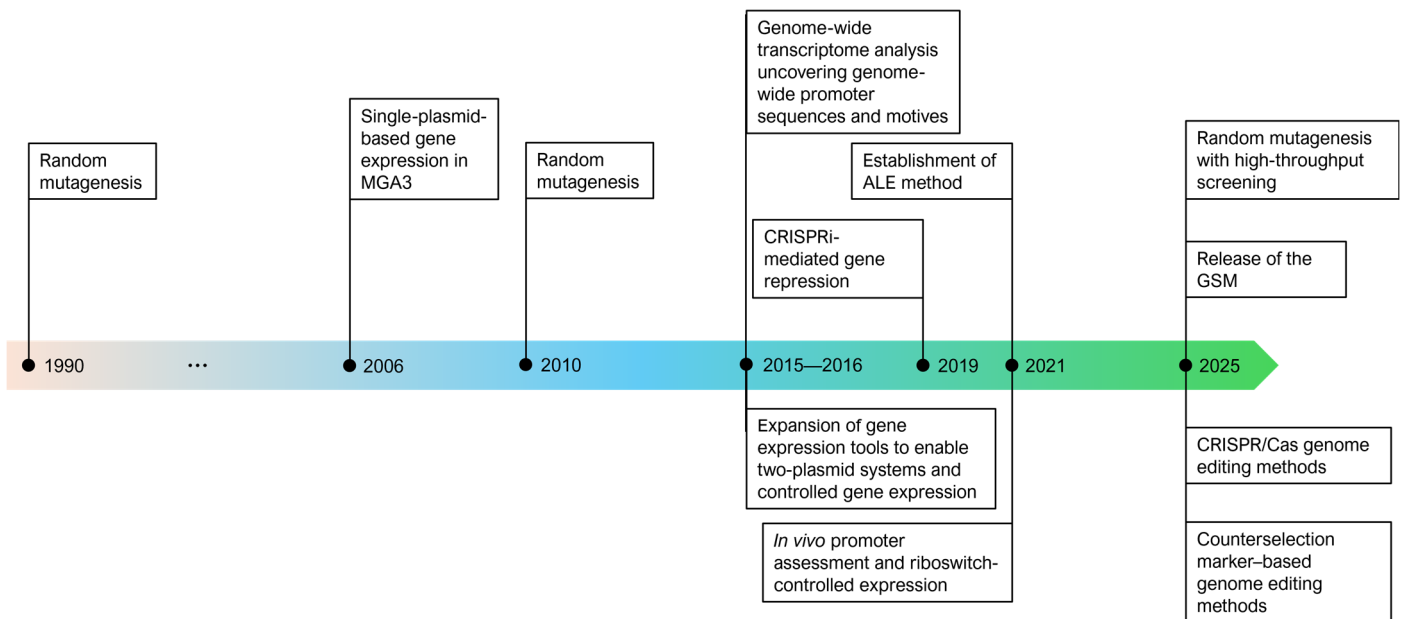
### 2.3. Gene Silencing and Deletion Technologies Opened Unexplored Opportunities for Strain Design and Characterization

In addition to gene overexpression, controlled repression is essential for fine-tuning metabolic pathways, reducing byproduct formation, and optimizing overall cellular performance. In this context, the *Streptococcus pyogenes* CRISPR-Cas9 system has emerged as a widely used tool. Gene repression is achieved via CRISPR interference (CRISPRi), in which a deactivated Cas9 protein (dCas9) binds to specific DNA sequences without generating double-stranded breaks, thereby blocking transcription initiation or elongation. This strategy is particularly advantageous for essential genes or pathways in which a genomic knockout would be deleterious, offering a level of precision and flexibility beyond traditional gene deletion methods [16]. The pNW33N plasmid was used as a basis to develop a CRISPRi system for *B. methanolicus*. The system has been successfully implemented to repress and functionally characterize genes involved in sporulation, H<sub>2</sub>O<sub>2</sub> detoxification, mannitol catabolism, L-glutamate biosynthesis and the RuMP cycle in *B. methanolicus* [17,18]. Beyond enabling the functional characterization of the *B. methanolicus* physiology, CRISPRi can provide a versatile platform for rational strain development. For instance, repression of the master regulator *spo0A* nearly completely suppressed the sporulation phenotype, demonstrating the high efficiency of this tool [18]. This is particularly valuable as suppression of sporulation is crucial for improving performance in large-scale industrial processes, where this trait is typically undesirable. However, gene repression using CRISPRi can be genetically unstable, as it depends on *dcas9* expression levels, which may fluctuate across growth phases or under varying environmental conditions. In contrast, biotechnological processes require genetically stable production hosts, making permanent genome editing a more reliable approach.

A CRISPR-Cas9 genome editing tool was developed for *B. methanolicus* based on the pCasPP plasmid originally designed for *Paenibacillus polymyxa*. The system uses a pUB110-based plasmid carrying the *S. pyogenes* Cas9 gene under the broad-host-range *sgsE* promoter from *Geobacillus stearothermophilus* and an sgRNA cassette driven by the constitutive *gapdh* promoter from *Streptomyces griseus*. A SpeI site enables the insertion of ~1 kb homologous arms for homology-directed repair [19]. This single-plasmid system induces Cas9-mediated double-strand breaks and leverages the native DNA repair pathways in *B. methanolicus*. Using this platform, the *spo0A* gene was deleted and replaced with a *mrfp* reporter gene. Moreover, deletions of the catalase-encoding *kata* and alanine dehydrogenase-encoding *ald* genes were verified by genome sequencing and corresponded to complete loss of the respective enzyme activities [20]. Furthermore, the ThermoCas9 system from *Geobacillus thermodenitrificans* was leveraged for better adaptation to the thermophilic nature of *B. methanolicus* [9]. Unlike the mesophilic *S. pyogenes* Cas9, ThermoCas9 exhibits broad thermal activity (20–70 °C), making it well-suited for the thermophilic physiology of *B. methanolicus* and enabling a robust CRISPR-based genome editing platform [9,21]. Additionally, the CRISPR/ThermoCas9 system was used to deregulate the arginine biosynthesis pathway through *argR* deletion, deletion of chromosomal RuMP cycle genes and integration of the pBM19 plasmid into the genome, and the physiological effects of those modifications are described in more detail in the following sections [9].

Genome modification in *B. methanolicus* could also be achieved using non-replicating plasmids carrying 2–6 kb homology regions to promote recombination through crossover events [15]. As proof of concept, the non-essential *mutS* gene was targeted using the temperature-sensitive plasmid pBMe01, which does not replicate at 60 °C, and *sfGFP* as a counter-selection marker [22]. In a different approach, a pUB110-derived non-replicating suicide vector was created by removing the repU gene, which encodes the plasmid replication initiation protein for *Bacillus* species [23]. The suicide vector carried much shorter, only ~1 kb homology regions along with selection and counterselection markers for scar-free

gene deletions. With this strategy, antibiotic resistance was used for selection, while the transport of the antimetabolite 5-fluoroorotate was used for counterselection. The chromosomal *upp* gene, essential for uracil metabolism, was deleted using this system, producing a strain with reduced sensitivity to 5-fluorouracil, demonstrating the practical utility of the counterselection approach for robust genome engineering in *B. methanolicus*. Although creation of reliable genome editing tools had been sought for over two decades in *B. methanolicus*, the past year has witnessed remarkable progress in this field and release of novel methods for precise and efficient genome engineering in this organism (Figure 1). The historically limited availability of genome modification methods is largely due to low transformation efficiency, which has been a major bottleneck. Remarkably, all successful studies have focused on enhancing transformation efficiency, enabling the successful implementation of advanced genome editing tools in this strain.



**Figure 1.** A timeline of genetic technology advancements in *B. methanolicus*.

#### 2.4. The Next Frontier: Genome-Scale Metabolic Model-Guided Rational Strain Design

Finally, as rational strain design in bacteria increasingly relies on genome-scale metabolic models (GSMs) to guide metabolic engineering strategies, this prompted the development of two GSMs for *B. methanolicus*, *iBmeth613* and *iBM822* [5,9]. The GSM of *B. methanolicus* comprises 613 genes, 1020 reactions, 899 metabolites and two compartments, being cytosol and extracellular space [5]. Systems-level analyses combined with this model provide detailed insights into methanol-based production pathways, such as L-glutamate and GABA [5]. Moreover, it has been applied in flux balance analysis-guided strategies to achieve putrescine overproduction in *B. methanolicus* [24]. The *iBM822* model comprises 822 protein-coding genes, 1730 compartmentalized reactions and 1479 metabolites, and it was used to increase L-arginine biosynthesis [9]. The recent models offer promising prospects for *B. methanolicus* strain engineering research.

