Minireview

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

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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.

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Introduction

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 (million 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 (2*S*, 3*R*, 4*S*)-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-pipecolic 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 CO_2 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 methylotrophic 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).

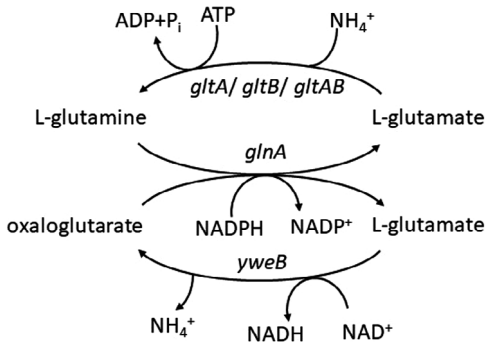
The mechanism of L-glutamate secretion in *B. methanolicus* is still not elucidated. In *C. glutamicum*, a known microbial L-glutamate producer, MscCG, a MscS-like channel, is the major L-glutamate export system (Nakamura *et al*., 2007; Nakayama *et al*., 2016; 2018; Wang *et al*., 2018). No homologues 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-pyrrolidone 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 thermolability 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

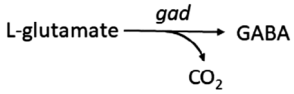
Table 1. Production of amino acids from methanol by fermentation. Characteristic of production strains and titres of illustrative processes are listed. For abbreviations see either text or below.

Glutamate production



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	-	0.8/ 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 [g l^{-1}]	Fermentation mode	References for established processes
B. methanolicus MGA3	Wild type	*gad*St	0.35/ 13.3	Shake flask/ Fed-batch	Irla *et al.* (2017)

Lysine production

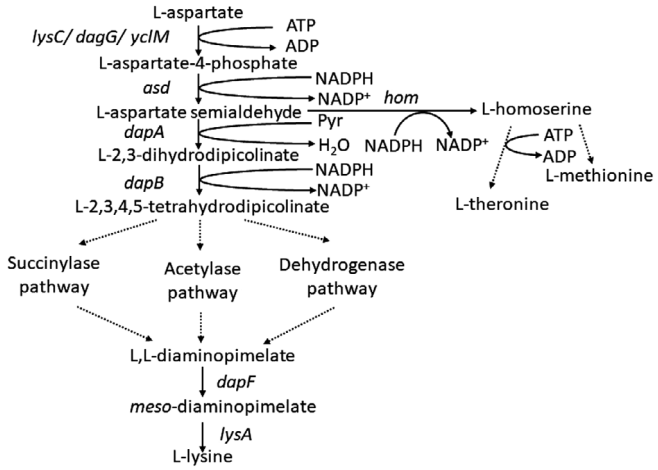
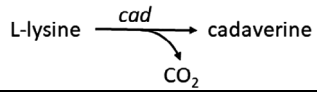


Table 1. (Continued)

