Minireview

Efficient cell factories for the production of \(N\)-methylated amino acids and for methanol-based amino acid production

Marta Irla\(^1\) and Volker F. Wendisch\(^2\)

\(^1\)Microbial Synthetic Biology, Department of Biological and Chemical Engineering, Aarhus University, Gustav Wieds Vej 10, Aarhus C, 8000, Denmark.

\(^2\)Genetics of Prokaryotes, Faculty of Biology and CeBiTec, Bielefeld University, Universit"atsstr. 25, Bielefeld, 33615, Germany.

Summary

The growing world needs commodity amino acids such as L-glutamate and L-lysine for use as food and feed, and specialty amino acids for dedicated applications. To meet the supply a paradigm shift regarding their production is required. On the one hand, the use of sustainable and cheap raw materials is necessary to sustain low production cost and decrease detrimental effects of sugar-based feedstock on soil health and food security caused by competing uses of crops in the feed and food industries. On the other hand, the biotechnological methods to produce functionalized amino acids need to be developed further, and titres enhanced to become competitive with chemical synthesis methods. In the current review, we present successful strain mutagenesis and rational metabolic engineering examples leading to the construction of recombinant bacterial strains for the production of amino acids such as L-glutamate, L-lysine, L-threonine and their derivatives from methanol as sole carbon source. In addition, the fermentative routes for bioproduction of \(N\)-methylated amino acids are highlighted, with focus on three strategies: partial transfer of methylamine catabolism, \(S\)-adenosyl-L-methionine dependent alkylation and reductive methylamination of 2-oxoacids.

Received 1 March, 2022; revised 12 April, 2022; accepted 18 April, 2022.

For correspondence. \*E-mail marta.irla@bce.au.dk; Tel. +49-521-1065611; Fax: +49-521-1065626. \**E-mail: volker.wendisch@unibiellefeld.de.

Microbial Biotechnology (2022) 15(8), 2145–2159
doi:10.1111/1751-7915.14067

© 2022 The Authors. Microbial Biotechnology published by Society for Applied Microbiology and John Wiley & Sons Ltd.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

Amino acid production by fermentation is a success story that started more than six decades ago (Lee and Wendisch, 2017). The market demand is steadily rising, even though African Swine Fever and the COVID-19 pandemic slowed the growth. The very efficient L-glutamate and L-lysine production processes that are operated at a huge scale (millions tons per year) benefit from the so-called “economy of scale” (Wendisch, 2020). However, since the margins are very low, two trends have emerged: a shift from commodities towards specialty amino acids (Ajinomoto, 2020) and a shift from traditional substrates towards alternatives carbon sources (Wendisch et al., 2016).

Traditional amino acid fermentation is based on sugars and molasses and costs for these feedstocks contribute notably to the operational expenditures. Considering substrate availability, costs and competing uses in the food and feed industries, a flexible feedstock concept was realized for amino acid producer strains enabling access to sustainable alternatives, for example, lignocellulosic, aqua- and agricultural sidestreams (Wendisch et al., 2022).

Specialty amino acids find applications in the pharmaceutical industry (e.g. infusions, injections, intermediates in active substance syntheses or as active pharmaceutical ingredients). Among others, *Escherichia coli* and *Corynebacterium glutamicum* strains have been engineered to produce the blood pressure-lowering L-arginine (Park et al., 2014), the insulinotropic (2S, 3R, 4S)-4-hydroxyisoleucine (Smirnov et al., 2010; Zhang et al., 2018), 5-hydroxy-L-tryptophan that can be used against depression and obesity (Mora-Villalobos and Zeng, 2018), and the cyclic amino acid L-pipeolic acid used as cell protectant and precursor of, for example, the immunosuppressant rapamycin and the antitumor agent swainsonine (Pérez-García et al., 2016; 2017; 2019).

In this review, we address these trends by focusing on how methanol, a feedstock without competing food and feed uses, can be harnessed for production of
L-glutamate, L-lysine, L-threonine and their derivatives by bacteria. In recent years, there has been substantial progress in the development of methods for methanol synthesis particularly through not only CO₂ hydrogenation but also isothermal methane conversion into methanol catalysed by copper-containing zeolites or production of methanol from crude glycerol (Haider et al., 2015; Tomkins et al., 2016; Mbatha et al., 2021). In this review methanol-based production of L-serine, an intermediate of serine cycle for formaldehyde assimilation, will not be presented as it has been thoroughly summarized elsewhere (Eggeling, 2007). Moreover, we cover how access to N-methylated amino acids, a particular class of specialty amino acids, has been gained by metabolic engineering.

Engineering cell factories for methanol-based amino acid production

Production of L-glutamate and its derivatives from methanol

Bacillus methanolicus MGA3 is a methanol-utilizing bacterium known for its capacity to overproduce L-glutamate up to 60 g l⁻¹ in methanol-controlled fed-batch fermentations (Table 1) (Schendel et al., 2000; Heggeset et al., 2012), and in flasks under magnesium or methanol limitation (Schendel et al., 2000; Brautaset et al., 2003). There are several factors that may contribute to L-glutamate accumulation in B. methanolicus: (1) overflow metabolism due to inactive tricarboxylic acid (TCA) cycle during methylo trophic growth, (2) production of L-glutamate as compatible solute in response to osmotic stress.

It is a widespread property of methylotrophs that they do not need a complete TCA to fulfil their energy requirements (Chistoserdova et al., 2009). While B. methanolicus is equipped with a full gene set for a functional TCA cycle and a functional glyoxylate shunt (Heggeset et al., 2012; Muller et al., 2015; Drejer et al., 2020), during growth on methanol the levels of some TCA enzymes were decreased and the carbon flux through the TCA cycle stopped after isocitrate with only small remaining flux needed to support the synthesis of biomass precursors (Müller et al., 2014; Delépine et al., 2020). For example, the activity of 2-oxoglutarate dehydrogenase (encoded by odhAB) in crude extract of B. methanolicus was lower than in other Bacillus species that do not overproduce L-glutamate, and its restoration through plasmid-based overexpression of odhAB decreased L-glutamate accumulation confirming importance of low carbon flux through TCA cycle for L-glutamate synthesis (Carlsson and Hederstedt, 1986; Brautaset et al., 2003; Krog et al., 2013). While being able to grow in seawater-based media (Komives et al., 2005), B. methanolicus possesses a restricted ability to cope with sustained osmotic stress through synthesis of the moderately effective compatible solute L-glutamate (Frank et al., 2021). The cellular L-glutamate pool increased concomitantly with increasing external osmolarity, and a large portion of the newly synthesized L-glutamate was excreted (Frank et al., 2021). The expression gltAB and gltA2 encoding two glutamate synthases was upregulated in response to high salinity along with that of gltC, which encodes a transcriptional activator of the glutamate synthase operon (Frank et al., 2021). Plasmid-based overexpression gltAB and gltA2 boosted secretion of L-glutamate by B. methanolicus, but not that of gltA2, yweB and glnA encoding glutamate synthase, glutamate dehydrogenase and glutamine synthetase (Table 1), respectively, indicating the major role of GltAB and GltA2 in L-glutamate biosynthesis (Krog et al., 2013).

The mechanism of L-glutamate secretion in B. methanolicus is still not elucidated. In C. glutamicum, a known microbial L-glutamate producer, MscCG, a MscS-like channel, is the major L-glutamate export system (Nakamura et al., 2007; Nakayama et al., 2016; 2018; Wang et al., 2018). No homologues of an MscCG channel are encoded in the genome of B. methanolicus and the question if the MscS-type channel-encoding gene (locus BMMGA3_16700) present in the genome is involved in L-glutamate secretion remains to be solved (Heggeset et al., 2012; Frank et al., 2021).