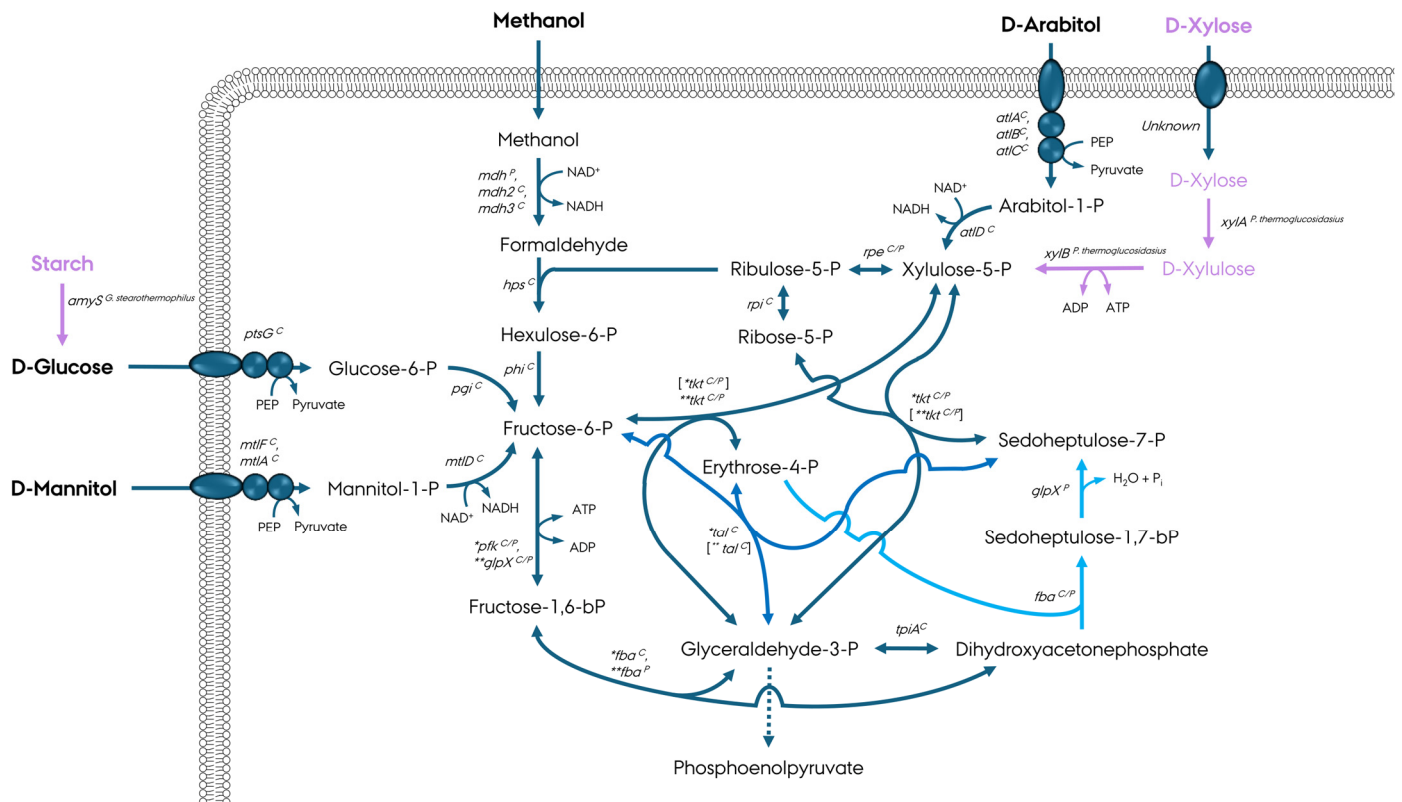
### 3. Native and Non-Native Carbon Sources of *B. methanolicus*

We describe and discuss the native catabolic pathways in *B. methanolicus*, with a particular focus on methanol metabolism. Recently, its substrate range, originally limited to methanol, mannitol, glucose, and arabinol, was expanded to include xylose and starch through the introduction of heterologous pathways. While methanol utilization is what makes *B. methanolicus* a unique microorganism for biotechnological applications, expanding its feedstock portfolio may increase the strain's flexibility in bioprocesses and potentially broaden its applications for co-utilization of methanol with various waste streams or other alternative feedstocks such as seaweed extracts.

#### 3.1. Methanol Is Oxidized to Formaldehyde, Which Is Assimilated via Two Parallel Versions of the Ribulose Monophosphate Cycle

*B. methanolicus* is a facultative methylotroph, which can grow rapidly in media containing  $0.25 \text{ g}\cdot\text{L}^{-1}$  yeast extract supplemented with 0.025 to 2 M methanol at growth rates of  $0.65 \pm 0.01 \text{ h}^{-1}$  to  $0.23 \pm 0.00 \text{ h}^{-1}$ , respectively [25]. In

methanol minimal medium, *B. methanolicus* grows at a rate of up to  $0.46 \pm 0.002 \text{ h}^{-1}$ . *B. methanolicus* assimilates methanol after its initial oxidation to formaldehyde followed by assimilation via the ribulose monophosphate (RuMP) cycle (Figure 2). Its genome consists of one chromosome (3.3 Mbp) and two plasmids, pBM19 (19 kbp) and pBM69 (69 kbp). Six of the eleven genes involved in methanol consumption have plasmid- and chromosome-borne copies. The pBM19 plasmid is essential for methylotrophic growth as its loss results in the inability to assimilate methanol [3]. Recently, pBM19 was integrated into the chromosome of *B. methanolicus* to prevent plasmid curing and preserve methylotrophic ability [9]. However, decreases in gene copy number and the choice of integration locus led to reduced growth rates, which were circumvented by strain development through ALE.



**Figure 2.** A schematic of uptake and assimilation of native (black font) and non-native (lilac) carbon sources in *B. methanolicus*. Genes: *mdh*, methanol dehydrogenase (EC 1.1.1.244); *hps*, 3-hexulose-6-phosphate synthase (EC 4.1.2.43); *phi*, 6-phospho-3-hexuloisomerase (EC 5.3.1.27); *pfk*, 6-phosphofruktokinase (EC 2.7.1.11); *fba*, fructose-1,6-bisphosphate aldolase (EC 4.1.2.13); *glpX*, fructose-1,6-bisphosphatase (EC 3.1.3.1); *tpiA*, triosephosphate isomerase (EC 5.3.1.1); *tkt*, transketolase (EC 2.2.1.1); *tal*, transaldolase (EC 2.2.1.2); *rpi*, ribose-5-phosphate isomerase (EC 5.3.1.6); *rpe*, ribulose 5-phosphate 3-epimerase (EC 5.1.3.1); *mtlF*, mannitol-specific phosphotransferase enzyme IIA component (EC 2.7.1.69); *mtlA*, PTS system mannitol-specific enzyme IIBC component (EC 2.7.1.69); *mtlD*, mannitol-1-phosphate 5-dehydrogenase (EC 1.1.1.17); *atlA*, IIA arabinol PTS component; *atlB*, IIB arabinol PTS component; *atlC*, IIC arabinol PTS component; *atlD*, arabinol phosphate dehydrogenase; *amyS*,  $\alpha$ -amylase (EC 3.2.1.1); *ptsG*, PTS-glucose-specific transporter subunit IICBA (EC 2.7.1.69); *pgi*, glucose-6-phosphate isomerase (EC 5.3.1.9); *xylA*, xylose isomerase (EC 5.3.1.5); *xylB*, xylulose kinase (EC 2.7.1.17). One asterisk “\*”, major glycolytic FBA; Two asterisks “\*\*”, major gluconeogenetic FBA. Superscript “C”, chromosomally encoded; superscript “P”, natural plasmid pBM19 encoded; superscript “*P. thermoglucosidasius*”, gene heterologously expressed and native to *Parageobacillus thermoglucosidasius*; superscript “*G. stearothermophilus*”, gene heterologously expressed and native to *Geobacillus stearothermophilus*. Bracket encapsulation “[...]”, putative direction. Lilac arrows, heterologous reactions; dark blue arrows, common reactions; blue arrows, transaldolase rearrangement variant reactions; light blue arrows, sedoheptulose-1,7-bisphosphatase rearrangement variant reactions. Dashed arrows indicate multiple enzymatic reactions.

