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Metabolic engineering advances and prospects for amino acid production

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A B S T R A C T

Amino acid fermentation is one of the major pillars of industrial biotechnology. The multi-billion USD amino acid market is rising steadily and is diversifying. Metabolic engineering is no longer focused solely on strain development for the bulk amino acids L-glutamate and L-lysine that are produced at the million-ton scale, but targets specialty amino acids. These demands are met by the development and application of new metabolic engineering tools including CRISPR and biosensor technologies as well as production processes by enabling a flexible feedstock concept, co-production and co-cultivation schemes. Metabolic engineering advances are exemplified for specialty proteinogenic amino acids, cyclic amino acids, omega-amino acids, and amino acids functionalized by hydroxylation, halogenation and *N*-methylation.

1. Introduction

Amino acid production by fermentation is a success story of biotechnology that started in Japan more than 50 years ago (Hashimoto, 2017; Nakamori, 2017) and developed into a business of an estimated US$19.5 billion in 2017 (Industry-Experts, 2019). Strain development for amino acid production was at the technological forefront throughout the classical (Leuchtenberger et al., 2005; Ohnishi et al., 2008), genetic engineering (Ikeda, 2003; Kirchner and Tauch, 2003), systems biology (Ma et al., 2017; Takors et al., 2007; Wendisch et al., 2006), synthetic biology (Lee and Wendisch, 2017; Wendisch, 2014), and systems metabolic engineering eras (Becker and Wittmann, 2012; Contador et al., 2009; Lee and Wendisch, 2017). This review focusses on metabolic engineering advances for the production of alpha-amino acids, omega-amino acids, cyclic amino acids and functionalized amino acids. The reader is referred to other reviews regarding metabolic engineering for the production of ketoacids as amino acid precursors (Wieschalka et al., 2013), amino acid derived amines (Kind and Wittmann, 2011; Wendisch et al., 2016b, 2018;), dipeptides (Yagasaki and Hashimoto, 2008) as well as regarding regulation, process intensification and downstream processing (Hermann, 2003; Ikeda, 2003; Lopez-Garzon and Straathof, 2014; Pfefferle et al., 2003; Roberts, 2016) or technoeconomic evaluation (Chen et al., 2019; Mueller and Huebner, 2003).

Amino acids can also be obtained by extraction from natural sources, e.g. L-cysteine from human hair (Joo et al., 2017a,b), or by chemical synthesis, e.g. synthesis of DL-methionine from methyl mercaptan, acrolein and hydrogen cyanide (Willke, 2014), a process that dominates the 4 billion USD DL-methionine market. Enzyme catalysis is the option of choice for some amino acids such as L-aspartic acid which is obtained from fumaric acid by means of L-aspartate ammonia lyase (Fusee et al., 1981; Gröger, 2019).

2. Uses of AA and market volumes

Worldwide demand for amino acids is expected to grow with a compound annual growth rate of 5.6% over 2017–2022 and will reach US$25.6 billion by 2022. Animal feed amino acids is the largest and fastest growing market for amino acids globally (growing by 6.9% from 2017 to 2022 to US$10.4 billion by 2022) (Industry-Experts, 2019).

The global amino acids market may see some changes in the near future. The chemical production process for racemic DL-methionine has experienced large expansion (Table 1) as e.g. Evonik doubled its production capacity in Singapore to 300,000 metric tons per year (Evonik, 2019a). However, it may face challenges from processes yielding the optically pure stereoisomer L-methionine. With an annual capacity of 90.000 tons, CJ runs a two-step process in which first acetylated or succinylated homoserine is produced by fermentation and then enzymatically converted to L-methionine with methyl mercaptan/methanethiol (Shim et al., 2017). On the other hand Metabolic Explorer has developed a *Corynebacterium glutamicum* L-methionine fermentation process which has been acquired by Evonik (Evonik, 2019b). Although both stereoisomers of racemic methionine can be utilized by animals and humans that have D-amino acid oxidase and L-specific transaminase, the optically pure stereoisomer L-methionine has 25% higher nutritional potency than racemic DL-methionine (Koga et al., 2017).

Another trend among the leading amino acid producing companies is the shift towards specialized amino acids. The “economy of scale” of the very efficient L-glutamate and L-lysine production processes meanwhile only compensates for the falling market prices. For example, although the demand for L-lysine and L-glutamate is rising, Ajinomoto K. K., the world market leader of amino acid production, has announced

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Table 1
Annual global production of amino acids and nucleotides with types of preferred production processes. Adapted and updated from (Ajinomoto, 2019a,b; Evonik, 2019a,b; Industry-Experts, 2019; Nikkei, 2019; Wendisch et al., 2015).

SUBSTANCE	PREDOMINANT PROCESS(ES)	ANNUAL GLOBAL PRODUCTION (METRIC TONS)
L-ALANINE	Enzymatic, extraction	500
L-ARGININE	Fermentation,	1200
L-ASPARAGINE	Extraction	100-1000
L-ASPARTIC ACID	Enzymatic	10,000
L-CYSTEINE	Enzymatic, extraction	3000
L-GLUTAMINE	Fermentation	1300
L-GLUTAMIC ACID	Fermentation	**3,210,000**
GLYCINE	Chemical	22,000
L-HISTIDINE	Fermentation	400
L-ISOLEUCINE	Fermentation	400
L-LEUCINE	Fermentation, extraction	500
L-LYSINE	Fermentation	**2,600,000**
DL-METHIONINE	Chemical; fermentation + biotransformation for L-METHIONINE	**1,100,000**
L-PHENYLALANINE	Fermentation, chemical	12,650
L-PROLINE	Fermentation	350
L-SERINE	Fermentation	350
L-THREONINE	Fermentation	**700,000**
L-TRYPTOPHAN	Enzymatic, fermentation	**41,000**
L-TYROSINE	Fermentation, extraction	165
L-VALINE	Fermentation	500
L-SELENOCYSTEINE	No process described for the 21st proteinogenic amino acid	
L-PYRROLYSINE	No process described for the 22nd proteinogenic amino acid	