Bacillus methanolicus is not the only methylotrophic candidate to become platform strain for methanol-based L-glutamate production, the classical mutant of Methylobacillus glycogenes, a Gram-negative obligate methylotroph, secreted 38.8 g l⁻¹ of L-glutamate in an 84-h 5-l methanol-based fermentation supplemented with 10 g l⁻¹ yeast extract (Table 1) (Libudzisz et al., 1983; Urakami and Komagata, 1986; Motoyama et al., 1993a).

Production of an L-glutamate-derivative, γ-aminobutyric acid (GABA), which is a precursor of a 2-pyrrolidinone building block of biodegradable polyamine, nylon 4, was established in B. methanolicus through heterologous overexpression of glutamate decarboxylase gene (gad) derived from Sulfobacillus thermosulfidooxidans (Table 1) (Irla et al., 2017; Fukuda and Sasanuma, 2018). While the choice of this thermophilic donor circumvented the issue of thermobility of E. coli-derived Gad, it did not alleviate the problem of its low activity at neutral pH (Irla et al., 2017; Fan et al., 2018). Bacterial Gads participate in acid stress response and are only active at low pH (Capitani et al., 2003). In two-phase, methanol-controlled fed batch fermentation 9 g l⁻¹ of GABA were produced by engineered B. methanolicus, however, this approach did not support full L-glutamate conversion, with almost 13 g l⁻¹ of L-glutamate left in fermentation broth (Irla et al., 2017). The purification of
| Host strain          | Phenotype/ Genotype or Relevant enzymatic characteristic | Overexpressed gene(s) | Titre \([\text{g l}^{-1}]\) | Fermentation mode | References for established processes |
|---------------------|----------------------------------------------------------|------------------------|-----------------------------|-------------------|-------------------------------------|
| *B. methanolicus* MGA3 Wild type                  | -                                         | 0.8/ 59                | Shake flask/ Fed-batch      | Heggeset *et al.* (2012); Krog *et al.* (2013) |
| *B. methanolicus* MGA3 Wild type                        | *gltAB*                                    | 1.1                    | Shake flask                 | Krog *et al.* (2013) |
| *B. methanolicus* MGA3 Wild type                        | *gltA2*                                    | 1.1                    | Shake flask                 | Krog *et al.* (2013) |
| *M. glycogenes* RV3 Phe* (auxotrophy revertant)       | -                                         | 9.3/ 38.8              | Test tubes/ Jar fermentor   | Motoyama *et al.* (1993a) |

**GABA production**

| Host strain          | Phenotype/ Genotype or Relevant enzymatic characteristic | Overexpressed gene(s) | Titre \([\text{g l}^{-1}]\) | Fermentation mode | References for established processes |
|---------------------|----------------------------------------------------------|------------------------|-----------------------------|-------------------|-------------------------------------|
| *B. methanolicus* MGA3 Wild type                  | *gad*                                      | 0.35/ 13.3             | Shake flask/ Fed-batch      | Irla *et al.* (2017) |

**Lysine production**
Table 1. (Continued)

| Host strain | Phenotype/ Genotype or Relevant enzymatic characteristic | Overexpressed gene(s) | Titre [g l⁻¹] | Fermentation mode | References for established processes |
|-------------|--------------------------------------------------------|----------------------|---------------|------------------|-----------------------------------|
| B. methanolicus MGA3 | Wild type | – | 0.01/0.4 | Fed-batch | Brautaset et al. (2010); Naerdel et al. (2011) |
| B. methanolicus MGA3 | Wild type | dapG | 0.01/0.4 | Shake flask | Jakobsen et al. (2009); Naerdel et al. (2011) |
| B. methanolicus MGA3 | Wild type | lysC | 0.06/0.38 | Shake flask/ Fed-batch | Jakobsen et al. (2009); Naerdel et al. (2011) |
| B. methanolicus MGA3 | Wild type | yclM | 0.14/1.8 | Shake flask | Naerdel et al. (2011) |
| B. methanolicus MGA3 | Wild type | dapG<sup>fr</sup> | 0.12 | Shake flask | Naerdel et al. (2011) |
| M. methylotrophus AS1 | Wild type | asd | 0.01 | Shake flask | Naerdel et al. (2011) |
| M. methylotrophus AS1 | Wild type | dapA | 0.01 | Shake flask | Naerdel et al. (2011) |
| M. methylotrophus AS1 | Wild type | lysC | 0.15 | Shake flask | Naerdel et al. (2011) |
| M. methylotrophus AS1 | Wild type | dapA<sup>fr</sup> - yclM | 0.21 | Shake flask | Naerdel et al. (2011) |
| M. methylotrophus MGA3 | Wild type | dapA - yclM - lysC<sup>E<sub>Ec</sub></sup> | 0.58 | Shake flask | Naerdel et al. (2011) |
| B. methanolicus MGA3 | Wild type | hom<sup>-</sup> | – | 0.15/11.0 | Shake flask/ Fed-batch | Brautaset et al. (2010) |
| M. methanolicus MGA3 | Wild type | dapG<sup>fr</sup> hom<sup>-</sup> P<sub>lys<sup>Es</sup></sub>mut<sup>−</sup> | 65 | Fed-batch | Brautaset et al. (2010) |
| M. methylotrophus AS1 | Wild type | lysC<sup>Ec</sup> | -0.01 | Test tube | Tsujimoto et al. (2006) |
| M. methylotrophus AS1 | Wild type | dapA<sup>Ec</sup> | 0.08 | Shake flask | Gunji and Yasueda (2006) |
| M. methylotrophus AS1 | Wild type | lysC<sup>Ec</sup> | 0.1 | Shake flask | Gunji and Yasueda (2006) |
| M. methylotrophus AS1 | Wild type | dapA<sup>Ec</sup> | 1.0 | Shake flask/ Fermentor | Gunji and Yasueda (2006) |
| M. methylotrophus G49 | Asd<sup>fr</sup>, DapA<sup>fr</sup> | - | 0.08 | Test tube | Tsujimoto et al. (2006) |
| M. methylotrophus G49 | Asd<sup>fr</sup>, DapA<sup>fr</sup> | dapA<sup>Ec</sup> - lysC<sup>Ec</sup> | 0.4/1.0 | Test tube/ Jar fermentor | Tsujimoto et al. (2006) |
| M. methylotrophus 102 | MetF<sup>-</sup> | lysC<sup>Ec</sup> | 1.2/9.0 | Shake flask/ Fermentor | Ishikawa et al. (2008a); Ishikawa et al. (2008b) |
| M. glyceogenes AL119 | Ak<sup>fr</sup>, DapA<sup>fr</sup> | dapA<sup>Ec</sup> | 0.4 | Test tube | Motoyama et al. (2001) |
| M. glyceogenes AL119 | Ak<sup>fr</sup>, DapA<sup>fr</sup> | dapA<sup>Ec</sup> - DHL122 | 1.1 | Test tube/ Jar fermentor | Motoyama et al. (2001) |
| M. glyceogenes DHL122 | Ak<sup>fr</sup>, DapA<sup>fr</sup> | dapA<sup>Ec</sup> | 8.0 | Test tube/ Jar fermentor | Motoyama et al. (1993a); Motoyama et al. (2001) |
| M. glyceogenes DHL122 | Ak<sup>fr</sup>, DapA<sup>fr</sup> | dapA<sup>Ec</sup> - DHL122 | 1.2/5.3 | Test tube/ Jar fermentor | Motoyama et al. (2001) |

Cadaverine production

L-lysine $\xrightarrow{\text{cad}}$ cadaverine

CO₂

| Host strain | Phenotype/ Genotype or Relevant enzymatic characteristic | Overexpressed gene(s) | Titre [g l⁻¹] | Fermentation mode | References for established processes |
|-------------|--------------------------------------------------------|----------------------|---------------|------------------|-----------------------------------|
| B. methanolicus MGA3 | Wild type | cadA | 0.45/10.2 | Shake flask/ Fed-batch | Irla et al. (2016) |

5AVA production

L-lysine $\xrightarrow{\text{cad}}$ cadaverine $\xrightarrow{\text{potA}}$ 5-aminopentanal $\xrightarrow{\text{patD}}$ 5AVA

CO₂

L-lysine $\xrightarrow{\text{raIP}}$ α-ketolysine $\xrightarrow{\text{patD}}$ 5AVA

O₂ + H₂O + NH₃ + H₂O₂ $\xrightarrow{\text{raIP}}$ α-ketolysine $\xrightarrow{\text{patD}}$ 5AVA
GABA from fermentation broth was achieved to 99.1% purity in a multistep process composed among others of flocculation, filtration, ultrafiltration, decolouration, ion exchange chromatography and lastly crystallization (Gao et al., 2013).