To date, methanol transport over the cytoplasmic membrane has not been investigated in *B. methanolicus*, but it can be assumed that methanol uptake is not rate-limiting as has been shown for ethanol uptake by *Zymomonas mobilis* [26]. In the Gram-positive non-methylotroph *Corynebacterium acetophilum*, methanol diffuses passively into the cell without any additional induction [27]. The ability of non-methylotrophic bacteria to take up methanol suggests that methanol may either passively diffuse across the cell membrane or that a dedicated uptake system has been conserved for energy production by methanol oxidation. A similar mechanism might function in *B. methanolicus*, although the

presence of methanol transporters cannot be excluded since no putative genes with this function were identified in the genome of *B. methanolicus* to date.

In Gram-negative methylotrophs, the methanol-oxidizing methanol dehydrogenase (Mdh) is located in the periplasmic space and requires pyrroloquinoline quinone (PQQ) as its redox co-factor [28]. Deletion of the PQQ-dependent Mdh-encoding gene in *Pseudomonas* sp. AM1 almost entirely halts the methanol metabolism [29]. In contrast, the Fe-NAD<sup>+</sup>-dependent Mdh of Gram-positive methylotrophic *Bacillus* species is located in the cytoplasm [30]. This means that methanol is oxidized by Mdh inside the cell rather than adjacent to the cell membrane, and that Mdh is most likely not associated with the transport of methanol [30].

Methanol enters the cytoplasm of *B. methanolicus* along its concentration gradient, which is sustained by the oxidation of methanol to formaldehyde by the Fe-NAD<sup>+</sup>-dependent Mdh [31]. Methanol dehydrogenases are key enzymes for methylotrophy, and their molecular properties and thermodynamics have been extensively reviewed elsewhere [32,33]. Methanol dehydrogenases have various cofactors, each with specific advantages and drawbacks [32]. The major merit of NAD<sup>+</sup> as a cofactor for methanol oxidation is that it serves as a carrier of high-energy electrons. This prevents the release of excess energy in form of heat or its storage as chemical bonds in toxic H<sub>2</sub>O<sub>2</sub>. On the other hand, the Gibbs free energy change ( $\Delta G'$ ) for methanol oxidation at ambient temperature with NAD<sup>+</sup> as a cofactor is positive, making the reaction thermodynamically favourable only at the elevated temperatures that match the optimal growth range of *B. methanolicus*. This organism possesses three genes encoding Mdh, one in the pBM19 plasmid (*mdh*) and two in the chromosome (*mdh2* and *mdh3*) [31]. These enzymes have a wide substrate specificity and comparable activity in the range of 0.01–0.06 U·mg<sup>-1</sup> with methanol, although the activator protein ACT only increases Mdh activity *in vitro* by 3.33–10 fold [31].

Deletions of chromosomal Mdh-encoding genes, both individually and in combination, slightly reduced growth in methanol minimal medium [9]. This suggests that the plasmid-encoded Mdh is the dominant enzyme for methanol growth, likely due to the higher copy number of the plasmid-borne gene. At the same time, it was confirmed that ACT plays a non-essential role *in vivo*, as the deletion of the *act* gene did not impact cell growth in methanol minimal medium. Similarly, the heterologous expression of *act* in *Escherichia coli* showed no effect on synthetically methylotrophic growth [9,34].

After methanol oxidation by Mdh, the resulting formaldehyde can be assimilated into biomass via the RuMP cycle. Alternatively, it is oxidized to CO<sub>2</sub> via one of the three formaldehyde dissimilation pathways, namely, the bacillithiol- and tetrahydrofolate-dependent linear pathways as well as the dissimilatory variant of RuMP cycle (reviewed in Klein et al., 2022) [35]. Those dissimilatory pathways serve as a source of energy and prevent the accumulation of lethal levels of formaldehyde.

The RuMP cycle is divided into three phases: fixation, cleavage and rearrangement [3,36]. In the first step of the fixation phase, formaldehyde is condensed with ribulose-5-phosphate (Ru5P) to hexulose-6-phosphate (H6P) by 3-hexulose-6-phosphate synthase (Hps). The next and last step of the fixation phase is isomerization of hexulose-6-phosphate to fructose-6-phosphate (F6P) by 6-phospho-3-hexuloisomerase (Phi) (Figure 2) [3]. The *B. methanolicus* *hps* and *phi* genes are co-transcribed as an operon essential for methanol assimilation, with their expression upregulated during methanol-based growth [4,9,10,37]. The essentiality of *hps-phi* operon was confirmed by deletion of the respective genes. Noteworthy, *hps* and *phi* deletions, along with all other chromosomal deletions of RuMP cycle genes mentioned in this review, were not complemented through plasmid-based expression to exclude effects of possible off-targets. *In vitro* studies showed that balancing the stoichiometric ratio of Mdh to Hps-Phi or creating an Mdh-Hps-Phi fusion protein shifts the pathway equilibrium toward F6P formation, showcasing that the supply of Ru5P or the combined Hps-Phi activity might be limiting for methylotrophic growth [38,39]. In fact, the *hps-phi* overexpression and increased Ru5P supply both increased the growth rate of *B. methanolicus* on methanol [10,15].

In nature, there are two variants of the cleavage phase of the RuMP cycle, wherein F6P is either phosphorylated to fructose-1,6-bisphosphate (F1,6BP) by phosphofructokinase (*pfk*) or converted to 2-keto-3-deoxy-6-phosphogluconate (KDPG) in a multi-reaction process catalyzed by Entner–Doudoroff enzymes. FBP and KDPG are then cleaved by aldolases to glyceraldehyde-3-phosphate (GA3P) and either dihydroxyacetone phosphate (DHAP) or pyruvate (Figure 2). Of those two variants, *B. methanolicus* utilizes the fructose-1,6-bisphosphate aldolase (FBA) variant. For this ability, *B. methanolicus* possesses a chromosomal *pfk*<sup>C</sup> and a pBM19-based *pfk*<sup>P</sup>. Deletion of the chromosomal *pfk*<sup>C</sup> nearly halts methylotrophic growth of *B. methanolicus* [9]. The pBM19-borne *pfk*<sup>P</sup> has not yet been deleted, but it seems to be unable to compensate for the loss of *pfk*<sup>C</sup>. *B. methanolicus* contains chromosomally (FBA<sup>C</sup>) and pBM19-(FBA<sup>P</sup>) encoded FBA [40]. Both versions of FBA can be activated by either manganese or cobalt ions, however, based on their catalytic properties, FBA<sup>C</sup> functions as the major glycolytic FBA as it exhibits 20-fold higher catalytic efficiency toward FBP

cleavage than FBA<sup>P</sup> [17,40]. The catalytic efficiency of FBA<sup>P</sup> for the aldol condensation reaction with DHAP and GA3P as substrates is about 3-fold higher than that of FBA<sup>C</sup>, thus, it can be considered the major gluconeogenic FBA [40].