to outsource production of these commodity amino acids in order to focus on production of specialty amino acids (Ajinomoto, 2019a,b). Thus, these are excellent times for metabolic engineering of *C. glutamicum* and *Escherichia coli* strains to overproduce, for example, L-arginine (Park et al., 2014a,b), branched-chain amino acids (Holatko et al., 2009; Vogt et al., 2014) or non-proteinogenic amino acids such as L-pipecolic acid (Perez-Garcia et al., 2016, 2017a). These chances are timely met by new developments regarding tools for engineering amino acid producing strains.

3. Tools development

Metabolic engineering of amino acid producing strains embraced new tools as soon as they emerged and helped to develop them further. Selected examples of using CRISPR technologies, flux enforcement and genetically encoded biosensors to improve amino acid production are discussed (Table 2).

3.1. CRISPR tools for genome editing and gene repression

The first application of the CRISPR technique to amino acid strain development has been described for gene repression by CRISPR interference (Wiedenheft et al., 2012) in *C. glutamicum* with respect to L-lysine production (Cleto et al., 2016). Before, synthetic small RNAs found application for gene repression in order to improve amino acid production (L-tyrosine production by *E. coli*), a technique that cannot be applied to *C. glutamicum* which lacks RNA chaperon Hfq (Na et al., 2013). Multiplexing of CRISPR mediated gene repression has been employed to improve L-lysine production by *C. glutamicum* (Park et al., 2018). Although developed earlier for *E. coli* (Qi et al., 2013), CRISPR interference has not been applied to amino acid production, however, it was used for pinosylvin and anthocyanins that can be derived from the aromatic amino acid metabolism (Cress et al., 2017; Liang et al., 2016). Orthogonally operating CRISPR/CRISPRi systems based on the Cas9 proteins from *Streptococcus pyogenes, Staphylococcus aureus* and *Streptococcus thermophilius* have been developed for *E. coli* (Sung et al., 2019). It has to be noted that the extent of gene repression by CRISPR interference varies with the targeted gene, its background expression, the location of the sgRNA within the targeted gene and with respect to the targeted strand (template vs. non-template strand) as well as with the expression level of dCas9. Moreover, CRISPR interference effects may be transient only.

Genome editing by CRISPR (Jinek et al., 2012) complements genome editing by two-step homologous recombination using the conditionally lethal levansucrase (*sacB*) for positive selection (Jäger et al., 1992). Of course, effects of gene deletions on product formation are identical and independent of their introduction by CRISPR genome editing or by two-step homologous recombination. CRISPR genome editing has been used widely in *C. glutamicum* (Cameron Coates et al., 2019; Cho et al., 2017; Jiang et al., 2017; Liu et al., 2017; Peng et al., 2019; Wang et al., 2018a,b,c,d,e,f) involving either Cpf1 endonuclease of *Francisella novicida* (Jiang et al., 2017) or Cas9 endonuclease from *S. pyogenes* in its native form (Liu et al., 2017) and in an optimized version (Cho et al., 2017). Expression control was crucial for both, Cpf1 and Cas9, genes, while the GC-rich PAM sequence of Cas9 is better adapted to the *C. glutamicum* genome GC content. The fact that *C. glutamicum* lacks efficient non-homologous end-joining makes CRISPR ideal for sequential or parallel targeted genome deletions and replacements. CRISPR applications dedicated to amino acid production include improving strains producing GABA (Cho et al., 2017), L-proline (Jiang et al., 2017), L-glutamate (Wang et al., 2018a,b,c,d,e,f), and L-arginine (Wang et al., 2018a,b,c,d,e,f). CRISPR genome editing with the aim to improve amino acid production has also been described for L-valine production by *Bacillus subtilis* (Westbrook et al., 2018) and L-ornithine production by the yeast *Saccharomyces cerevisae* (Liu et al., 2018c).

3.2. Flux enforcement for amino acid production

Flux enforcement refers to coupling a biosynthetic production pathway to a metabolite pathway required for growth. This strategy has been applied to amino acid production by *E. coli* and *C. glutamicum*. Coupling of a production pathway involving 2-oxoglutarate dependent hydroxylase to growth by deletion of 2-oxoglutarate dehydrogenase subunit gene *sucA* has first been described for *E. coli* strains producing 4-hydroxy-L-isoleucine (Smirnov et al., 2010) and later for *E. coli* strains producing 4-hydroxy-L-proline (Theodosiou et al., 2017). Thus, these production pathways become part of an artificial TCA cycle. This concept was extended in succinyl-CoA synthetase-negative (Δ*sucCD*), lysine producing *C. glutamicum* where the succinylase branch of L-lysine production metabolically complemented the disrupted TCA cycle (Kind et al., 2013). A third variant has been developed for glutarate producing *C. glutamicum* by coupling growth in the absence of the major ammonium assimilating enzyme glutamate dehydrogenase to transamination reactions (cadaverine/putrescine transaminase PutA and GABA/5AVA amino transferase GabT) in glutarate production (Perez-Garcia et al., 2018).