Methanol-based production of L-lysine, its derivatives, and L-threonine

B. methanolicus wild type produced up to 0.4 g l\(^{-1}\) of L-lysine in high cell density fed-batch fermentations, and its mutant strain NOA2#13A52-8A66 up to 65 g l\(^{-1}\) under the same conditions which is caused by several mutation in its genome (Hanson et al., 1996; Brautaset et al., 2010). The amino acid exchange in one of its three aspartokinases (AKs), catalysing the phosphorylation of L-aspartate to L-aspartate-4-phosphate, encoded by \(dapG\) abolished feedback inhibition by meso-diaminopimelic acid (DAP) (Naerdal et al., 2011; 2017). Due to mutation in homoserine dehydrogenase (Hom) gene Hom activity decreased and metabolic flux was redirected from reduction of aspartate 4-semialdehyde to homoserine catalysed by Hom towards synthesis of 4-hydroxytetrahydrodipicolinate by its synthase (DapA) in L-lysine biosynthetic pathway (Naerdal et al., 2011; 2017). Finally, the mutation of the region upstream of lysA (Table 1) increased its expression in comparison to the wild-type strain, presumably enhancing the decarboxylation of meso-DAP to L-lysine by LysA (Naerdal et al., 2011; 2017). Apart from mutations in L-lysine biosynthesis pathway, NOA2#13A52-8A66 strain exhibits changes in enzyme activities in central carbon metabolism. Decreased pyruvate dehydrogenase activity in comparison to the wild type caused by point mutation in \(pdhD\) gene can potentially decrease carbon flux towards TCA cycle and direct it towards oxaloacetate through activity of pyruvate decarboxylase instead (Brautaset et al., 2003; Naerdal et al., 2017).

Methylphilus methylotrophus AS1 is an obligate methylotroph with a ribulose monophosphate (RuMP) pathway for formaldehyde assimilation (Jenkins et al., 1987; Gunji et al., 2004). \(M.\ methylphilus\) wild type naturally produces less than to 0.01 g l\(^{-1}\) of L-lysine in test tube cultivations (Jenkins et al., 1987; Gunji et al., 2004), however, L-lysine titre was increased to 0.08 g l\(^{-1}\) in strain G49 due to mutations in \(asd\) and \(dapA\) genes (Table 1) that caused partial resistance to feedback inhibition of aspartate semialdehyde dehydrogenase (Asd) catalysing formation of L-aspartate-semialdehyde in the reductive dephosphorylation of L-aspartate-4-phosphate, and DapA further converting L-aspartate-semialdehyde to 4-hydroxy-tetrahydrodipicolinate (Gunji et al., 2004; Tsujimoto et al., 2006).

Table 1. (Continued)

| Host strain | Phenotype/ Genotype or Relevant enzymatic characteristic | Overexpressed gene(s) | Titre [g l\(^{-1}\)] | Fermentation mode | References for established processes |
|-------------|----------------------------------------------------------|-----------------------|----------------------|------------------|-------------------------------------|
| B. methanolicus MGA3 | Wild type | cadA-patA-patD | 0.02 | Shake flask | Brito et al. (2021) |
| B. methanolicus MGA3 | Wild type | raiP | 0.02 | Shake flask | Brito et al. (2021) |

Methylated amino acids and amino acids from methanol

| Host strain | Phenotype/ Genotype or Relevant enzymatic characteristic | Overexpressed gene(s) | Titre [g l\(^{-1}\)] | Fermentation mode | References for established processes |
|-------------|----------------------------------------------------------|-----------------------|----------------------|------------------|-------------------------------------|
| M. glycogenes AL119 | AK\(^{br}\), Dap\(^{Abr}\) | - | 11.0 | Jar fermentor | Motoyama et al. (1993b) |
| M. glycogenes ATR80 | AK\(^{br}\), HK\(^{br}\), Dap\(^{Abr}\) | - | 8.5 | Jar fermentor | Motoyama et al. (1993b) |
| M. glycogenes TR80 | AK\(^{br}\), HK\(^{br}\), Dap\(^{Abr}\) | hom-thrC | 12.3 | Jar fermentor | Motoyama et al. (1994) |
| M. glycogenes A513 | AEC\(^{R}\), Thr\(^{R}\), Phe\(^{+}\), Ile\(^{-}\) | hom-thrC | 16.3 | Jar fermentor | Motoyama et al. (1994) |

AEC\(^{R}\), S-(2-aminoethyl)-L-cysteine resistance; Thr\(^{R}\), L-threonine resistance; Phe\(^{+}\), phenylalanine auxotrophy; Phe\(^{-}\), phenylalanine prototrophy; Ile\(^{-}\), isoleucine auxotrophy; fbr, feedback inhibition resistance.
The mutant DHL122 derived from *M. glycoeneges* ATCC 21276 produced 5.6 g l\(^{-1}\) of L-threonine and 3.1 g l\(^{-1}\) of L-lysine in 72-h, 5-liter jar fermentation (Table 1) (Motoyama *et al.*, 1993a). The AK\(^{\text{DHL122}}\) was completely insensitive to inhibition by L-lysine in contrast to that of parental strain ATCC 21276, and it was activated with increasing concentrations of L-threonine (Motoyama *et al.*, 1993b, 2001). Moreover, the L-lysine feedback inhibition of DapA\(^{\text{DHL122}}\) was partially alleviated compared to wild type, possibly due to amino acid exchanges located in the region relevant for interaction with the allosteric effector, L-lysine (Motoyama *et al.*, 1993b, 2001).

One strategy to increase the L-lysine titre in *B. methanolicus* is plasmid-based overexpression of genes belonging to L-lysine biosynthesis pathway (Table 1). Upon overexpression of AK-encoding genes *dapG*, *lysC* and *yclM* in *B. methanolicus* L-lysine titres either did not increase or increased 8- and 20-fold in flask cultivation, and 2-, 10- and 60-fold in high cell density methanol fed-batch fermentations, respectively, with a final titre in the fed-batch fermentation for a yclM-expressing strain of 11 g l\(^{-1}\) (Jakobsen *et al.*, 2009; Naerdal *et al.*, 2011). Interestingly, overexpression of NOA2\#13A52-8A66-derived mutated *dapG* coding for a previously mentioned AK desensitized to feedback inhibition led to 17-fold increase in L-lysine titre compared to the control strain in flask cultivation (Naerdal *et al.*, 2011).

While overexpression of *asd* and *dapA* had no positive effect on L-lysine production in *B. methanolicus* wild type, the L-lysine titre increased almost two- and fivefold in L-lysine producing mutant *B. methanolicus* strain M168-20 overexpressing *asd* and *dapA* in comparison to empty vector control (Naerdal *et al.*, 2011). Similarly, the overexpression of the gene encoding feedback inhibition resistant DapA\(^{\text{DHL122}}\) in DHL122 and its parent strain AL119 elevated the specific activity of DapA 20-fold in both strains and L-lysine production two- and threefold, respectively, with concomitant reduction of L-threonine accumulation in test tube cultures. AL119 overexpressing *dapA\(^{\text{DHL122}}\)* produced 8 g l\(^{-1}\) of L-lysine in a 5-liter jar fermentor from methanol as a substrate (Motoyama *et al.*, 2001).