Rearrangement is the last phase of the RuMP cycle, and it serves to regenerate Ru5P for the fixation step [15]. *B. methanolicus* is the only known bacterium which possesses genes encoding both known variants of the RuMP cycle rearrangement phase [41]. In both variants, the first step is the transfer of a glycolaldehyde group from F6P to GA3P by transketolase (TKT), forming erythrose-4-phosphate (E4P) and xylulose-5-phosphate (Xu5P) (Figure 2) [42]. Thus, TKT plays a major role in the RuMP cycle with *B. methanolicus* harbouring two TKT encoding genes, one in the chromosome (*tkt<sup>C</sup>*) and the other in pBM19 (*tkt<sup>P</sup>*) [42]. While both TKTs are dependent on bivalent cations such as Mn<sup>2+</sup>, Mg<sup>2+</sup>, and Ca<sup>2+</sup>, and exhibit similar kinetic parameters for F6P, deletion of *tkt<sup>C</sup>* does not affect methanol metabolism [9,42]. TKT<sup>P</sup> might therefore be the major TKT for methylotrophy; however, it cannot be ruled out that both enzyme versions contribute to this function. The sedoheptulose-1,7-bisphosphatase (SBPase) variant of the regeneration phase in RuMP cycle was confirmed to be active through <sup>13</sup>C-labelling experiments [43]. Its first step is the condensation of E4P with DHAP to sedoheptulose-1,7-bisphosphate (S1,7BP) catalyzed by an aldolase (Figure 2). Both FBA enzymes have been experimentally confirmed *in vitro* to be bifunctional, also acting as sedoheptulose-1,7-bisphosphate aldolase (SBA). As deletion of *fbac* does not affect methanol metabolism in *B. methanolicus* and only pBM19-encoded FBA is upregulated in methylotrophic conditions, it can be concluded that FBA<sup>P</sup> is sufficient for methylotrophy *in vivo*. Interestingly, CRISPRi-mediated repression of *fbac* and *fbap* leads to compensatory upregulation of their paralogs, suggesting their interchangeable functionality in formaldehyde assimilation. In the next step, S1,7BP undergoes dephosphorylation to sedoheptulose-7-phosphate (S7P) catalyzed by sedoheptulose-1,7-bisphosphatase (SBPase) (Figure 2) [44]. While *B. methanolicus* possesses two copies of the SBPase gene *glpX*, chromosome- and pBM19-borne, only *glpX<sup>P</sup>* is upregulated during methylotrophic growth and encodes an enzyme which catalyses dephosphorylation of S1,7BP. On the other hand, both the pBM19- and chromosome-borne *glpX* genes encode enzymes active as fructose-1,6-bisphosphatases (FBPases) [44]. Both FBPases are activated by Mn<sup>2+</sup>, but as GlpX<sup>C</sup> has higher catalytic efficiency and a lower K<sub>M</sub> for F1,6BP than GlpX<sup>P</sup> [44], it is considered the major FBPase in *B. methanolicus* [44]. Moreover, methanol-based growth of *B. methanolicus*  $\Delta$ *glpX<sup>C</sup>* was unaffected, indicating that GlpX<sup>C</sup> is not required for methylotrophic growth [9].

In the transaldolase (TA) rearrangement phase variant, TA catalyses the transfer of a dihydroxyacetone group from F6P to E4P, producing S7P and GA3P (Figure 2) [41]. Unlike most of the enzymes of the RuMP cycle, *B. methanolicus* possesses only one TA encoding gene located on the chromosome (*tal<sup>C</sup>*). While the TA variant of rearrangement phase is consistently shown to be active in metabolic flux analyses, and the TA activity is present in *B. methanolicus* crude extract, *tal<sup>C</sup>* in *B. methanolicus* encodes a truncated protein without TA activity *in vitro* leaving an unanswered question regarding genetic background of TA activity [5,41]. Thus, in *B. methanolicus*, both the SBPase and TA variant likely function in parallel or compensate one another in case of inactivation, which is a unique feature of this bacterium [5,41].

S7P generated in both variants of the RuMP cycle rearrangement phase serves as a substrate for the reaction catalyzed by TKT. To that extent, TKT is assumed to catalyse the transfer of a glycolaldehyde unit from S7P to GA3P, but this has not been experimentally confirmed [42]. Lastly, Ru5P can be produced from ribose-5-phosphate or Xu5P by ribose-5-phosphate isomerase (*rpi*) and ribulose 5-phosphate 3-epimerase, respectively (*rpe*) (Figure 2) [45]. RPI is encoded solely by a chromosomal gene, while RPE is encoded both chromosomally (*rpe<sup>C</sup>*) and on plasmid pBM19 (*rpe<sup>P</sup>*) [4,45]. Deletion of *rpe<sup>C</sup>* deemed this gene to be essential for methylotrophic growth, even though only *rpe<sup>P</sup>* is upregulated in such conditions [9,37]. It remains to be shown whether RPE<sup>P</sup> is required for methylotrophic growth or instead contributes by enhancing growth with methanol above the level supported by RPE<sup>C</sup>.

### 3.2. Rapid Growth of *B. methanolicus* on Mannitol Emphasizes Its Potential as a Cell Factory Using Seaweed Extracts as Feedstock

Mannitol is a 6-carbon sugar alcohol which is as a source of carbon and energy for *B. methanolicus* with a maximal growth rate of  $0.37 \pm 0.01 \text{ h}^{-1}$  [46]. Mannitol is taken up from the environment into the *B. methanolicus* cell through the mannitol-specific phosphotransferase system (PTS) with subunits IIA and IIBC encoded by *mtlF* and *mtlA*, respectively [46]. The mannitol uptake occurs with concomitant phosphorylation to mannitol-1-phosphate with phosphoenolpyruvate (PEP) serving as a phosphate group donor, catalyzed by phosphoenolpyruvate-protein phosphotransferase and phosphocarrier protein [46]. Mannitol-1-phosphate is oxidized to F6P by NAD<sup>+</sup>-dependent mannitol-1-phosphate 5-dehydrogenase encoded by *mtlD* [46] (Figure 2). The organization in the genome suggests monocistronic transcription of *mtlA* and co-transcription of *mtlRFD* or *mtlFD* as an operon and its suboperon [47]. The

*mtlR* gene encodes a mannitol-dependent transcriptional activator MtlR, which represses transcription of *mtlRFD* from its upstream promoter  $P_{mtlR}$ . Only mannitol, but no other sugar alcohol such as arabitol, serves as an inducer [7]. Similarly to growth on methanol, deletion of *pfk<sup>C</sup>* completely halts mannitol-based growth [9]. In addition, deletion of *fba<sup>C</sup>* and *phi* moderately reduced the cell growth in minimal medium with mannitol. While FBA<sup>C</sup> plays a major role in glycolytic reactions, it is unclear why deletion of *phi* affects growth on mannitol, however, it must be stated that the *phi* deletion mutant was not genetically complemented, thus, unwanted secondary off target effects may confuse conclusions about PHI function. Recent work has demonstrated that *B. methanolicus* can grow on the mannitol-rich extracts of brown seaweed (*Saccharina latissima*) [48]. *B. methanolicus* strain heterologously expressing dehydrosqualene synthase and dehydrosqualene desaturase produced C30 terpenoids during mannitol- and seaweed-based growth [48]. Furthermore, during shake flask cultivations on 50% v/v seaweed extract and 10 g·L<sup>-1</sup> mannitol, recombinant *B. methanolicus* strains were able to secrete  $287 \pm 13$  mg·L<sup>-1</sup> cadaverine, a titer slightly higher compared to when cultivated on methanol [48]. Moreover, when cultivated under mannitol fed-batch fermentation this strain secreted 6.3 g·L<sup>-1</sup> of cadaverine, underscoring the potential of *B. methanolicus* as a host for future seaweed-based fermentations [48].

### 3.3. *B. methanolicus* Can Utilize the Sugar Alcohol Arabitol by PTS-Mediated Uptake and Phosphorylation

The 5-carbon compound arabitol is another sugar alcohol that *B. methanolicus* can use as a carbon and energy source, with a maximal growth rate of  $0.20 \pm 0.01$  h<sup>-1</sup> [46]. Arabitol is transported into the cell via an arabitol-specific PTS with three subunits encoded by *atla*, *atlb*, and *atlc* [46]. During its import via PTS, arabitol is phosphorylated by phosphoenolpyruvate–protein phosphotransferase and phosphocarrier protein to arabitol-1-phosphate with PEP serving as a phosphate group donor [46]. Arabitol-1-phosphate is then oxidized by the NAD<sup>+</sup>-dependent arabitol phosphate dehydrogenase, encoded by *atld*, to Xu5P as confirmed experimentally through metabolic flux analysis (Figure 2) [43,46]. In addition, *atld* from *B. methanolicus* was shown to restore growth of a *Corynebacterium glutamicum mtlD* mutant with arabitol as the sole carbon source. The *atlabcd* suboperon belongs to a larger operon composed of nine genes, which likely contains an additional copy of the arabitol phosphate dehydrogenase gene.