3.3. Biosensors

Genetically encoded biosensors are of help for screening amino acid overproducing mutants (Bott and Eggeling, 2017). They work based on the assumption that overproduction of a secreted product is reflected by an increased intracellular concentration of that product. Of course, this is not true for mutants lacking an export system for that product. Genetically encoded biosensors come in two flavors: riboswitches and transcriptional repressors. Both regulatory elements have been evolved in nature to sense intracellular concentrations of low-molecular weight compounds such as amino acids. Fine-tuning biosensors is required to adjust the threshold concentrations required to switch a transcriptional repressor or riboswitch to the requirements of strain development. To develop biosensors responding to nonnative ligands requires structure-guided approaches or mutations and selection schemes. Biosensor

Table 2
Advanced tools applied to amino acid production strains and processes.

PROTEIN	GENE/ENZYME TARGET	AMINO ACID PRODUCTION	REFERENCE
CRISPR			
dCas9	Phosphoglucoisomerase gene *pgi*	L-lysine by *C. glutamicum*	Cleto et al. (2016)
dCas9	Pyruvate kinase gene *pyk*; PEP carboxykinase gene *pck*	L-glutamate by *C. glutamicum*	Cleto et al. (2016)
Cpf1	γ-glutamyl kinase gene *proB*	L-proline by *C. glutamicum*	Jiang et al. (2017)
Cas9, RecET	Arginine repressor gene *argR;* repressor gene *farR;* acetyl-glutamate kinase gene *argB;* hosphoglucoisomerase gene *pgi*	L-arginine by *C. glutamicum*	Wang et al. (2018a,b,c,d,e,f)
Cas9, AID	Pyruvate kinase gene *pyk*; fermentative L-lactate dehydrogenase *ldhA*	L-glutamate by *C. glutamicum*	Wang et al. (2018a,b,c,d,e,f)
Cas9	2-isopropylmalate synthase gene *leuA*; threonine dehydratase gene *ilvA*; pyruvate dehydrogenase subunit E1α gene *pdhA*	L-valine by *B. subtilis*	Westbrook et al. (2018)
Cas9, RecT	L-glutamate exporter gene *yggB*, GABA transaminase gene *gabT* and GABA permease gene *gabP*	GABA by *C. glutamicum*	Cho et al. (2017)
FLUX ENFORCEMENT			
2-oxoglutarate depended L-isoleucine hydroxylase IDO	Deletion of 2-oxoglutarate dehydrogenase subunit gene *sucA*	4-hydroxy-L-isoleucine by *E. coli*	Smirnov et al. (2010)
Succinylase branch of L-lysine biosynthesis	Deletion of succinyl-CoA synthetase genes *sucCD*	L-lysine by *C. glutamicum*	Kind et al. (2013)
2-oxoglutarate depended L-proline 4-hydroxylase	Deletion of 2-oxoglutarate dehydrogenase subunit gene *sucA*	4-hydroxy-L-proline by *E. coli*	Theodosiou et al. (2017)
cadaverine/putrescine transaminase PutA and GABA/5AVA amino transferase GabT	Deletion of glutamate dehydrogenase	5AVA, glutarate by *C. glutamicum*	Perez-Garcia et al. (2018)
BIOSENSOR			
LysG	Fluorescent reporter gene	L-lysine production by *C. glutamicum*	(Binder et al., 2012, 2013)
Lrp	Fluorescent reporter gene	L-valine production by *C. glutamicum*	(Mahr et al., 2015; Mustafi et al., 2012, 2014)
LysG	Fluorescent reporter gene	L-arginine production by *C. glutamicum*	Schendzielorz et al. (2014)
ArgP	Beta-lactamase gene *bla* (allows for selection rather than screening)	L-arginine production by *E. coli*	Ginesy et al. (2015)
AraC-ect	Fluorescent reporter gene	Ectoine production by *E. coli*	Chen et al. (2015)
Cg0702	Fluorescent reporter gene	L-serine production by *C. glutamicum*	Zhang et al. (2018a,b,c,d,e)
Lysine-OFF riboswitch	citrate synthase gene *gltA*	L-lysine production by *C. glutamicum*	Zhou and Zeng (2015a,b)
Lysine-ON riboswitch	lysine export gene *lysE*	L-lysine production by *C. glutamicum*	Zhou and Zeng (2015a,b)
fusion of lysine-/arginine-/ornithine-binding protein (LAO-BP) to two fluorescent proteins (enhanced cyan fluorescent protein and citrine)		L-lysine production by *C. glutamicum*	Steffen et al. (2016)

technology for amino acid strain development has been commercialized by SenseUp (Rupprecht et al., 2017).

Based on the characterization of the transcriptional regulators LysG (Vrljic et al., 1996) and Lrp (Lange et al., 2012) from *C. glutamicum*, biosensors for L-lysine and branched-chain amino acids have been developed and used for live cell imaging (Mustafi et al., 2014), high-throughput screening (Eggeling et al., 2015) and strain optimization by adaptive laboratory evolution (Mahr et al., 2015). Biosensors have also been constructed for L-arginine (based on *E. coli* ArgP) (Ginesy et al., 2015) and L-serine (based on *C. glutamicum* cg0702) (Zhang et al., 2018e). AraC of *E. coli* was engineered to recognize the non-proteinogenic amino acid ectoine as its non-natural effector. The AraC-ect biosensor activates transcription upon ectoine binding in a linear range of 1 mM to 1 M (Chen et al., 2015). The biosensor was used in high-throughput screening of ectoine overproducing strains. Amino acid biosensors typically show relatively high threshold concentrations (millimolar range) as compared to biosensors responding to (sub)micromolar concentrations of antibiotics or flavonoids (Cheng et al., 2018a). L-Lysine riboswitches that responded to intracellular L-lysine concentrations as low as 50 μM were used for selecting L-lysine overproducing *C. glutamicum* mutants in a setup where the L-lysine responsive riboswitch controlled expression either of citrate synthase gene *gltA* ("OFF" mode) or of lysine export permease gene *lysE* ("ON" mode) (Zhou and Zeng, 2015a,b). Biosensors for the intermediate of aromatic amino acid biosynthesis shikimate, of the central metabolite cAMP and of the reduced redox cofactor NADPH have been developed and used to improve amino acid production (Goldbeck et al., 2018; Liu et al., 2018a; Schulte et al., 2017; Siedler et al., 2014; Spielmann et al., 2018). NADPH biosensors have been developed as NADPH auxotrophic *E. coli* or as FRET-based biosensors (Lindner et al., 2018; Zhao et al., 2016). A series of FRET-based biosensors with K_D values between 3 and 100 μM has also been developed for L-lysine detection by using fusion of lysine-/arginine-/ornithine-binding protein (LAO-BP) to two fluorescent proteins (enhanced cyan fluorescent protein (ECFP) and citrine) (Steffen et al., 2016). Interestingly, most amino acid specific biosensors are based on regulation of amino acid transport: either a binding protein of an amino acid transport system is used (LAO-BP) or transcriptional regulators governing amino acid transport gene expression (Lrp, LysG, ArgP).