Finally, through the overexpression of the gene encoding diaminopimelate decarboxylase (LysA), the last enzyme of the L-lysine biosynthesis pathway, 20-fold increase in L-lysine accumulation was achieved in *B. methanolicus* wild type in comparison to empty vector control (Naerdal *et al.*, 2011). In this respect, it has to be noted that expression of *lysA* was increased in NOA2\#13A52-8A66 due to previously mentioned point mutation in promoter region (Naerdal *et al.*, 2011).

Co-expression of several genes of L-lysine biosynthesis had a cumulative effect on L-lysine production in *B. methanolicus* (Table 1), when *dapA* was overexpressed together with *yc/lM* the L-lysine titre increased 30-fold, and addition of *lysA* to this pair resulted in an 83-fold rise in comparison to the wild-type strain (Naerdal *et al.*, 2011). Heterologous expression of mutated versions of *E. coli*-derived *dapAEc24* and *lysECm20* encoding enzymes with reduced sensitivity to feedback inhibition and wild-type version of dihydrodipicolinate reductase gene (*dapB\(^{\text{Ec}}\)*) in *M. methylotrophus* G49 improved L-lysine titre to 0.4 g l\(^{-1}\) in test tube cultivation compared to 0.08 g l\(^{-1}\) for empty vector strain, with final titre of 1 g l\(^{-1}\) in jar fermentor (Tsujimoto *et al.*, 2006).

Another strategy to increase L-lysine titres is the overexpression of exporter encoding gene (*lysE*) (Table 1). Heterologous expression of mutated *lysE\(^{\text{Cm24}}\)* gene derived from *C. glutamicum* in *M. methylotrophus* AS1 increased L-lysine titre eightfold in the test tube in comparison to empty vector control strain (Gunji and Yasueda, 2006). The strain AS1 overexpressing *lysE\(^{\text{Cm24}}\)* with *dapA\(^{\text{24}}\)* produced 1 g l\(^{-1}\) L-lysine in shake flask cultivation and 11.3 g l\(^{-1}\) in 72 h jar fermentation (Gunji and Yasueda, 2006). A methionine auxotrophic *M. methylotrophus* mutant with deletion of 10-methyltetrahydrofolate reductase gene (*metF*) overexpressing *lysE\(^{\text{Cm24}}\)* and *dapA\(^{\text{24}}\)* produced 1.2 g l\(^{-1}\) L-lysine in shake flasks and more than 9.0 g l\(^{-1}\) in 1-liter jar fermentors (Ishikawa *et al.*, 2008a, 2008b). The *metF* deletion presumably positively affected L-lysine biosynthesis due to homocysteine accumulation that inhibited activity of homoserine kinase (HK) encoded by *thkB* (Ishikawa *et al.*, 2008a). Inhibition of HK activity decreased accumulation of intracellular L-threonine, an AK inhibitor, subsequently averting feedback inhibition of AK by L-threonine and increasing L-lysine production (Gunji *et al.*, 2004; Ishikawa *et al.*, 2008a). *B. methanolicus* strain co-expressing *lysC* with *lysE\(^{\text{G9}}\)* produced almost sevenfold more L-lysine in flask cultivation in comparison to strain expressing only *lysC*, while expression on native *lysE* had no effect on L-lysine titres, leading to question whether the latter protein serves as L-lysine exporter in *B. methanolicus* MGA3 (Naerdal *et al.*, 2017).

Based on the presented results for three different methylotrophic bacterial species, several approaches seem to be particularly successful in strain engineering for L-lysine production: (i) expression of the genes encoding for the enzymes relieved from feedback inhibition or introduction of genomic modifications to alleviate the feedback inhibition, (ii) overexpression of genes coding for L-lysine export systems and (iii) deactivation of competing pathways. Furthermore, it seems that overexpression of genes of enzymes of the pathways that are not feedback regulated brings the least positive effects.
L-Lysine can be converted to cadaverine, a monomer for bio-polymer synthesis, in one reaction catalysed by lysine decarboxylase encoded by cadA (Table 1). Cadaverine, called also 1,5-diaminopentane, has a plethora of applications in agriculture, medicine, and industry (Wendisch et al., 2018b). It can be purified from fermentation broth by solvent extraction followed by a subsequent two-step distillation process (Kind et al., 2014). Polymerization of bio-based cadaverine with appropriate bio-blocks, such as succinic acid or sebacic acid yields completely bio-based polyamides PA-5,4 and PA-5,10 respectively (Kind et al., 2014; Yang et al., 2019). Overexpression of E. coli-derived cadA in B. methanolicus wild type resulted in full conversion of L-lysine to cadaverine and accumulation of the latter to a final titre of 6.5 g l\(^{-1}\) in a high cell density methanol-controlled fed-batch fermentation (Table 1), later improved to 10.2 g l\(^{-1}\) through use of stable episomal vector for expression of cadA (Naerdaal et al., 2015; Irla et al., 2016).

5-Aminovalerate (5AVA) is one of the intermediates of different L-lysine degradation pathways. It is a precursor of valerolactam which can be used for the development of novel polyamides (PAs), and can be separated from its precursor, L-lysine, through chromatography (Kim et al., 2020). Evaluation of five pathways for 5AVA biosynthesis in B. methanolicus resulted in the establishment of its production either using activity of lysine \(\alpha\)-oxidase (RaiP) (Table 1) or via a pathway with cadaverine as intermediate composed of CadA, putrescine transaminase (PatA), and 5-aminopentanal dehydrogenase (PatD) (Table 1) (Brito et al., 2021). Initial titre of 0.02 g l\(^{-1}\) for the latter pathway in flask cultivation was increased fourfold through external supplementation with cadaverine (Brito et al., 2021). While B. methanolicus wild type exhibited low tolerance to 5AVA, mutant stains with increased 5AVA tolerance were selected by adaptive laboratory evolution (ALE) (Haupka et al., 2021).

Strains AL119 (derived from M. glycogenes ATCC 21276) and ATR80 (derived from ATCC 21371) produced 11.0 g l\(^{-1}\) and 8.5 g l\(^{-1}\) of L-threonine (Table 1), respectively, in 5-liter jar fermentors at 72 h (Motoyama et al., 1994). While the AKs of ATCC 21276 and ATCC 21371 were sensitive to L-threonine and partially to L-lysine, AK\(^{AL119}\) was completely insensitive to inhibition by L-lysine and its activity was gradually enhanced with increasing concentrations of L-threonine similarity to AK\(^{DHL122}\), whereas AK\(^{ATR80}\) was completely insensitive to inhibition by L-lysine, and partially inhibited by L-threonine (Motoyama et al., 1993b).

The inhibition of the HK\(^{ATR80}\) activity by L-threonine was slightly reduced compared with that of parental wild-type strain, and the DapA of both AL119 and ATR80 were somewhat desensitized to L-lysine inhibition in comparison to parental strains (Motoyama et al., 1993b). The expression of the hom-thr\(^{C}\) genes, encoding homoserine dehydrogenase and threonine synthase (Table 1), respectively, in ATR80 and its L-isoleucine auxotroph, A513, led to up to 12-fold elevated activities of respective enzymes (Motoyama et al., 1994). The hom-thr\(^{C}\) expressing A513 strain produced about 40% more L-threonine in test tube cultivation in comparison to empty vector control with final titre of 16.3 g l\(^{-1}\) after 72 h in 5-liter jar fermentors (Motoyama et al., 1994).

### Engineering cell factories for production of N-methylated amino acids

Amino acids are functionalized, for example, by phosphorylation, acetylation, hydroxylation or halogenation. These modifications may affect either the free amino acid or an amino acid residue in a protein, and they typically alter bioactivity. For example, phosphorylation of free aspartic acid yields aspartyl-phosphate, an activated intermediate of the lysine, methionine and threonine biosynthesis pathways (Wittmann and Becker, 2007). However, specific aspartyl residues in regulatory proteins are phosphorylated to control their activities, for example, the response regulator PhoR of C. glutamicum is activated upon phosphorylation of aspartic acid residue 59 (Kocan et al., 2006).