### 3.4. PTS-Mediated Glucose Uptake in *B. methanolicus* Allows for Growth with Glucose and, Upon Recombinant $\alpha$ -Amylase Production, Enables Starch Catabolism

*B. methanolicus* can also utilize glucose as a source of carbon and energy, though the maximum growth rate on glucose is lower than that on methanol and mannitol and reaches  $0.16 \pm 0.01$  h<sup>-1</sup> [49]. Similarly to mannitol and arabitol, glucose is transported into the cell via PTS system composed of phosphoenolpyruvate–protein phosphotransferase, phosphocarrier protein, and glucose-specific transporter subunit IICBA encoded by *ptsG*. Concomitant with its uptake, glucose is phosphorylated to glucose-6-phosphate, which then enters glycolysis (Figure 2) [46]. The native glucose metabolism was successfully extended to starch through recombinant  $\alpha$ -amylase production and secretion in *B. methanolicus*, supporting a growth rate of  $0.05 \pm 0.01$  h<sup>-1</sup>. The maximum OD<sub>600</sub> reached when grown on 9 g·L<sup>-1</sup> soluble starch is approximately  $1.1 \pm 0.1$  compared to  $7.7 \pm 0.4$  on 9 g·L<sup>-1</sup> glucose [4,49]. This indicates a potential for utilization of other sugar and sugar alcohol-based polysaccharides by *B. methanolicus* through heterologous production of various hydrolytic enzymes [49].

### 3.5. Xylose Is Established as a Non-Native Carbon Source for Ribulose-5-Phosphate Regeneration

Xylose is a 5-carbon sugar, which cannot natively be catabolized by *B. methanolicus* [15]. Therefore, xylose is commonly used as a gratuitous inducer for heterologous gene expression in *B. methanolicus* from a synthetic cloning vector pBV2xp, which contains a *B. megaterium*-derived xylose-inducible promoter [7,15,50]. This reveals that *B. methanolicus* can take xylose up from the environment, although the enzymatic repertoire responsible for this process are still unknown. Recently, xylose co-utilization with methanol was established in *B. methanolicus* through heterologous expression of two genes from *P. thermoglucosidasius*: *xylA* and *xylB* coding thermophilic xylose isomerase and xylulose kinase, respectively [15]. Xylose isomerase catalyzes the conversion of xylose to xylulose, while xylulose kinase phosphorylates xylulose to Xu5P using ATP as a phosphate group donor (Figure 2). *B. methanolicus* supplemented with 2.5 g·L<sup>-1</sup> xylose achieved growth rates of 0.46–0.67 h<sup>-1</sup> with methanol at concentrations between 5–55 g·L<sup>-1</sup> compared to 0.27–0.51 h<sup>-1</sup> without xylose supplementation [15]. As previously mentioned, this is likely due to a higher Ru5P supply indicating that its regeneration might be limiting for methanol-based growth [15].



## 4. Expanding Portfolio of Methanol-Based Value-Added Compounds

We have recently reviewed *B. methanolicus* as a cell factory for the production of amino acids and their derivatives from methanol (see Irla and Wendisch (2022)). Since then, our understanding of carbon fluxes during L-glutamate production and mechanism of its export has increased substantially. Additionally, the product portfolio was expanded beyond amino acids and their derivatives (Table 1).

### 4.1. Production of Metabolites Derived from TCA Cycle Based Amino Acids

*B. methanolicus* MGA3 can secrete  $59 \text{ g}\cdot\text{L}^{-1}$  of L-glutamate in methanol fed-batch fermentations [4]. To better understand the carbon fluxes underlying L-glutamate production, a systems-level study was recently conducted comparing the wild-type strain and the L-glutamate overproducing strain ABBM4307 generated through chemical mutagenesis [5]. The whole-genome sequencing, transcriptomics,  $^{13}\text{C}$ -metabolic flux analysis, and genome-scale metabolic modelling unravelled several metabolic changes in the mutant strain. These include increased carbon flux through (1) the regeneration phase of the RuMP cycle supporting recycling of Ru5P, the formaldehyde acceptor, (2) the pyruvate carboxylase, which replenishes oxaloacetate in the TCA cycle, and (3) upper reactions of the TCA cycle from oxaloacetate to  $\alpha$ -ketoglutarate, leading to improved precursor supply. The transcriptomic analysis indicated changes in expression levels of genes involved in various metabolic processes such as cell wall metabolism, biosynthesis of vitamins, cofactors, fatty acids, and nucleotides, electron transport chain and flagella structure and function. This study suggested that L-glutamate appears to be synthesized primarily via transamination reactions that use branched-chain amino acids rather than through the classical glutamate dehydrogenase and glutamate synthase in *B. methanolicus*. However, the fundamental question of the specific role of the glutamate synthase (encoded by *gltAB* and *gltA2*) or glutamate dehydrogenase (encoded by *yweB*) in L-glutamate biosynthesis remained unanswered. Nonetheless, knock-down and overexpression data points toward glutamate synthase playing a major role in this process as the targeted repression of the *gltAB* operon effectively decreased L-glutamate accumulation whereas *gltAB* or *gltA2* overexpression increased it (Figure 3) [51,52].

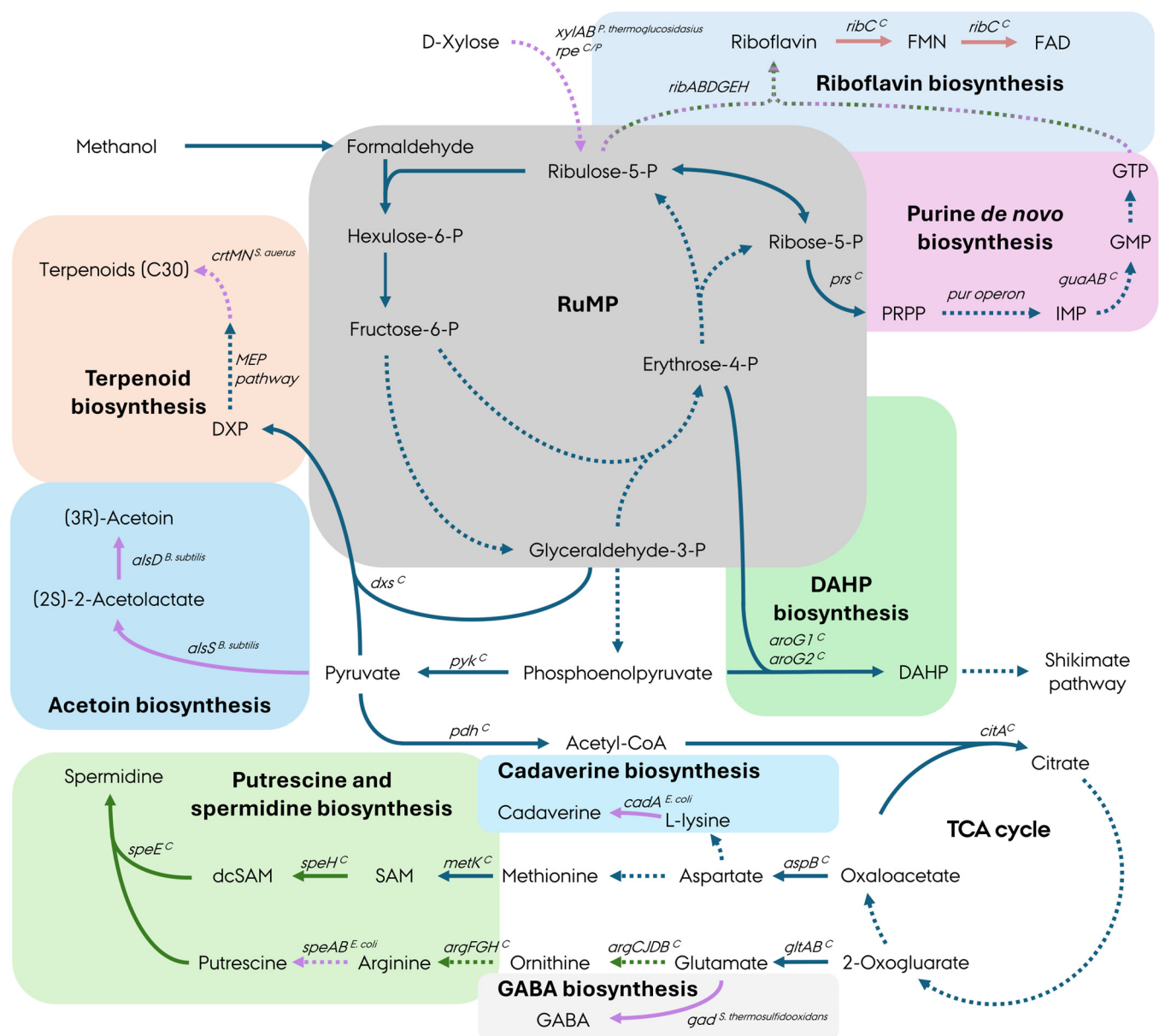
A small-conductance mechanosensitive channel (MscS) has been identified as playing a role in L-glutamate export in *B. methanolicus* [53]. Transcriptome analyses during L-glutamate overproduction revealed that the bacterium modulates lipid metabolism, suggesting changes in membrane fluidity that facilitate channel opening [53]. This hypothesis was supported by the use of surfactants in the fermentation medium, which enhanced L-glutamate secretion [54]. *In silico* analysis indicated that the channel is homologous to the *E. coli* MscS protein. Functional studies, including targeted gene repression and overexpression, demonstrated corresponding loss- and gain-of-function effects on L-glutamate export, confirming the role of the MscS channel in this process [1]. Identification of the L-glutamate export mechanism in *B. methanolicus* is a significant milestone for future engineering efforts, particularly for compounds derived from L-glutamate.