4. Transport engineering

Transport engineering aims to improve either product export and substrate uptake or to avoid loss of intermediates by excretion (Perez-Garcia and Wendisch, 2018) (Table 3). These strategies are based on the discovery and biochemical characterization of proteins for substrate uptake and product excretion (Eggeling, 2017; Marin and Krämer, 2007). First, two ways of manipulating L-lysine export catalyzed by LysE (Vrljic et al., 1996) will be presented: improving production of L-lysine as final product and avoiding export of L-lysine as intermediate to improve production of L-lysine derived products. L-Lysine productivity was improved in genome reduced strains by a second chromosomally encoded copy of *lysE* (Henke et al., 2018; Unthan et al., 2015a,b). On the other hand, deletion of *lysE* improved production of L-lysine derived products such as cadaverine (Kind et al., 2014), 5-aminovalerate (Rohles et al., 2016) and L-pipecolic acid (Perez-Garcia et al., 2017a). With respect to substrate utilization, endogenous and heterologous uptake systems may be employed (refer to section 6 for further examples). Here, import of hexosamines by phosphoenolpyruvate:carbohydrate phosphotransferase systems (PTS) is discussed. The hexosamines glucosamine and *N*-acetylglucosamine (GlcNAc) are subunits of chitin, and *N*-acetylmuraminic acid (MurNAc) and GlcNAc are subunits of cell wall peptidoglycan. *C. glutamicum* imports glucosamine via a side activity of the glucose-specific PTS component PtsG (Uhde et al., 2013). For uptake of GlcNAc an exogenous PTS was needed: the GlcNAc-specific PTS NagE from *C. glycinophilum* (Matano et al., 2014). Finally, uptake of MurNAc required MurNAc-specific PTS component MurP from *E. coli*. When general PTS component Crr from *E. coli* was present in addition, faster growth with MurNAc resulted (Sgobba et al., 2018a).

5. Prospects on new cultivation concepts

Amino acid production processes have been modified in order to either co-produce a secreted amino acid with a cell-bound second product or to subdivide production of an amino acid from a feedstock into two consecutive steps catalyzed by two different bacterial species or strains (Fig. 1).

5.1. Co-production

Metabolic engineering typically aims at maximizing yield, titer and productivity of a single product. This is particularly true for large volume processes where substrate costs may limit economic feasibility. Moreover, this facilitates downstream processing which may be best exemplified by the recent demonstration by Ajinomoto of L-glutamate production by *Pantoea* species where the product formed large crystals that could be separated easily from the liquid medium and the cells (Hara et al., 2012). However, this scenario also highlights the possibility to co-produce a soluble product such as an amino acid with a cell-bound product such as a lipid. Both products can be purified in two streams, namely the cell pellet obtained by centrifugation, for example, and the supernatant resulting from the centrifugation.

The concept of co-producing an amino acid with a second value-added compound has recently been demonstrated for the co-production of L-lysine with carotenoids (Henke et al., 2018) (Fig. 1). A genome-reduced L-lysine producing *C. glutamicum* strain was engineered for accumulation of the carotenoid astaxanthin which could be extracted from the cell pellet while L-lysine could be isolated from the supernatant. In a fed-batch bioreactor culture, 48 g/L L-lysine and 10 mg/L astaxanthin were co-produced (Henke et al., 2018).

This co-production scheme has, however, not been chosen for providing two separated product streams (L-lysine in the supernatant, astaxanthin in the cell pellet) (Henke et al., 2018). Rather, this process makes use of the fact that, on the one hand, both L-lysine and astaxanthin have applications in the same market segment (poultry feed and aquaculture with astaxanthin as egg yolk or fish flesh colorant). On the other hand, spray-dried L-lysine fermentation broth (including cells) has been commercialized as an L-lysine formulation by at least two of the major L-lysine producers (Kelle et al., 2005).

5.2. Co-cultures

Amino acid overproducing strains are typically used in monoculture in industry. In nature, however, division of labor prevails and ecological niches are rarely dominated by a single microbial species. Microbial consortia do not only exist abundantly in nature, but have been used by mankind for ages, mainly in food biotechnological processes. Knowledge accumulated on how to maintain these consortia for a plethora of fermented foods. However, this is done without knowing the exact species composition of the consortia and oftentimes the consortia are handled like a black box and only product outcome is monitored.