Alkylation and in particular methylation of the amino group of free or protein-bound amino acids is abundant in nature. For example, N-methylated amino acids are components of secondary metabolites such as the anticancer compound actinomycin D (Mindt et al., 2020), or they have bioactivity themselves, such as the flavour compound of green tea, L-theanine (Benninghaus et al., 2021). In peptide-based drugs, alkylated amino acids provide stabilization against proteolytic attack and they increase lipophilicity for better membrane permeability and pharmacokinetics (Di Gioia et al., 2016), as shown, for example, for the anti-prostate and anti-breast cancer drug leuproide (Haviv et al., 2002).

Due to the incomplete stereoselectivity, use of genotoxic alkylating agents and low yields of chemical synthesis of N-methylated amino acids, enzymatic (Hyslop et al., 2019; Yao et al., 2021) and fermentative (Mindt et al., 2020) routes for their bioproduction have been developed. Three strategies for the fermentative production of N-methylated amino acids (Table 2) will be discussed.

#### Fermentative production by partial transfer of methylimine catabolism

N-Methylglutamate is an intermediate in monomethylamine catabolism of some methylotrophs such as...
**Methylobacterium extorquens.** Assimilation of a C1 compound, methylamine as the sole carbon and nitrogen source by *M. extorquens* involves three specific enzymes (Ochsner et al., 2015). **γ-**Glutamylmethylamide synthetase (GMAS) first methylamidates glutamate at its C5 position before N-methylglutamate synthase (NMGS) transfers the N-methyl group of **γ-**glutamylmethylamide to 2-oxoglutarate yielding glutamate and N-methylglutamate.
Next, *N*-methylglutamate dehydrogenase (NMGDH) catalysed oxidative demethylation of *N*-methylglutamate to glutamate and formaldehyde, the latter being fixed in the serine cycle. Upon expression of the *M. extorquens*-derived GMAS and NMGS genes in the non-methylotrophic *P. putida*, *N*-methylglutamate was produced to about 18 g l\(^{-1}\) in 2-liter bioreactor fed-batch cultivation with a yield of 0.11 g g\(^{-1}\) and a volumetric productivity of about 0.13 g l\(^{-1}\) h\(^{-1}\), if methylvamine was added to the growth medium (Mindt et al., 2018b).

When the C2 compound monoethylamine was provided to one of the non-methylotrophic hosts *E. coli*, *C. glutamicum* and *P. putida* that expressed a GMAS-encoding gene, efficient production of L-theanine resulted with differences in final titres of L-theanine resulting from the supply of precursor, L-glutamate, with extensive genetic work performed for *E. coli*, and only limited changes, deletion of *L*-glutamate exported or overexpression of *gdh*, for *C. glutamicum* and *P. putida*, respectively (Fan et al., 2020; Ma et al., 2020; Benninghaus et al., 2021). The ethylamide L-theanine is the major free amino acid and bioactive component of green tea and it is known for its favourable physiological and pharmacological effects (Vuông et al., 2011). The L-theanine-producing *E. coli* strain, for example, expressed the GMAS-encoding gene from *Paracoccus aminovorans*. It had improved glutamate availability due to overexpression of the endogenous citrate synthase gene, the glutamate dehydrogenase and pyruvate carboxylase genes from *C. glutamicum*, the phosphoenolpyruvate carboxykinase gene from *Mannheimia succiniciproducens*, and a deletion of the succinyl-CoA synthetase genes (Fan et al., 2020). The resulting *E. coli* strain produced about 71 g l\(^{-1}\) L-theanine in a 5-liter bioreactor fed-batch cultivation with a yield of 0.42 g g\(^{-1}\) glucose and a volumetric productivity of about 2.7 g l\(^{-1}\) h\(^{-1}\) (Fan et al., 2020).

Notably, the addition of the C2 compound ethylamine could be circumvented by metabolic engineering of its biosynthesis in *E. coli* (Hagihara et al., 2021). To this end, acetyl-CoA, a central carbon metabolite, was reduced to acetaldehyde by endogenous acetaldehyde dehydrogenase EuuE and the \(\omega\)-transaminase SpuC-II from *P. putida* transferred the amino group of the co-substrate L-alanine to acetaldehyde yielding pyruvate and ethylamine. The resulting *E. coli* strain produced about 16 g l\(^{-1}\) L-theanine without the requirement to add ethylamine to the growth medium (Hagihara et al., 2021).

**Fermentative production via S-adenosyl-L-methionine-dependent alkylation**

*S*-Adenosyl-L-methionine (SAM) is a universal cofactor of cellular metabolism. SAM-dependent methyltransferases that catalyse regioselective methylation reactions and show a defined substrate spectrum have found wide applications in enzyme catalysis (Struck et al., 2012; Zhang and Zheng, 2015). For amino acids, SAM-dependent methylation of *C*, *N* and *O*-atoms has been described, for example, in the synthesis of *N*-methylarginine, an inhibitor of nitric oxide synthase (Stefanovic-Racic et al., 1994), 3-methyl-arginine, a suppressor of bacterial blight of soybean (Braun et al., 2008), and the grape flavours O-methyllyanthranilate and *N*-methyl-O-methyllyanthranilate (Lee et al., 2019). Anthranilate can be methylated to *N*-methyl-O-methyllyanthranilate by sequential reactions of an *N*-methyltransferase and an *O*-methyltransferase (Table 2). The intermediate may be either *N*-methyllyanthranilate or *O*-methyllyanthranilate (Table 2). While only little *N*-methyl-O-methyllyanthranilate was produced by a recombinant *E. coli* strain (Lee et al., 2019), *E. coli* and *C. glutamicum* have recently been engineered for efficient production of O-methyllyanthranilate (Luo et al., 2019). About 5.7 g l\(^{-1}\) O-methyllyanthranilate was produced by a recombinant *C. glutamicum* strain with a yield of 0.02 g g\(^{-1}\) glucose and a volumetric productivity of 0.052 g l\(^{-1}\) h\(^{-1}\), and about 4.5 g L\(^{-1}\) by an *E. coli* strain with a yield and productivity of 0.02 g g\(^{-1}\) glucose and of 0.052 g L\(^{-1}\) h\(^{-1}\) respectively (Luo et al., 2019). To this end, the gene coding for anthranilic acid methyltransferase 1 (AAMT1) from the plant *Zea mays* was expressed in strains that were engineered for overproduction of the immediate precursor anthranilate, an intermediate of L-tryptophan biosynthesis, and for improved regeneration of SAM. Product toxicity was avoided by using a tributyrin overlay as second AAMT1 phase that captured the product O-methyllyanthranilate leading to its in situ extraction which can facilitate downstream purification (Luo et al., 2019). In both strains, accumulation of the precursor, anthranilate, was observed suggesting that the methylation reaction was limiting for formation of O-methyllyanthranilate, either due to arability of co-substrate SAM or activity of AAMT1. The other monomethylated anthranilate, *N*-methyllyanthranilate, is a precursor in plant secondary metabolism leading to acridone alkaloids and avenacin, which have anticancer, cytotoxic and antimicrobial properties relevant for pharmaceutical and therapeutic applications purposes (Rohde et al., 2007). Expression of the gene for *N*-methyltransferase (ANMT) from the plant herb-of-grace *Ruta graveolens* enabled a genome-reduced *C. glutamicum* chassis strain engineered for overproduction of anthranilate as precursor and for improved regeneration of SAM to produce 0.5 g l\(^{-1}\) of *N*-methyllyanthranilate with a yield of about 0.005 g g\(^{-1}\) glucose and a volumetric productivity of 0.01 g l\(^{-1}\) h\(^{-1}\) (Walter et al., 2020). Similarly, in this process an excess of precursor, anthranilate, accumulated suggesting that
methylation was a limiting step of the N-methylanthranilate production.