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); Naerdal *et al.* (2011)
B. methanolicus MGA3	Wild type	*dapG*	0.01/ 0.38	Shake flask	Jakobsen *et al.* (2009); Naerdal *et al.* (2011)
B. methanolicus MGA3	Wild type	*lysC*	0.06/ 1.8	Shake flask/ Fed-batch	Jakobsen *et al.* (2009); Naerdal *et al.* (2011)
B. methanolicus MGA3	Wild type	*yclM*	0.14/ 11	Shake flask/ Fed-batch	Jakobsen *et al.* (2009); Naerdal *et al.* (2011)
B. methanolicus MGA3	Wild type	$dapG^{fbr}$	0.12	Shake flask	Naerdal *et al.* (2011)
B. methanolicus MGA3	Wild type	*asd*	0.01	Shake flask	Naerdal *et al.* (2011)
B. methanolicus MGA3	Wild type	*dapA*	0.01	Shake flask	Naerdal *et al.* (2011)
B. methanolicus MGA3	Wild type	*lysA*	0.15	Shake flask	Naerdal *et al.* (2011)
B. methanolicus MGA3	Wild type	*dapA-yclM*	0.21	Shake flask	Naerdal *et al.* (2011)
B. methanolicus MGA3	Wild type	*dapA-yclM-lysA*	0.58	Shake flask	Naerdal *et al.* (2011)
B. methanolicus MGA3	Wild type	$lysC\text{-}lysE^{Cg}$	0.38	Shake flask	Naerdal *et al.* (2017)
B. methanolicus M168-20	hom^{-}	–	0.15/ 11.0	Shake flask/ Fed-batch	Brautaset *et al.* (2010)
B. methanolicus M168-20	hom^{-}	*asd*	0.28	Shake flask	Naerdal *et al.* (2011)
B. methanolicus M168-20	hom^{-}	*dapA*	0.70	Shake flask	Naerdal *et al.* (2011)
B. methanolicus M168-20	hom^{-}	*dapA-yclM*	0.66	Shake flask	Naerdal *et al.* (2011)
B. methanolicus M168-20	hom^{-}	$lysE^{Cg}$	0.25	Shake flask	Naerdal *et al.* (2017)
B. methanolicus NOA2#13A52-8A66	$dapG^{fbr}$ $hom^{-}P_{lysA}{}^{mut}$	-	65	Fed-batch	Brautaset *et al.* (2010)
M. methylotrophus AS1	Wild type	-	<0.01	Test tube	Tsujimoto *et al.* (2006)
M. methylotrophus AS1	Wild type	$lysE^{Cg24}$	0.08	Shake flask	Gunji and Yasueda (2006)
M. methylotrophus AS1	Wild type	$dapA^{24}$	0.1	Shake flask	Gunji and Yasueda (2006)
M. methylotrophus AS1	Wild type	$lysE^{Cg24}$-$dapA^{24}$	1.0/ 11.3	Shake flask/ Jar fermentor	Gunji and Yasueda (2006)
M. methylotrophus G49	Asd^{fbr}, $DapA^{fbr}$	-	0.08	Test tube	Tsujimoto *et al.* (2006)
M. methylotrophus G49	Asd^{fbr}, $DapA^{fbr}$	$dapA^{Ec24}$-$lysC^{Ec80}$-$dapB^{Ec}$	0.4/ 1.0	Test tube/ Jar fermentor	Tsujimoto *et al.* (2006)
M. methylotrophus 102	$MetF^{-}$	$lysE^{Cg24}$-$dapA^{24}$	1.2/ 9.0	Shake flask/ Jar fermentor	Ishikawa *et al.* (2008a); Ishikawa *et al.* (2008b)
M. glycogenes AL119	AK^{fbr}, $DapA^{fbr}$	-	0.4	Test tube	Motoyama *et al.* (2001)
M. glycogenes AL119	AK^{fbr}, $DapA^{fbr}$	$dapA^{DHL122}$	1.1/ 8.0	Test tube/ Jar fermentor	Motoyama *et al.* (2001)
M. glycogenes DHL122	AK^{fbr}, $DapA^{fbr}$	-	0.6/ 3.1	Test tube/ Jar fermentor	Motoyama *et al.* (1993a); Motoyama *et al.* (2001)
M. glycogenes DHL122	AK^{fbr}, $DapA^{fbr}$	$dapA^{DHL122}$	1.2/ 5.3	Test tube/ Jar fermentor	Motoyama *et al.* (2001)