L-Glutamate serves as a precursor of various non-proteinogenic and proteinogenic amino acids, for example its decarboxylation leads to the formation of  $\gamma$ -aminobutyric acid (GABA) (Figure 3). The increased flux towards L-glutamate production in the mutant strain ABBM4307 strain was harnessed for GABA biosynthesis resulting in a titer of  $13.2 \text{ g}\cdot\text{L}^{-1}$  during fed-batch fermentation using this mutant as host (Table 1) [5]. L-Glutamate is also a precursor in an eight-step L-arginine biosynthesis pathway regulated at the transcriptional level by a repressor ArgR. Deletion of *argR* leads to accumulation of arginine at  $0.2 \text{ g}\cdot\text{L}^{-1}$  during methanol-based growth in shake flasks (Table 1) [9]. The overexpression of *argB*, encoding acetylglutamate kinase, known to be the rate-limiting step in arginine biosynthesis, increased L-arginine 4-fold to  $0.8 \text{ g}\cdot\text{L}^{-1}$ , while overexpression of other genes of the L-arginine biosynthetic pathway did not lead to changes in its biosynthesis.

The biosynthetic capabilities of *B. methanolicus* were recently expanded towards production of polyamines derived from L-glutamate, *i.e.*, putrescine and spermidine [24]. Putrescine biosynthesis at  $47.5 \pm 0.8 \mu\text{M}$  was achieved through heterologous expression of *E. coli*-derived *speAB*, encoding arginine decarboxylase and agmatinase, respectively (Figure 3). This titer was improved 3-fold to  $137.7 \pm 1.8 \mu\text{M}$  ( $0.012 \text{ g}\cdot\text{L}^{-1}$ ) upon overexpression of *argCJDB* encoding the endogenous ornithine biosynthesis pathway in the putrescine-producing strain (Table 1). The spermidine production was achieved with the co-expression of native *speE* and *speH* encoding spermidine synthase and *S*-adenosylmethionine (SAM) decarboxylase, respectively, to enable aminopropyl group transfer via decarboxylated SAM (dcSAM) (Figure 3). To enhance precursor supply for spermidine biosynthesis, *E. coli*-derived *speAB* was overexpressed in spermidine-producing strain, leading to a 2-fold increase in spermidine production to a final titer of  $83.9 \pm 2.7 \mu\text{M}$  ( $0.012 \text{ g}\cdot\text{L}^{-1}$ )

(Table 1). Future efforts may explore the biosynthesis of higher-chain or functionalized polyamines with applications in polymer chemistry, plant growth promotion, and biomedical materials.

Besides L-glutamate and its derivatives, L-lysine is another highly relevant target for methanol-based production, particularly considering that stoichiometrically the maximum methanol-based L-lysine production of  $0.82 \text{ g}\cdot\text{g}^{-1}$  in *B. methanolicus* is comparable to the maximum glucose-based L-lysine production in *C. glutamicum* [55]. While *B. methanolicus* wild-type only secretes  $0.4 \text{ g}\cdot\text{L}^{-1}$  L-lysine in optimized fed-batch methanol fermentations, the classically obtained mutant NOA2#13A52-8A66 overproduces L-lysine up to  $65 \text{ g}\cdot\text{L}^{-1}$  [11]. Not only is L-lysine itself a product of interest, but it also serves as a precursor for various valuable compounds like 1,5-diaminopentane (cadaverine), a monomer of bioplastics. The wild-type *B. methanolicus* heterologously expressing *E. coli*-derived *cadA* produces cadaverine titers of  $10.2 \text{ g}\cdot\text{L}^{-1}$  without L-lysine accumulation in a high-cell density fed-batch methanol fermentation [7,56]. The L-lysine biosynthesis is strongly regulated in the wild-type *B. methanolicus*, and accumulations of high titers of cadaverine in this genetic background strain can be explained by the metabolic pull caused by the activity of lysine decarboxylase, which deregulates feedback inhibition [56]. Furthermore, the feasibility of mannitol and seaweed as feedstocks for cadaverine production by recombinant *B. methanolicus* was demonstrated [48].



**Figure 3.** Biosynthesis overview of value-added compounds derived from central carbon metabolism in *B. methanolicus*. Pathways: RuMP cycle, ribulose monophosphate cycle; TCA, tricarboxylic acid cycle; MEP, methylerythritol-phosphate pathway. Metabolites: GTP, guanosine-5'-triphosphate; GMP, guanosine-5'-monophosphate; IMP, inosine-5'-monophosphate; PRPP, 5-phospho- $\alpha$ -D-ribose-1-pyrophosphate; DAHP, 3-deoxy-arabinoheptulosonate-7-phosphate; DXP, 1-deoxy-xylulose-5-phosphate; SAM, S-adenosylmethionine; dcSAM, decarboxylated S-adenosylmethionine. Genes: *mdh*, methanol dehydrogenase (EC 1.1.1.244); *hps*,

3-hexulose-6-phosphate synthase (EC 4.1.2.43); *phi*, 6-phospho-3-hexuloisomerase (EC 5.3.1.27); *pfk*, 6-phosphofructokinase (EC 2.7.1.11); *fba*, fructose-1,6-bisphosphate aldolase (EC 4.1.2.13); *rpi*, ribose-5-phosphate isomerase (EC 5.3.1.6); *xyIA*, xylose isomerase (EC 5.3.1.5); *xyIB*, xylulose kinase (EC 2.7.1.17); *prs*, 5-phospho- $\alpha$ -D-ribose-1-pyrophosphate synthetase (EC 2.7.6.1); *pur* operon, purine biosynthesis operon; *guaA*, guanosine 5'-monophosphate synthetase (EC 6.3.5.2); *guaB*, inosine 5'-monophosphate dehydrogenase (EC 1.1.1.205); *ribAB*, bifunctional enzyme guanosine 5'-triphosphate cyclohydrolase II/3,4-DHBP synthase (EC 4.1.99.12/3.5.4.25); *ribDG*, bifunctional diaminohydroxyphosphoribosylaminopyrimidine deaminase/5-amino-6-(5-phosphoribosylamino)uracil reductase (EC 3.5.4.26/1.1.1.193); *ribE*, riboflavin synthase (EC 2.5.1.9); *ribH*, 6,7-dimethyl-8-(1-D-ribityl)lumazine synthase (EC 2.5.1.78); *ribC*, bifunctional riboflavin kinase/FMN adenylyltransferase (EC 2.7.1.26); *aroG1* and *aroG2*, 3-deoxy-arabinoheptulosonate-7-phosphate synthase (EC 2.5.1.54); *pyk*, pyruvate kinase (EC 2.7.1.40); *dxs*, 1-deoxy-xylulose-5-phosphate synthase (EC 2.2.1.7); *crtM*, dehydrosqualene synthase (EC 2.5.1.96); *crtN*, dehydrosqualene desaturase (EC 1.3.8.-); *alsS*, acetolactate synthase (EC 2.2.1.6); *alsD*, acetolactate decarboxylase (EC 4.1.1.5); *pdh*, pyruvate dehydrogenase (EC 1.2.4.1); *citA*, citrate synthase (EC 2.3.3.16); *aspB*, aspartate aminotransferase (EC 2.6.1.1); *gltAB*, glutamate synthase (EC 1.4.1.13); *argC*, N-acetyl- $\gamma$ -glutamyl-phosphate reductase (EC 1.2.1.38); *argJ*, bifunctional glutamate N-acetyltransferase (EC 2.3.1.35); *argD*, acetylornithine aminotransferase (EC 2.6.1.11); *argB*, acetylglutamate kinase (EC 2.7.2.8); *argF*, ornithine carbamoyltransferase (EC 2.1.3.3); *argG*, argininosuccinate synthase (EC 6.3.4.5); *argH*, argininosuccinate lyase (EC 4.3.2.1); *speA*, arginine decarboxylase (EC 4.1.1.19); *speB*, agmatinase (EC 3.5.3.11); *metK*, S-adenosylmethionine synthetase (EC 2.5.1.6); *speH*, S-adenosylmethionine decarboxylase (EC 4.1.1.50); *speE*, putrescine aminopropyltransferase (EC 2.5.1.16); *cadA*, lysine decarboxylase (EC 4.1.1.18); *gad*, glutamate decarboxylase (EC 4.1.1.15). Superscript “C”, chromosomally encoded; superscript “P”, natural plasmid pBM19 encoded; superscript “*P. thermoglucosidasius*”, gene heterologously expressed and native to *Parageobacillus thermoglucosidasius*; superscript “*S. aureus*”, gene heterologously expressed and native to *Staphylococcus aureus*; superscript “*B. subtilis*”, gene heterologously expressed and native to *Bacillus subtilis*; superscript “*E. coli*”, gene heterologously expressed and native to *Escherichia coli*; superscript “*S. thermosulfidooxidans*”, gene heterologously expressed and native to *Sulfobacillus thermosulfidooxidans*. Pink arrows, heterologous reactions; dark blue arrows, common reactions; green arrows, native reactions with overexpressed genes; red arrows, native reactions with gene knockdown; green-pink arrows, both native and heterologous genes have been overexpressed. Dashed arrows indicate multiple enzyme reactions.