**Fermentative production via reductive methylation of 2-oxoacids**

2-Oxo acids are converted to the respective amino acids by transamination or reductive amination using ammonium as substrate. The enzyme DpkA from *P. putida* has been described to catalyse reductive alkylation of 2-oxo acids with methylamine or ethylamine instead of ammonium as substrate. In nature, DpkA reduces the imine bond of piperideine-2-carboxylate to yield L-pipecolic acid in D-lysine catabolism (Muramatsu et al., 2005a; 2005b). 2-Oxo acids and methylamine spontaneously form imines that are reduced by DpkA to yield the respective N-methylated amino acids, for example, N-methyl-L-alanine from pyruvate or N-methyl-L-leucine from 2-oxoisocaproate (Mihara et al., 2005).

Expression of *dpkA* in *C. glutamicum* strains engineered to overproduce glyoxylate, pyruvate or phenylpyruvate as 2-oxoacid precursor (Wieschalka et al., 2012; 2013; Zahoor et al., 2014) enabled fermentative production of about 37 g l⁻¹ N-methyl-L-alanine (Mindt et al., 2018a), about 8.7 g l⁻¹ sarcosine (Mindt et al., 2019b), about 1.6 g l⁻¹ N-ethylglycine (Mindt et al., 2019a) and about 0.7 g l⁻¹ N-methyl-L-phenylalanine (Kerbs et al., 2021) upon addition of (m)ethylamine to the growth medium (Table 2). Production of N-methyl-L-phenylalanine did not only require systems metabolic engineering for provision of phenylpyruvate as substrate but also engineering of the enzyme DpkA. Native DpkA from *P. putida* prefers pyruvate over phenylpyruvate, however, upon introduction of the amino acid exchanges P262A and M141L in the substrate binding pocket of DpkA comparable catalytic efficiencies with phenylpyruvate and pyruvate resulted (Kerbs et al., 2021). When the xylose isomerase gene *xylA* from *Xanthomonas campestris* and the endogenous xylulokinase gene *xylB* were expressed, sustainable production of N-methyl-L-phenylalanine from the lignocellulosic pentose sugar xylose to a titre of 0.6 g l⁻¹ with a yield of 0.05 g g⁻¹ xylose was achieved (Kerbs et al., 2021). Further extension of this concept is possible, but has not been realized experimentally.

**Concluding remarks**

In this review, we have presented how the C1 metabolism can be harnessed for the production of amino acids or their methylated derivatives, either by use of methylotrophic cell factories or activity of specific enzymes involved in methylotrophy. Regarding biosynthesis of methylated amino acids, we focused on three strategies relying on the activity of different enzymes or enzymatic cascades (i) GMAS and NMGS derived from methylotrophic *M. extorquens* where they function as part of methylamine assimilation pathway, (ii) ANMT and AAMT derived from plants or (iii) DpkA derived from *P. putida* where it functions in D-lysine degradation. Here, supply of precursors and co-factors, as well as the activity of the biosynthetic enzymes seem to play major roles in the process efficiency, becoming major strain engineering targets. As an outlook, we foresee that the development of methylated amino acids may respond to market needs to a certain extent. N-Methylated amino acids do not only play a role as free bioactives or in peptide drugs, but they may also be co-translationally incorporated into proteins at specific locations by codon engineering (Hoesl and Budisa, 2012). For example, translational amber stop codons have been re-coded using an evolved pyrrolysyl-tRNA synthetase-pylT pair (Blight et al., 2004) to incorporate meta-nitrophenylacetate-photocaged N2-L-lysine residues. Upon photolysis in vivo, the labelled proteins were converted to proteins with monomethylated lysine residues (Wang et al., 2010).

The strategies used for methanol-based production of amino acids by natural methylotrophs generally include use of classical mutagenesis and selection of best-performing strains, or expression of genes encoding feedback inhibition alleviated enzymes or amino acid exporters. In case of non-natural products, such as the diamine cadaverine, or the non-proteinogenic amino acids 5AVA and GABA, expression of heterologous pathways was necessary. Considering that all these compounds are bulk chemicals, with L-glutamate and L-lysine serving as food and feed additives, and cadaverine, 5AVA and GABA as building blocks of polyamines of platform chemicals, it is worthwhile to investigate their methanol-based productions. Methanol is considered a promising raw material for bioprocesses due to its stable prices, easiness of transport and storage and the fact that it can be produced sustainably from non-food sources.

We foresee that the development of new and more efficient processes for production of amino acids from methanol will be driven by a technology push. Specifically, we anticipate that the use of various CRISPR technologies will revolutionize producer strain development (Schultenkamper et al., 2019; 2020). Adaptive laboratory evolution (Hu et al., 2016; Sandberg et al., 2019; Hennig et al., 2020; Wang et al., 2020) and enforcement of production by coupling it to growth (Haupka et al., 2020) will allow for efficient selection procedures of superior strains (Prell et al., 2021). Moreover, development of novel genetic tools will facilitate strain engineering of
methylotrophic production hosts (Irla et al., 2016; Irla et al., 2021). In addition, synthetic consortia of different microorganisms may be developed to divide labour, for example, between conversion of a substrate such as methanol to an intermediate by one microorganism and product formation from the intermediate by another (Sgobba and Wendisch, 2020). In this respect it has to be noted that methanol initially is oxidized to formaldehyde and there are other sources of formaldehyde that may be used as substrates for fermentation. However, formaldehyde has to be liberated from these, for example, by degradation of formaldehyde oligomers such as trioxymethylene and hexamethylenetetramine (Kaszycki and Koloczek, 2002) or by demethylation of vanillin and other methylated aromatic compounds that are present in lignin (Wendisch et al., 2018a; Costa et al., 2021). Albeit attractive, this is clearly unchartered terrain and it is questionable whether these compounds will be available at reasonable cost and quantities.

Taken together, production of amino acids from methanol and production of N-methylated amino acids has seen substantial success. It is anticipated that future developments driven by technology push and/or market demand will shape this existing field of microbial biotechnology.

Funding information

VFW is a self-funded partner in the ERA CoBioTech project MCM4SB (327216).

Conflict of interest

The authors declare no competing financial interest.

References

Ajinomoto (2020) URL https://www.ajinomoto.co.jp/company/en/ir/event/medium_term/main/014/teaserItems1/0/linkList/00/link/Amino%20Science%20Business_E.pdf.

Benninghaus, L., Walter, T., Mindt, M., Risseur, J.M., and Wendisch, V.F. (2021) Metabolic engineering of Pseudomonas putida for fermentative production of L-theanine. J Agric Food Chem 69: 9849–9858.

Blight, S.K., Larue, R.C., Mahapatra, A., Longstaff, D.G., Chang, E., Zhao, G., et al. (2004) Direct charging of tRNA_{CUA} with pyrrolysine in vitro and in vivo. Nature 431: 333–335.

Brautaset, T., Jakobsen, O.M., Degnes, K.F., Netzer, R., Naerdal, I., Krog, A., et al. (2010) Bacillus methanolicus pyruvate carboxylase and homoserine dehydrogenase I and II and their roles for L-lysine production from methanol at 50 degrees C. Appl Microbiol Biotechnol 87: 951–964.

Brautaset, T., Williams, M.D., Dillingham, R.D., Kaufmann, C., Bennaars, A., Crabbe, E., and Flickinger, M.C. (2003) Role of the Bacillus methanolicus citrate synthase II gene, citY, in regulating the secretion of glutamate in L-lysine-secreting mutants. Appl Environ Microbiol 69: 3986–3995.

Brito, L.F., Irla, M., Naerdal, I., Le, S.B., Delepine, B., Heux, S., and Brautaset, T. (2021) Evaluation of heterologous biosynthetic pathways for methanol-based 5-aminovalerate production by thermophilic Bacillus methanolicus. Front Bioeng Biotechnol 9: 868319.

Capitani, G., De Biase, D., Aurizi, C., Gut, H., Bossa, F., and Grüter, M.G. (2003) Crystal structure and functional analysis of Escherichia coli glutamate decarboxylase. EMBO J 22: 4027–4037.