Recently, metabolic engineering has demonstrated that microbial consortia can be designed synthetically to "divide labor between two strains or species in a combined biotech process" in order to produce a desired compound. Importantly, metabolic engineering design principles take center stage. A number of fine chemicals can be produced by designed microbial consortia such as resveratrol by *E. coli*-based consortia (Camacho-Zaragoza et al., 2016), paclitaxel precursors by *E. coli*-*S. cerevisiae* (Minty et al., 2013), or vitamin C production from either a recombinant *Erwinia herbicola* strain or by consortia consisting of

Table 3
Transport systems relevant to metabolic engineering of amino acid producing *E. coli* and *C. glutamicum* strains. Adapted and updated from (Eggeling, 2017; Marin and Krämer, 2007; Perez-Garcia and Wendisch, 2018).

PROTEIN	SUBSTRATE(S)	TRANSPORTER CLASS	ORIGIN	REFERENCE
ADIC	L-arginine, agmatine	MFS antiporter	*E. coli*	Gong et al. (2003)
ALAE	L-alanine		*E. coli*	Hori et al. (2011)
ARAE	L-arabinose	Proton symporter	*C. glutamicum* ATCC31831	Kawaguchi et al. (2009)
ARAE	L-arabinose, D-xylose	Proton symporter	*E. coli*	Chen et al. (2017)
ARGO	L-arginine, canavanine	ABC transporter	*E. coli*	Pathania et al. (2016)
ARGTHISPQM	L-arginine	ABC transporter	*E. coli*	Wissenbach et al. (1995)
AROP	L-phenylalanine, L-tyrosine	MFS uptake	*E. coli*	Chye et al. (1986)
AROP	L-phenylalanine, L-tryptophan, L-tyrosine, L-histidine,	Proton symporter	*C. glutamicum*	Wehrmann et al. (1995)
ARTIMPQ	L-arginine	ABC transporter	*E. coli*	Wissenbach et al. (1993)
BCR	L-cysteine	MFS	*E. coli*	Yamada et al. (2006)
BRNEF	L-leucine, L-methionine, L-valine, L-isoleucine	Proton symporter	*C. glutamicum*	Kennerknecht et al. (2002)
CADB	L-lysine, cadaverine	MFS antiporter	*E. coli*	Meng and Bennett (1992)
CGMA	L-arginine, putrescine, cadaverine	Permease of the major facilitator superfamily	*C. glutamicum*	Kennerknecht et al. (2002)
CYCA	L-alanine, L-serine, glycine	MFS uptake	*E. coli*	Schneider et al. (2004)
CYDDC		ABC transporter	*E. coli*	Pittman et al. (2002)
CYSXYZ		ABC transporter	*E. coli*	Berger and Heppel (1972)
EAMD	L-cysteine	DMT exporter	*E. coli*	Dassler et al. (2000)
GABP	L-glutamate, GABA	MFS antiporter	*E. coli*	Metzer and Halpern (1990)
GLNHPQ		ABC transporter	*E. coli*	Nohno et al. (1986)
GLPF	Glycerol	Uptake facilitator	*E. coli*	Meiswinkel et al. (2013a,b)
GLTIJKL		ABC transporter	*E. coli*	Deguchi et al. (1989)
HISJMPQ	L-histidine	ABC transporter	*E. coli*	Liu and Ames (1997)
IOLT1, IOLT2	Glucose, fructose, *myo*-inositol, D-xylose	Proton symporters	*C. glutamicum*	(Bäumchen et al., 2009; Brüsseler et al., 2018; Lindner et al., 2011)
LACY	Lactose	Lactose permease	*E. coli*	Brabetz et al. (1991)
LEUE	L-leucine	LysE type exporter	*E. coli*	Kutukova et al. (2005)
LIVFGHJM		ABC transporter	*E. coli*	Ovchinnikov et al. (1977)
LYSE	L-lysine, L-arginine, L-citrulline	Proton antiporter	*C. glutamicum*	Vrljic et al. (1996)
LYSO	L-lysine	CACA	*E. coli*	Pathania and Sardesai (2015)
LYSP	L-lysine import	MFS uptake	*E. coli*	Steffes et al. (1992)
METD		ABC transporter	*E. coli*	Merlin et al. (2002)
MURP, (CRR)	*N*-acetylmuraminic acid	*N*-acetylmuraminic acid-specific PTS permease	*E. coli*	Sgobba et al. (2018a,b)
NAGE	*N*-acetylglucosamine	*N*-acetylglucosamine-specific PTS permease	*C. glycinophilum*	Matano et al. (2014)
PHEP	L-phenylalanine	MFS uptake	*E. coli*	Pi and Pittard (1996)
PHEP	L-phenylalanine	amino acid transporter, AAT family	*C. glutamicum*	Zhao et al. (2011)
PROU		ABC transporter	*E. coli*	Gowrishankar et al. (1986)
PTSG	Glucose, glucosamine	Glucose-specific PTS permease	*C. glutamicum*	Lindner et al. (2011)
RHTA	L-threonine, homoserine, aminolevulinic acid	DMT exporter	*E. coli*	Livshits et al. (2003)
RHTB	L-threonine	LysE-type exporter	*E. coli*	Zakataeva et al. (1999)
SUCE	Succinic acid	permease	*C. glutamicum*	Huhn et al. (2011)
THRE	L-threonine, L-serine	Proton symporter	*C. glutamicum*	Simic et al. (2001)
YDDG	L-tryptophan	DMT exporter	*E. coli*	Tsuchiya et al. (2016)
YGAZH	L-isoleucine	LIV-E	*E. coli*	Park et al. (2012)
YGGB	L-glutamate	Mechanosensitive channel	*C. glutamicum*	Nakamura et al. (2007)
YTFF	L-alanine	LysE-type exporter	*E. coli*	Hori et al. (2011)

Gluconobacter oxydans and *Ketogulonicigenium vulgare* (Wang et al., 2016). Besides subdivision of two biosynthesis modules, other consortia were designed to subdivide upstream degradation of polymeric feedstock from the actual production, e.g. by co-culturing the cellulase-secreting fungus *Trichoderma reesei* with an isobutanol producing *E. coli* strain for production of isobutanol from lignocellulosic feedstock (Minty et al., 2013).