Cadaverine production



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

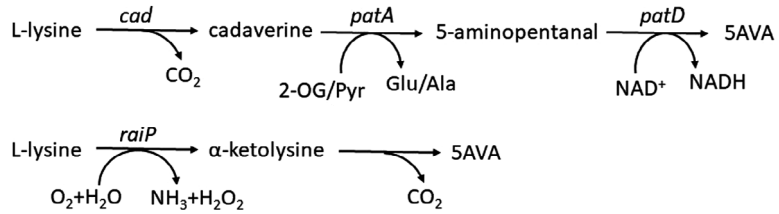
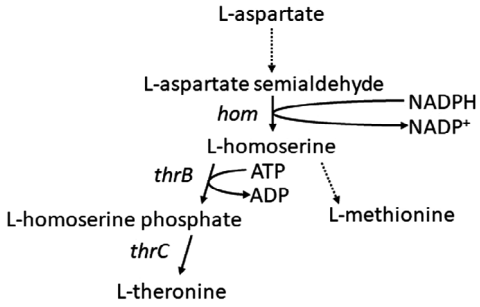


Table 1. (Continued)

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)

Threonine production



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^{fbr}, $DapA^{fbr}$	-	11.0	Jar fermentor	Motoyama *et al.* (1993b)
M. glycogenes ATR80	AK^{fbr}, HK^{fbr} $DapA^{fbr}$	-	8.5	Jar fermentor	Motoyama *et al.* (1993b)
M. glycogenes TR80	AK^{fbr} HK^{fbr} $DapA^{fbr}$	*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.

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-hydroxy-tetrahydrodipicolinate 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).

Methylophilus methylotrophus AS1 is an obligate methylotroph with a ribulose monophosphate (RuMP) pathway for formaldehyde assimilation (Jenkins *et al.*, 1987; Gunji *et al.*, 2004). *M. methylotrophus* 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).

The mutant DHL122 derived from *M. glycogenes* 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^{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 $DapA^{DHL122}$ 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 $DapA^{DHL122}$ 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 $dapA^{DHL122}$ 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 *yclM* 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 $dapA^{Ec24}$ and $lysC^{Ec80}$ encoding enzymes with reduced sensitivity to feedback inhibition and wild-type version of dihydrodipicolinate reductase gene ($dapB^{Ec}$) 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 $lysE^{Cg24}$ 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 $lysE^{Cg24}$ with $dapA^{24}$ 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-methylenetetrahydrofolate reductase gene (*metF*) overexpressing $lysE^{Cg24}$ and $dapA^{24}$ 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 *thrB* (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 $lysE^{Cg}$ 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 Θ-replication vector for expression of *cadA* (Naerdal *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 α-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 fementors at 72 h (Motoyama *et al*., 1993a). 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 similarly 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-thrC* 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-thrC* 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 leuprolide (Haviv *et al*., 2002).

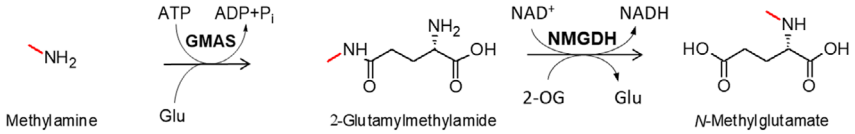
Due to the incomplete stereoselectivity, use of genotoxic alkylating agents and low yields of chemical synthesis of *N*-methylated amino acids, enzymatic (Hyslop *et al*., 2019; Yao *et al*., 2021) and fermentative (Mindt *et al*., 2020) routes for their bioproduction have been developed. Three strategies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

Fermentative production by partial transfer of methylamine catabolism

N-Methylglutamate is an intermediate in monomethylamine catabolism of some methylotrophs such as