Carlsson, P., and Hederstedt, L. (1986) In vitro complementation of Bacillus subtilis and Escherichia coli 2-oxoglutarate dehydrogenase complex mutants and genetic mapping of B. subtilis citK and citM mutations. FEMS Microbiol Lett 37: 373–378.

Chistoserdova, L., Kaluzhnyaya, M.G., and Lidstrom, M.E. (2009) The expanding world of methylotrophic metabolism. Annu Rev Microbiol 63: 477–499.

Costa, C.A.E., Vega-Aguilar, C.A., and Rodrigues, A.E. (2021) Added-value chemicals from lignin oxidation. Molecules 26: 4602.

Delepêne, B., Gil López, M., Carnicer, M., Vicente, C.M., Wendisch, V.F., and Heux, S. (2020) Charting the metabolic landscape of the facultative methylotroph Bacillus methanolicus. mSystems 5: e00745–00720.

Di Gioia, M.L., Leggio, A., Malagrinò, F., Romio, E., Siciliano, C., and Liguori, A. (2016) N-methylated alpha-amino acids and peptides: synthesis and biological activity. Mini Rev Med Chem 16: 683–690.

Drejer, E.B., Chan, D.T.C., Haupka, C., Wendisch, V.F., Brautaset, T., and Irla, M. (2020) Methanol-based acetoin production by genetically engineered Bacillus methanolicus. Green Chem 22: 788–802. doi: https://doi.org/10.1039/C9GC03950C

Eggeling, L. (2007) t-Serine and glycine. In Amino Acid Biosynthesis – Pathways, Regulation and Metabolic Engineering. Wendisch, V.F. (ed.). Berlin, Heidelberg, Germany: Springer Berlin Heidelberg, pp. 259–272.

Fan, L.Q., Li, M.W., Qiu, Y.J., Chen, Q.M., Jiang, S.J., Shang, Y.J., and Zhao, L.M. (2018) Increasing thermal stability of glutamate decarboxylase from Escherichia coli by site-directed saturation mutagenesis and its application in GABA production. J Biotechnol 278: 1–9.

Fan, X., Zhang, T., Ji, Y., Li, J., Long, K., Yuan, Y., et al. (2020) Pathway engineering of Escherichia coli for one-step fermentative production of L-theanine from sugars and ethylamine. Metab Eng Commun 11: e00151.

Frank, C., Hoffmann, T., Zelder, O., Felle, M.F., and Bremer, E. (2021) Enhanced glutamate synthesis and export by the thermotolerant emerging industrial workhorse Bacillus methanolicus in response to high osmolality. Front Microbiol 12: 640980.

Fukuda, Y., and Sasanuma, Y. (2018) Computational characterization of nylon 4, a biobased and biodegradable
polyamide superior to nylon 6. ACS Omega 3: 9544–9555.

Gao, Q., Duan, Q., Wang, D., Zhang, Y., and Zheng, C. (2013) Separation and purification of gamma-aminobutyric acid from fermentation broth by flocculation and chromatographic methodologies. J Agric Food Chem 61: 1914–1919.

Gunji, Y., Tsujimoto, N., Shimaoka, M., Ogawa-Miyata, Y., Sugimoto, S., and Yasueda, H. (2004) Characterization of the L-lysine biosynthetic pathway in the obligate methylotroph Methylobacterium methylophilus. Biosci Biotechnol Biochem 68: 1449–1460.

Gunji, Y., and Yasueda, H. (2006) Enhancement of L-lysine production in methylotroph Methylobacterium methylophilus by introducing a mutant LysE exporter. J Biotechnol 127: 1–13.

Hagihara, R., Ohno, S., Hayashi, M., Tabata, K, and Endo, H. (2021) Production of l-Theanine by Escherichia coli in the absence of supplemental ethylamine. Appl Environ Microbiol 87: e00031-21.

Haider, M.H., Dummer, N.F., Knight, D.W., Jenkins, R.L., Howard, M., Moulijn, J., et al. (2015) Efficient green methanol synthesis from glycerol. Nat Chem 7: 1028–1032.

Hanson, R., Dillingham, R., Olson, P., Lee, G., Cue, D., Schendel, F., et al. (1996) Production of L-lysine and some other amino acids by mutants of B. methanolicus. In Microbial Growth on C1 Compounds. Lidstrom, M.E., and Tabita, F.R (eds.) Dordrecht: Kluwer Academic Publishers, pp. 227–236.

Haupka, C., Brito, L.F., Busche, T., Wibberg, D., and Wendisch, V.F. (2021) Genomic and transcriptomic investigation of the physiological response of the methylotroph Bacillus methanolicus to 5-aminolevulinate. Front Microbiol 12: 664598.

Haupka, C., Delepine, B., Irla, M., Heux, S., and Wendisch, V.F. (2020) Flux enforcement for fermentative production of 5-aminolevulinate and glutarate by Corynebacterium glutamicum. Catalysts 10: 1065.

Haviv, F., Fitzpatrick, T.D., Swenson, R.E., Nichols, C.J., Mort, N.A., Bush, E.N., et al. (2002) Effect of N-methyl substitution of the peptide bonds in luteinizing hormone-releasing hormone agonists. J Med Chem 36: 363–369.

Heggeset, T.M., Krog, A., Balzer, S., Wentzel, A., Ellingsen, T.E., and Brautaset, T. (2012) Genome sequence of the thermotolerant Bacillus methanolicus: features and regulation related to methylotrophy and production of L-lysine and L-glutamate from methanol. Appl Environ Microbiol 78: 5170–5181.

Hennig, G., Haupka, C., Brito, L.F., Ruckert, C., Cahoreau, E., Heux, S., and Wendisch, V.F. (2020) Methanol-essential growth of Corynebacterium glutamicum: adaptive laboratory evolution overcomes limitation due to methanethiol assimilation pathway. Int J Mol Sci 21: 3617.

Hoels, M.G., and Budisa, N. (2012) Recent advances in genetic code engineering in Escherichia coli. Curr Opin Biotechnol 23: 751–757.

Hu, B., Yang, Y.M., Beck, D.A., Wang, Q.W., Chen, W.J., Yang, J., et al. (2016) Comprehensive molecular characterization of Methylobacterium extorquens AM1 adapted for 1-butanol tolerance. Biotechnol Biofuels 9: 84.

Hyslop, J.F., Lovelock, S.L., Watson, A.J.B., Sutton, P.W., and Roiban, G.D. (2019) N-Alkyl-alpha-amine acids in Nature and their biocatalytic preparation. J Biotechnol 293: 56–65.

Irla, M., Hakvag, S., and Brautaset, T. (2021) Developing a riboswitch-mediated regulatory system for metabolic flux control in thermophilic Bacillus methanolicus. Int J Mol Sci 22: 4686.

Irla, M., Heggeset, T.M., Naerdal, I., Paul, L., Haugen, T., Le, S.B., et al. (2016) Genome-based genetic tool development for Bacillus methanolicus: theta- and rolling circle-replicating plasmids for inducible gene expression and application to methanol-based cadaverine production. Front Microbiol 7: 1481.

Irla, M., Naerdal, I., Brautaset, T., and Wendisch, V.F. (2017) Methanol-based γ-aminobutyric acid (GABA) production by genetically engineered Bacillus methanolicus strains. Ind Crops Prod 106: 12–20. doi: https://doi.org/10.1016/j.indcrop.2016.11.050.

Ishikawa, K., Asahara, T., Gunji, Y., Yasueda, H., and Asano, K. (2008a) Disruption of metF increased L-lysine production by Methylobacterium methylophilus from methanol. Biosci Biotechnol Biochem 72: 1317–1324.

Ishikawa, K., Toda-Murakoshi, Y., Ohnishi, F., Kondo, K., Osumi, T., and Asano, K. (2008b) Medium composition suitable for L-lysine production by Methylobacterium methylophilus in fed-batch cultivation. J Biosci Bioeng 106: 574–579.