Recently, synthetic *C. glutamicum-E. coli* consortia have been designed for amino acid production (Sgobba et al., 2018b). As proof-of-concept of a commensal consortium, an L-lysine auxotrophic, sucrose negative *E. coli* strain was co-cultivated with an L-lysine producing *C. glutamicum* that secretes fructose to feed the *E. coli* strain when grown with sucrose as consequence of deletion of the fructose importer gene *ptsF* (Sgobba et al., 2018b). With sucrose minimal medium, neither strain grew as monoculture, but the consortium grew and produced L-lysine (Sgobba et al., 2018b). Thus, a net production of L-lysine only results when the L-lysine producing *C. glutamicum* strain produces more L-lysine than required to support growth of the L-lysine auxotrophic *E. coli* strain. In this commensal consortium, the *E. coli* strain is not beneficial for the *C. glutamicum* strain.

In a mutualistic binary consortium, both strains draw a benefit from each other. Such a mutualistic *C. glutamicum-E. coli* consortium was designed for L-lysine production from starch. One strain was designed for degrading the polymeric feedstock starch to glucose, the other for conversion of glucose to L-lysine (Sgobba et al., 2018b). Starch-based L-lysine production was possible with a consortium consisting of an α-amylase secreting, L-lysine auxotrophic *E. coli* strain and a naturally amylase-negative, L-lysine overproducing *C. glutamicum* strain. Neither strain grew with starch in single culture, whereas the consortium catabolized starch and produced L-lysine (Sgobba et al., 2018b). A 25 L

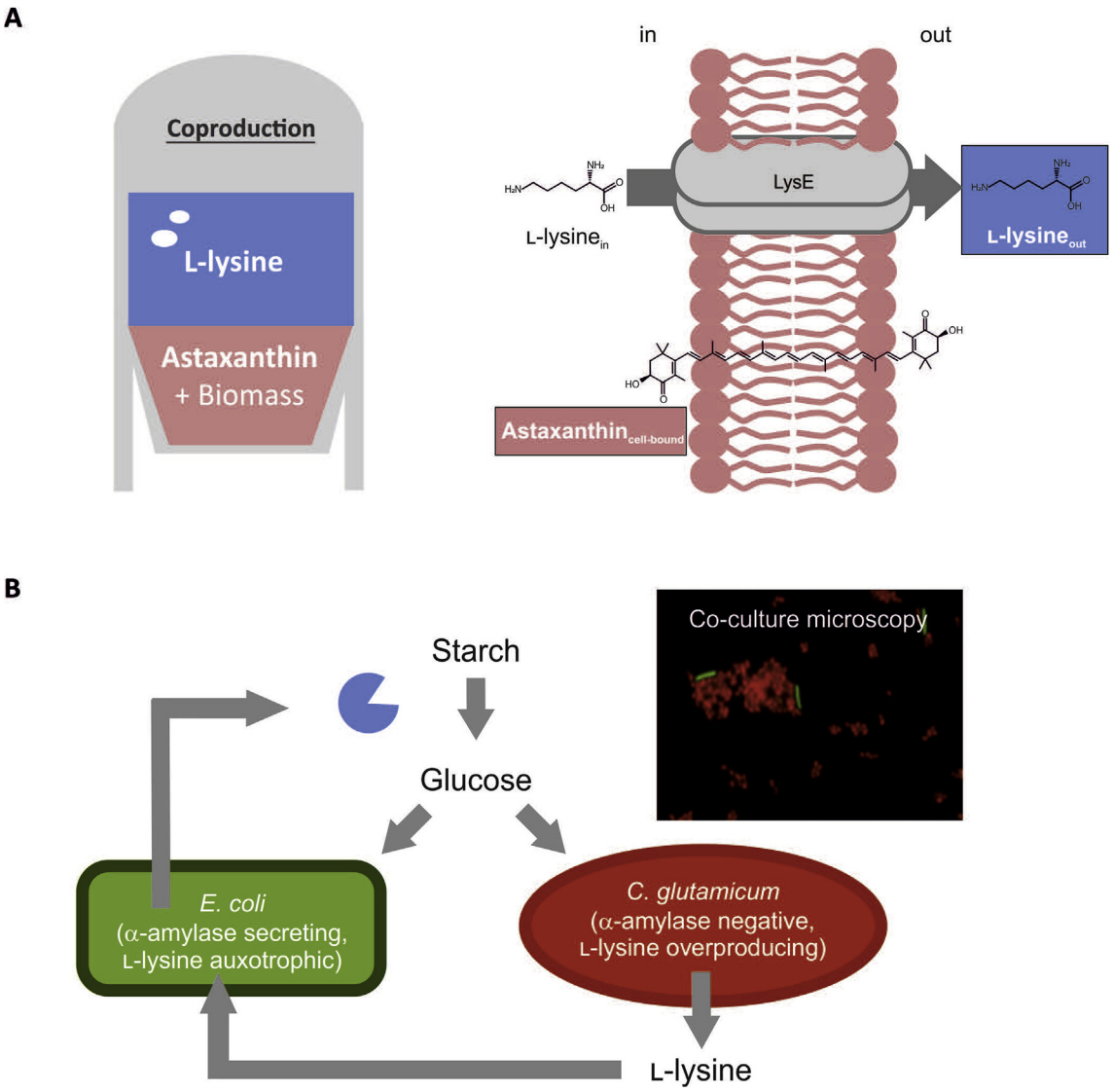

Fig. 1. Amino acid production processes based on co-production with the carotenoid astaxanthin (A) or based on cu-cultivation of *E. coli* and *C. glutamicum* strains (B). An L-lysine overproducing C. glutamicum strains engineered for co-production of astaxanthin secretes L-lysine into the fermenter medium and accumulates cell-bound astaxanthin (A). An α-amylase secreting, L-lysine auxotrophic, green fluorescent *E. coli* strain and an α-amylase-negative, L-lysine overproducing, red fluorescent *C. glutamicum* strain are mutually dependent in starch minimal medium. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