#### 4.2. Production of Value-Added Compounds Derived from RuMP Cycle Intermediates

Riboflavin (vitamin B<sub>2</sub>) plays a central role in redox metabolism as the precursor of the FMN and FAD cofactors and its commercial production is well established in *B. subtilis* [57]. Riboflavin is synthesized *de novo* in some microorganisms in a pathway that begins with guanosine triphosphate (GTP) and Ru5P, the latter being an intermediate of the RuMP cycle in *B. methanolicus*. However, methanol-based riboflavin production has only recently been realized [50]. While overexpression of native riboflavin biosynthesis *rib* operon increased riboflavin titers, the heterologous expression of *B. subtilis* and *P. thermoglucosidasius*-derived *rib* operons improved the titers up to  $0.180 \pm 0.002 \text{ g}\cdot\text{L}^{-1}$  and  $0.202 \text{ g}\cdot\text{L}^{-1}$ , respectively, in shake flask experiments (Table 1) [15,50]. Further improvement to  $369 \text{ mg}\cdot\text{L}^{-1}$  was achieved by (1) increasing Ru5P supply via supplementation of xylose in *xyLAB*-expressing strain, (2) increasing purine supply via exchange of native promoter of the purine operon to a strong synthetic promoter and (3) decreasing riboflavin conversion to FMN and FAD via genome-based point mutation that lowered activity of bifunctional riboflavin kinase/FMN adenylyl transferase RibC (Figure 3) [15]. The engineered strain expressing the xylose metabolism operon, *xyLAB*, was cultivated in a fed-batch fermentation with minimal xylose and methanol medium, and produced riboflavin to a high titer of  $2.58 \text{ g}\cdot\text{L}^{-1}$  (Table 1) [15]. Future work will reveal if increased supply of the precursor GTP will enhance riboflavin yields even more.

**Table 1.** Products synthesized by genetically engineered *B. methanolicus* strains.

Products	Genetic Modifications	Carbon Source	Titer in Shake Flask ( $\text{g}\cdot\text{L}^{-1}$ )	Titer of Fed-Batch Fermentation ( $\text{g}\cdot\text{L}^{-1}$ )	Yield ( $\text{g}\cdot\text{g}^{-1}$ Methanol)	Productivity ( $\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ )	References
	Expression of <i>ribDGEABH</i> from <i>B. subtilis</i>	Methanol	0.18	0.52	-	-	[50]
Riboflavin	Expression of <i>xyLAB</i> and <i>ribDEAH</i> from <i>P. thermoglucosidasius</i> , mutation of genomic <i>ribC</i> <sup>G199D</sup> , replacement of the purine operon promoter with pH27 promoter in the genome;	Methanol and xylose	0.37	2.58	-	-	[15]

Acetoin	Expression of <i>alsSD</i> operon from <i>B. subtilis</i> , <i>mae<sup>Gs</sup></i> from <i>Geobacillus stearothermophilus</i> 10 and <i>B. methanolicus</i> -derived <i>aceA</i>	Methanol	0.42	-	0.07	-	[12]
GABA	Chemical mutagenesis and screening for enhanced L-glutamate production; expression of <i>gad</i> from <i>S. thermosulfidooxidans</i> ;	Methanol	0.16	13.2	0.04	-	[5]
L-Arginine	Deletion of <i>argR</i> and overexpression of <i>argB</i>	Methanol	0.8	-	-	-	[9]
Putrescine	Expression of <i>speA</i> , <i>speB</i> from <i>E. coli</i> and <i>argCJDB</i> from <i>B. methanolicus</i>	Methanol	0.012	-	-	-	[24]
Spermidine	Expression of <i>speA</i> , <i>speB</i> from <i>E. coli</i> and <i>speE</i> , <i>speH</i> from <i>B. methanolicus</i>	Methanol	0.012	-	-	-	[24]
Cadaverine	Expression of <i>cadA</i> from <i>E. coli</i>	Methanol	0.45	10.2	-	-	[7,56]

The shikimate pathway is another metabolic route that utilizes RuMP cycle intermediates such as E4P as its precursors [58]. It serves as the biosynthetic pathway to aromatic amino acids (L-phenylalanine, L-tyrosine, L-tryptophan) and numerous secondary metabolites. The first committed step is the formation of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) from E4P and PEP by DAHP synthase (*aroG*) (Figure 3). E4P is an intermediate of the RuMP cycle and PEP is an intermediate of the lower glycolysis, which begins with GA3P derived from the RuMP cycle. The two native DAHP synthases from *B. methanolicus* encoded by *aroG1*, *aroG2* are thermostable and exhibit substrate specificity [59]. Interestingly, AroG1 is not feedback inhibited, whereas the activity of AroG2, which contains an active chorismate mutase domain, is inhibited by chorismate and prephenate. Although no production data for DAHP or downstream shikimate derivatives in *B. methanolicus* have yet been reported, the availability of functional native enzymes lays the groundwork for pathway extension. Since both PEP and E4P are readily derived from glycolytic and RuMP cycle reactions, it is feasible to reconstitute and amplify the entire shikimate pathway in this host, with potential challenges such as tight regulatory feedback control. Engineering efforts could involve introducing feedback-resistant variants of DAHP synthase (*aroG<sup>fb</sup>*), and integrating heterologous downstream modules for L-tyrosine or L-phenylalanine biosynthesis [60].

#### 4.3. Production of Value-Added Compounds Derived from the Precursor Molecule Pyruvate

Acetoin (3-hydroxy-2-butanone) is a valuable precursor for solvents, food flavourings, and polymer precursors derived from pyruvate. An acetoin-producing *B. methanolicus* strain was constructed by heterologous expression of the *alsS* and *alsD* genes from *B. subtilis* [12], which encode enzymes for the conversion of pyruvate to acetolactate and subsequently to acetoin (Figure 3). In minimal methanol medium, the engineered strain reached an acetoin titer of about 0.42 g·L<sup>-1</sup>, with a yield of 0.07 g·g<sup>-1</sup> methanol (Table 1). Acetoin synthesis competes with the TCA cycle for pyruvate, which may be mitigated by fine-tuning flux through pyruvate-forming and consuming reactions, or by dynamic regulation using inducible systems to decouple growth and production phases.