Jakobsen, O.M., Brautaset, T., Degnes, K.F., Heggeset, T.M., Balzer, S., Fleckinger, M.C., et al. (2009) Overexpression of wild-type aspartokinase increases L-lysine production in the thermotolerant methylotrophic bacterium Bacillus methanolicus. Appl Environ Microbiol 75: 652–661.

Jenkins, O., Byrom, D., and Jones, D. (1987) Methylophilus: a new genus of methanol-utilizing bacteria. Int J Syst Evol Microbiol 37: 446–448.

Kaszycki, P., and Koloczek, H. (2002) Biodegradation of formaldehyde and its derivatives in industrial wastewater with methylotrophic yeast Hansenula polymorpha and with the yeast-bioaugmented activated sludge. Biodegradation 13: 91–99.

Kerbs, A., Mindt, M., Schwarmann, L., and Wendisch, V.F. (2021) Sustainable production of N-methylphenylalanine by reductive methylation of phenylpyruvate using engineered Corynebacterium glutamicum. Microorganisms 9: 824.

Kim, S., Ahn, J.O., Kim, K.-M., and Lee, C.-H. (2020) Effects of the mobile phase on the chromatographic separation of L-lysine and 5-aminolevulinic acid. Microchem J 152: 104369.

Kind, S., Neubauer, S., Becker, J., Yamamoto, M., Volkert, M., Abendroth, G., et al. (2014) From zero to hero - production of bio-based nylon from renewable resources using engineered Corynebacterium glutamicum. Metab Eng 25: 113–123.

Kocan, M., Schaffer, S., Ishige, T., Sorgo-Herrmann, U., Wendisch, V.F., and Bott, M. (2006) Two-component systems of Corynebacterium glutamicum: deletion analysis and involvement of the PhoS-PhoR system in the phosphate starvation response. J Bacteriol 188: 724–732.
gene, encoding a mechanosensitive channel homolog, induce L-glutamic acid production. *Appl Environ Microbiol* 73: 4491–4498.

Nakayama, Y., Becker, M., Ebrahimian, H., Konishi, T., Kawakazi, H., Kramer, R., and Martinac, B. (2016) The impact of the C-terminal domain on the gating properties of MscCG from *Corynebacterium glutamicum*. *Biochim Biophys Acta* 1858: 130–138.

Nakayama, Y., Komazawa, K., Bavi, N., Hashimoto, K.I., Kawakazi, H., and Martinac, B. (2018) Evolutionary specialization of MscCG, an MscS-like mechanosensitive channel, in amino acid transport in *Corynebacterium glutamicum*. *Sci Rep* 8: 12893.

Ochsner, A.M., Sonntag, F., Buchhaupt, M., Schrader, J., and Vorholt, J.A. (2015) *Methylbacterium extorquens*: methylotrophy and biotechnological applications. *Appl Microbiol Biotechnol* 99: 517–534.

Park, S.H., Kim, H.U., Kim, T.Y., Park, J.S., Kim, S.S., and Lee, S.Y. (2014) Metabolic engineering of *Corynebacterium glutamicum* for L-arginine production. Nat Commun 5: 4618.

Pérez-García, F., Brito, L.F., and Wendisch, V.F. (2019) Function of L-pipeolic acid as compatible solute in *Corynebacterium glutamicum* as basis for its production under hyperosmolar conditions. *Front Microbiol* 10: 340.

Pérez-García, F., Peters-Wendisch, P., and Wendisch, V.F. (2016) Engineering *Corynebacterium glutamicum* for fast production of L-lysine and L-pipeolic acid. *Appl Microbiol Biotechnol* 100: 8075–8090.

Pérez-García, F., Rissé, J.M., Friehs, K., and Wendisch, V.F. (2017) Fermentative production of L-pipeolic acid from glucose and alternative carbon sources. *Biotechnol J* 12: 1600646.

Prel, C., Busche, T., Ruckert, C., Nolte, L., Brandenbusch, C., and Wendisch, V.F. (2021) Adaptive laboratory evolution accelerated glutarate production by *Corynebacterium glutamicum*. *Microb Cell Fact* 20: 97.

Rohde, B., Hans, J., Martens, S., Baumert, A., Hunziker, P., and Matern, U. (2007) Anthranilate N-methyltransferase, a branch-point enzyme of acridone biosynthesis. *Plant J* 53: 541–553.

Sandberg, T.E., Salazar, M.J., Weng, L.L., Palsson, B.O., and Feist, A.M. (2019) The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. *Metab Eng* 56: 1–16.

Schendel, F.J., Dillingham, R., Hanson, R.S., Sano, K., and Matsui, K. (2000) Production of glutamate using wild type Bacillus methanolicus. United States patent application 08/953,265.

Schultenkämper, K., Brito, L.F., López, M.G., Brautaset, T., and Wendisch, V.F. (2019) Establishment and application of CRISPR interference to affect sporulation, hydrogen peroxide detoxification, and mannitol catabolism in the methylotrophic thermophile *Bacillus methanolicus*. *Appl Microbiol Biotechnol* 103: 5879–5889.

Schultenkämper, K., Brito, L.F., and Wendisch, V.F. (2020) Impact of CRISPR interference on strain development in biotechnology. *Biotechnol Appl Biochem* 67: 7–21.

Sgobba, E., and Wendisch, V.F. (2020) Synthetic microbial consortia for small molecule production. *Curr Opin Biotechnol* 62: 72–79.
bacteria: recent progress, applications, and perspectives. Appl Microbiol Biotechnol 102: 3583–3594.
Wendisch, V.F., Nampoothiri, K.M., and Lee, J.-H. (2022) Metabolic engineering for valorization of agri-and aquaculture sidestreams for production of nitrogenous compounds by Corynebacterium glutamicum. Frontiers in Microbiology 13: 835131. doi: https://doi.org/10.3389/fmicb.2022.835131
Wieschalka, S., Blombach, B., and Eikmanns, B.J. (2012) Engineering Corynebacterium glutamicum for the production of pyruvate. Appl Microbiol Biotechnol 94: 449–459.
Wieschalka, S., Blombach, B., Bott, M., and Eikmanns, B.J. (2013) Bio-based production of organic acids with Corynebacterium glutamicum. Microb Biotecnol 6: 87–102.
Wittmann, C., and Becker, J. (2007) The L-lysine story: from metabolic pathways to industrial production. In Amino Acid Biosynthesis – Pathways, Regulation and Metabolic Engineering. Wendisch, V.F. (ed). Heidelberg, Germany: Springer, pp. 39–70.
Yang, P., Li, X., Liu, H., Li, Z., Liu, J., Zhuang, W., et al. (2019) Thermodynamics, crystal structure, and characterization of a bio-based nylon 54 monomer. CrystEngComm 21: 7069–7077.
Yao, P., Marshall, J.R., Xu, Z., Lim, J., Charnock, S.J., Zhu, D., and Tumer, N.J. (2021) Asymmetric synthesis of N-substituted α-amino esters from α-ketoesters via imine reductase-catalyzed reductive amination. Angew Chem Int Ed 60: 8717–8721.
Zahoor, A., Otten, A., and Wendisch, V.F. (2014) Metabolic engineering of Corynebacterium glutamicum for glycolate production. J Biotechnol 192(Pt B): 366–375.
Zhang, C., Li, Y., Ma, J., Liu, Y., He, J., Li, Y., et al. (2018) High production of 4-hydroxyisoleucine in Corynebacterium glutamicum by multistep metabolic engineering. Metab Eng 49: 287–298.
Zhang, J., and Zheng, Y.G. (2015) SAM/SAH analogs as versatile tools for SAM-dependent methyltransferases. ACS Chem Biol 11: 583–597.

© 2022 The Authors. Microbial Biotechnology published by Society for Applied Microbiology and John Wiley & Sons Ltd., Microbial Biotechnology, 15, 2145–2159