batch-fermentation with this consortium demonstrated that the process was scalable. As the *E. coli* and *C. glutamicum* strains expressed different fluorescent proteins, the composition of the consortium could be followed. Single cell analysis by fluorescence microscopy revealed that the *C. glutamicum* strain outnumbered the *E. coli* strain by far. Likely few α-amylase secreting *E. coli* cells are sufficient to maintain starch hydrolysis in the consortium (Sgobba et al., 2018b). When comparing lysine productivity, the consortium's performance falls short of that of α-amylase secreting, L-lysine overproducing *C. glutamicum* strains (Seibold et al., 2006; Tsuge et al., 2013). Future research will have to improve process performance based on designed consortia combining specifically dedicated metabolic engineering and process control strategies.

Accordingly, a microfluidic co-cultivation system has been designed and characterized for the analysis of growth and interactions within microbial consortia at single-cell resolution (Burmeister et al., 2018). Two strains were co-cultivated, but were physically separated to isolate interactions by diffusible compounds from cell-cell contact interactions. A simple commensalistic consortium was used and it comprised two strains of a single species: an L-lysine-producing *C. glutamicum* strain and an L-lysine auxotrophic mutant *C. glutamicum* strain. This approach allowed to characterize the interaction due to the diffusible compound L-lysine without interference of interactions due to cell-cell contact (Burmeister et al., 2018).

The design and characterization of synthetic microbial consortia will not only further our understanding of naturally occurring microbial consortia, but is expected to have its major impact within the biorefinery concept going beyond the current proof-of-principle studies. The challenges facing a biorefinery such as flexible conversion of renewable feedstocks to a multitude of value-added compounds irrespective of seasonal or other variations of the complex biomass substrates may be met either by many individually optimized strains to be used in monoculture or by a modularized approach where a series of biomass degrading strains can be operated with a set of strains producing the value-added compounds in demand by the customer. As a first step in this direction, the microbial consortium developed for starch-based L-lysine production was modularized: the starch-degrading L-lysine auxotrophic *E. coli* strain was combined with three

starch-negative *C. glutamicum* producer strains (Sgobba et al., 2018b). In addition to the L-lysine producing *C. glutamicum* strain, a strain overproducing cadaverine and a strain overproducing L-pipecolic acid was used (Sgobba et al., 2018b). For each combination starch-based production of the desired product could be shown only for the designed consortium, but not for the individual strains alone. However, this extension of the original concept was only possible, because cadaverine and L-pipecolic acid are derived from L-lysine and the amylase-negative *C. glutamicum* strains produced enough L-lysine to maintain growth of the *E. coli* strain in addition to the main product cadaverine or L-pipecolic acid. Production by dedicated monocultures is superior to that by the first described consortia (Jorge et al., 2016; Mimitsuka et al., 2007; Perez-Garcia et al., 2016, 2017a,b; Tateno et al., 2009). Thus, in addition to new metabolic engineering and process control strategies to improve performance design concepts have to be developed to decouple formation of the final product from providing a supplement for interaction with the partner strain in the consortium.

6. Realizing a flexible feedstock concept for amino acid production

Amino acid production varies in scale and reaches annual production volumes of million tons e.g. for L-lysine. At this scale, substrate cost and steady availability is relevant. In addition, market demand for production processes that do not interfere with utilization of agricultural products for human and animal nutrition is on the rise. Thus, amino acid production from alternative feedstocks that do not have competing uses in the food and feed industries is sought after. In this respect, amino acid production may benefit from and contribute to second generation ethanol production. This is due to the fact that second generation feedstocks contain proteins and, thus, nitrogen, which either can be liberated as ammonium when producing biofuels (Huo et al., 2011) or may be used for production of nitrogenous products such as amino acids. Moreover, a flexible feedstock concept will ease integration of amino acid production into biorefineries that have to respond to fluctuations in feedstock availability and market demand (De Schouwer et al., 2019).

E. coli possesses many degradation systems for sugars, sugar alcohols and organic acids, while *C. glutamicum* has a relatively narrow substrate spectrum (Blombach and Seibold, 2010). Table 4 lists the metabolic engineering strategies followed that enabled access to various biotechnologically relevant monomeric and polymeric carbon sources. The engineering concepts applied to *E. coli* and *C. glutamicum* are exemplified below for amino acid production based on glycerol, hexosamines, xylans and xylose as well as C1 compounds.

Glycerol is an abundant stoichiometric waste product of biodiesel manufacturing, however, its price is volatile (Khanna et al., 2012). *E. coli* naturally utilizes glycerol, both aerobically and anaerobically. To increase glycerol utilization fast growing *E. coli* strains with mutations in glycerol kinase gene *glpK* and in RNA polymerase subunit gene *rpoC* were selected (Cheng et al., 2014). *C. glutamicum* possesses transcriptionally silent genes for glycerol kinase and glycerol-3-phosphate dehydrogenase (*glpD*), but only grew if these endogenous genes or their heterologous counterparts from *E. coli* were overproduced (Meiswinkel et al., 2013b; Rittmann et al., 2008). Glycerol uptake was accelerated upon expression of the *E. coli* glycerol facilitator gene *glpF* while deletion of the gene encoding glycerol 3-phosphatase, which is responsible for formation of glycerol as by-product, was not required (Lindner et al., 2012). Glycerol-based production of L-glutamate, L-lysine, L-ornithine, L-arginine, succinate and putrescine by recombinant *C. glutamicum* has been described (Litsanov et al., 2013; Meiswinkel et al., 2013b).