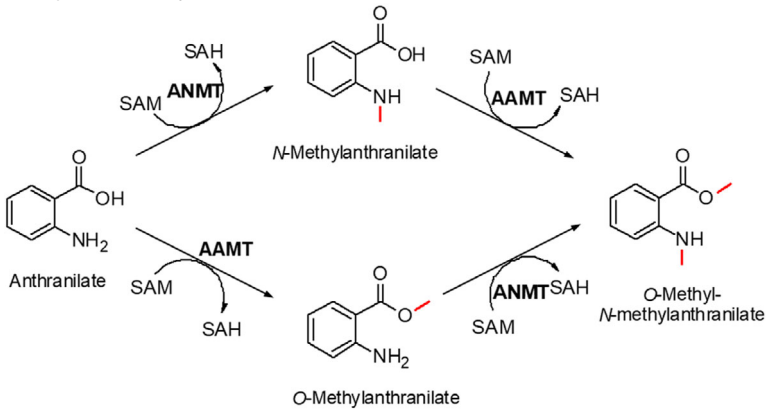
Table 2. Production of *N*-methylated amino acids by fermentation. Key enzymatic reactions of reductive methylamination, SAM-dependent methylation and partial transfer of methylamine catabolism are depicted. Titres, yields and productivities of illustrative processes are listed. For abbreviations see text.

Partial transfer of methylamine catabolism



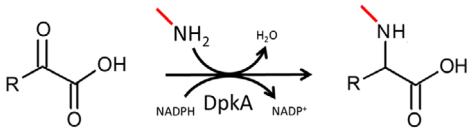
Product	Titre [g l^{-1}]	Yield [g g^{-1}]	Productivity [g l^{-1} h^{-1}]	Fermentation mode	References for established processes
N-methyl-L-glutamate	17.9	0.11	0.13	Fed-batch	Mindt *et al.* (2018b)
L-Theanine					
by *E. coli*	70.6	0.42	2.72	Fed-batch	Fan *et al.* (2020)
by *C. glutamicum*	42.0	0.20	0.88	Fed-batch	Ma *et al.* (2020)
by *P. putida*	21.0	0.03	0.38	Fed-batch	Benninghaus *et al.* (2021)

SAM-dependent methylation



Product	Titre [g l^{-1}]	Yield [g g^{-1}]	Productivity [g l^{-1} h^{-1}]	Fermentation mode	References for established processes
N-methyl-anthranilate	0.5	0.005	0.01	Fed-batch	Walter *et al.* (2020)
O-methyl-anthranilate					
by *C. glutamicum*	5.7	0.02	0.05	Fed-batch	Luo *et al.* (2019)
by *E. coli*	4.5	0.05	0.06	Fed-batch	Luo *et al.* (2019)

Reductive methylamination



Product	Titre [g l^{-1}]	Yield [g g^{-1}]	Productivity [g l^{-1} h^{-1}]	Fermentation mode	Reference
N-methyl-L-alanine	31.7	0.71	0.35	Fed-batch	Mindt *et al.* (2018a)
Sarcosine	9.1	0.26	0.16	Shake flask	Mindt *et al.* (2019b)
N-ethylglycine	6.1	0.17	0.11	Shake flask	Mindt *et al.* (2019a)
N-methyl-L-phenylalanine	0.7	0.05	0.01	Shake flask	Kerbs *et al.* (2021)

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

(Table 2). 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} glycerol and a volumetric productivity of about 0.13 g l^{-1} h^{-1}, if methylamine 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 (Vuong *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 EutE and the ω-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*-methylanthranilate and *N*-methyl-*O*-methylanthranilate (Lee *et al.*, 2019). Anthranilate can be methylated to *N*-methyl-*O*-methylanthranilate by sequential reactions of an *N*-methyltransferase and an *O*-methyltransferase (Table 2). The intermediate may be either *N*-methylanthranilate or *O*-methylanthranilate (Table 2). While only little *N*-methyl-*O*-methylanthranilate 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*-methylanthranilate (Luo *et al.*, 2019). About 5.7 g l^{-1} *O*-methylanthranilate 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*-methylanthranilate 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*-methylanthranilate, either due to arability of co-substrate SAM or activity of AAMT1. The other monomethylated anthranilate, *N*-methylanthranilate, 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*-methytransferase (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*-methylanthranilate 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 methylamination 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 alkylamination 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 *N*ε-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 exiting field of microbial biotechnology.

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Conflict of interest

The authors declare no competing financial interest.

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