Finally, terpenoids are among the most structurally diverse natural products, with wide applications as pharmaceuticals, nutraceuticals, and biofuels. In *B. methanolicus*, terpenoid biosynthesis occurs via the methylerythritol phosphate (MEP) pathway, which uses pyruvate and GA3P as precursors. The production of C30 terpenoids in an engineered *B. methanolicus* was demonstrated in a proof-of-concept study, using not only methanol but also mannitol and seaweed extracts as raw materials [48]. Though production of C30 terpenoids was only confirmed qualitatively, not quantitatively, the functionality of the endogenous MEP pathway at 50 °C suggests high potential for future engineering. Typically, key strategies to increase terpenoid production in bacteria include: (1) overexpression of rate-limiting MEP enzymes (e.g., 1-deoxy-D-xylulose-5-phosphate (DXP) synthase, DXP reductoisomerase, isopentenyl diphosphate isomerase) and (2) introduction of heterologous terpene synthases tailored to desired products (Figure 3) [61]. In addition, the engineering of NADPH and ATP supply is crucial to support the reductive steps of MEP and terpene biosynthesis [61].

## 5. *B. methanolicus* as Source and Host for Protein Production

The robustness, thermophilic lifestyle, and natural methylotrophy of *B. methanolicus* make its proteins attractive for synthetic biology applications, particularly for constructing methanol-utilizing production platforms in mesophilic hosts. *B. methanolicus* enzymes are catalytically active at elevated temperatures, which can confer certain advantages when these genes are heterologously expressed in various bacterial species.

### 5.1. *B. methanolicus* Is a Valuable Source of Novel Enzymes

Much research on *B. methanolicus* has focused on characterizing genes and enzymes involved in methylotrophy, more specifically in methanol oxidation and formaldehyde assimilation and dissimilation pathways. The genome of this bacterium was sequenced in 2012 [4,47], providing access to its complete collection of genes. Transcriptome [62], proteome [37], and metabolome [63] analyses have respectively identified RNAs, proteins, and metabolites with increased abundance during methylotrophic growth. The three different methanol dehydrogenases Mdh, Mdh2, and Mdh3, and the activator protein ACT, were purified and biochemically characterized [31], as well as multiple enzymes representing the RuMP pathway [17,40–42,44,45]. On the one hand, this research was important for understanding methylotrophy in *B. methanolicus*, as described in detail above. But on the other hand, these enzymes represent valuable candidates for engineering methylotrophy into non-methylotrophic hosts, typically referred to as synthetic methylotrophy.

*B. methanolicus* has been instrumental in engineering synthetic methylotrophy in non-methylotrophic hosts. In particular, its methanol dehydrogenase genes have been introduced into *E. coli*, *B. subtilis*, and *C. glutamicum* to enable methanol oxidation into formaldehyde; the first and crucial step in bacterial methylotrophy [34,64,65]. But also the genes encoding the key RuMP cycle enzymes responsible for formaldehyde assimilation, Hps and Phi, have been co-expressed in *E. coli* [66]. Although most bacteria possess native enzymes representing several of the RuMP pathway reactions, ribulose-phosphate 3-epimerase (*rpe*), ribose-5-phosphate isomerase (*rpi*), transketolase (*tkt*), and transaldolase (*tal*) genes have still been heterologously expressed in these hosts to facilitate regeneration of Ru5P for continuous formaldehyde assimilation needed for functional methylotrophy (reviewed by Antoniewicz) [67].

In addition to the methylotrophy genes, several additional genes from various *B. methanolicus* strains have been heterologously expressed and characterized in bacterial hosts. This includes the peroxygenase P450 enzyme CYP152K6 [68], a novel cold-adapted type I pullulanase for efficient amylopectin debranching hydrolysis [69], a milk clotting enzyme MCE [70], a  $\beta$ -fructofuranosidase for biosynthesis of lactosucrose [71], and two DAHP synthases AroG1 and AroG2 [59].

### 5.2. The Methylotrophic and Thermophilic Nature of *B. methanolicus* Makes It a Promising Host for the Production of Proteins

Although most metabolic engineering approaches using *B. methanolicus* have focused on enabling bioproduction of small molecules such as amino acids and their derivatives (reviewed by Pfeifenschneider et al.) [34,72], this organism has also been explored as a potential host for heterologous protein production. While still under-developed compared to traditional hosts like *E. coli* and *B. subtilis*, *B. methanolicus* offers certain distinct advantages for specific applications, particularly those aiming for methanol-based bioproduction. Also, the thermophilic nature of *B. methanolicus* enables expression of proteins that require elevated temperatures for proper folding or catalytic activity. Over years of research, the genetic toolbox of *B. methanolicus* has been gradually expanded. Molecular tools such as high- and low copy number replicative plasmids, strong constitutive and inducible promoters (e.g., mannitol- and xylose-inducible expression systems), and CRISPR/Cas9-based genome editing tools have been developed to support its engineering [7,49,73]. Together, these tools enable strong and controlled gene expression and the production of functional proteins at 50 °C. Examples of model and reporter proteins that have been functionally expressed in *B. methanolicus* are GFP, LacZ, sfGFP, and  $\alpha$ -amylase [49,74].

The protein secretion capabilities of this bacterium are currently under investigation, with some success in secreting heterologous proteins into the culture medium, which simplifies downstream processing. Moreover, efforts are ongoing to optimize codon usage, improve translation efficiency, and expand the host range of expression elements. Challenges remain in protein yield optimization, secretion efficiency, and expanding the genetic toolbox. Methods for methanol fed-batch cultivations in bio reactors are well established for *B. methanolicus* [34] that will also be highly useful for scale-up bioprocess development for heterologous protein production.

## 6. Major Challenges in the Establishment of *B. methanolicus* as an Industrial Workhorse

From vitamin and bulk chemicals to the potential synthesis of complex natural products, *B. methanolicus* provides a robust and versatile platform for methanol-based bioproduction. Currently, one of the major limitations in the scaling-up of methanol-based bioprocesses is securing a sufficient oxygen supply for methylotrophic microorganisms. Methanol has a high energy density due to a carbon reduction degree of 6, compared to the commonly used feedstock such as glucose with a carbon reduction degree of 4. While use of methanol as a feedstock generates the surplus of reducing power for biosynthetic pathways in comparison to glucose, methanol requires at least a double oxygen supply leading to issues with large scale fermentations [75]. However, technological advancements together with the application of high throughput approaches to optimize fermentation conditions are likely to provide rapid solutions to these practical challenges [76]. Moreover, currently there is limited knowledge about transport proteins in *B. methanolicus*, regarding substrate uptake, export of low-molecular-weight compounds, or protein secretion, which is a limitation for the strain engineering. The thermophilic nature of *B. methanolicus* and accordingly, high membrane fluidity may reveal new transport systems and, consequently, new roads for metabolic engineering of transport proteins as well as membrane composition.

However, despite current constraints, the expanding genetic accessibility and demonstrated production capacity should make *B. methanolicus* a valuable alternative candidate for future biotechnological applications. Besides its well-established position as an effective cell factory for the production of various value-added compounds from methanol, *B. methanolicus* is an important source for novel genes encoding thermostable proteins useful to engineer methylotrophy into non-methylotrophic hosts and other applications, and a promising host for heterologous production of proteins especially suited for methanol-based biomanufacturing under thermophilic conditions. Interestingly, this organism can also grow well on mannitol and seaweed extracts [48], and recently was engineered toward co-utilization of the seaweed carbohydrate xylose with methanol [15], further confirming its potential for sustainable bioproduction purposes. As tools for genome editing, pathway balancing, and dynamic control have greatly matured for this organism in the past year [5,15,20,23], the landscape of possible methanol-derived products will likely expand. Moreover, the integration of ALE, and systems biology approaches will help unlock high-yield production routes for a broader range of chemicals. The future of *B. methanolicus* as a flagship chassis for methanol-based synthetic biology is not just feasible; it is already unfolding.

### Author Contributions

Conceptualization, L.F.B. and M.I.; Writing—Original Draft Preparation, L.F.B., M.K.F., H.Z., T.B., V.F.W., M.I.; Writing—Review & Editing, L.F.B., M.K.F., H.Z., T.B., V.F.W., M.I.; Visualization, L.F.B., M.K.F.; Funding Acquisition, L.F.B., T.B., V.F.W., M.I.

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### 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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