The hexosamines glucosamine, *N*-acetylglucosamine (Glc-NAc) and *N*-acetylmuraminic acid (MurNAc) occur throughout nature and are monomeric constituents of chitin (abundantly available e.g. in shrimp shell waste) and/or fungal and bacterial cell walls (a potential source from the fermentation industries). The fact that these hexosamines serve as sources of carbon, energy and nitrogen makes them particularly interesting for production of nitrogenous compounds such as amino acids. While *E. coli* readily utilizes all three hexosamines, only glucosamine is a natural *C. glutamicum* substrate/carbon source (Uhde et al., 2013). Heterologous GlcNAc-specific PTS NagE from *C. glycinophilum* (Matano et al., 2014) was combined with overexpression or derepression of the endogenous *nagB* coding for Glc-NAc-6-P deaminase (Uhde et al., 2016). Access to MurNAc required heterologous expression of the *E. coli* genes *murP, crr,* and *murQ* that encode a MurNAc-specific PTS component, a general PTS component and an etherase for cleavage of MurNAc-6-phosphate to GlcNAc-6-phosphate and lactate (Sgobba et al., 2018a). The amino acid L-lysine was produced from these three hexosamines (Matano et al., 2014; Sgobba et al., 2018a,b; Uhde et al., 2013).

Access to polymeric feedstocks is more complex since typically both breakdown of the polymer to monomers and their subsequent utilization as substrates for amino acid production is required. Direct access to cellulose required secretion or surface display of cellulases. Upon synthesis of the scaffolding protein CpbA from *Clostridium cellulovorans* and secretion of *C. thermocellum* endoglucanase carboxymethyl cellulose was utilized (Hyeon et al., 2011). Surface display of a cellulase complex containing endoglucanase CelE and beta-glucosidase BglA from *C. thermocellum* anchored to the mechanosensitive channel Msc enabled saccharification of cellulose (Kim et al., 2014). Similarly, direct lysine fermentation from cellobiose was shown (Anusree et al., 2016).

Hemicellulose contains xylans. Xylan breakdown by *C. glutamicum* strain was achieved with endoxylanase (XlnA) from *Streptomyces coelicolor* A3 and xylosidase (XynB) from *Bacillus pumilus* (Imao et al., 2017; Kuge et al., 2017; Watanabe et al., 2015; Yim et al., 2016). The generated xylose can be imported into the *C. glutamicum* cell via IolT1 and/or IolT2, permeases that facilitate uptake of *myo*-inositol (Krings et al., 2006), fructose (Bäumchen et al., 2009), glucose (Lindner et al., 2011) and xylose (Brüsseler et al., 2018) and derepression of their genes via deletion of repressor gene *iolR* accelerates xylose utilization (Klaffl et al., 2013). Xylose uptake was also supported by heterologous uptake systems: XylE from *E. coli* (Yim et al., 2017) or AraE from *C. glutamicum* ATCC31831 or *B. subtilis* (Mao et al., 2018; Sasaki et al., 2009). Two pathways for xylose utilization have been installed in *C. glutamicum*: the isomerase pathway (Kawaguchi et al., 2006) and the Weimberg pathway (Radek et al., 2014, 2017). To install the isomerase pathway only heterologous xylose isomerase XylA was required (Kawaguchi et al., 2006) since *C. glutamicum* possesses xylulokinase XylB as part of arabitol catabolism (Laslo et al., 2012). Alternative xylose isomerase genes, genome integration, ALE, transcriptional regulator engineering, expression of a phosphoketolase gene further improved xylose catabolism (Jo et al., 2017; Lange et al., 2018; Meiswinkel et al., 2013a,b; Radek et al., 2017; Wang et al., 2018a,b,c,d,e,f). To install the Weimberg pathway in *C. glutamicum* five genes from *Caulobacter crescentus* had to be expressed: xylose dehydrogenase (*xylB*), xylonolactonase (*xylC*), xylonate dehydratase (*xylD*), a 2-keto-3-desoxyxylonate dehydratase (*xylX*), and 2-oxoglutarate semialdehyde dehydrogenase (*xylA*) (Radek et al., 2014). This pathway lends itself ideal for production of 2-oxoglutarate dependent products such as L-glutamate or GABA (Baritugo et al., 2018).

Mannitol, the major constituent of macroalgae, does not support growth of wild-type *C. glutamicum* as sole carbon source (Laslo et al., 2012). This is due to the fact that the arabitol catabolic operon is derepressed only by arabitol, in the derepressed state mannitol can be utilized due to the promiscuity of the arabitol uptake permease and arabitol dehydrogenase that allow conversion of mannitol to fructose (Laslo et al., 2012). Deletion of the arabitol operon repressor gene *atlR* enabled mannitol catabolism which subsequently was improved based on carbon flux analysis to support production of lysine to a titer of about 2 g/L at a yield of 0.19 g/g (Hoffmann et al., 2018).

Methanol and other C1 compounds are interesting carbon sources

Table 4
Examples of *E.coli* and *C. glutamicum* strains engineered for amino acid production from nonnative carbon sources. Adapted and updated from (Wendisch et al., 2016a).

FEEDSTOCK	HETEROLOGOUS ENZYME	AMINO ACID PRODUCTION	REFERENCE
STARCH	α-amylase from *S. griseus*	L-lysine	Seibold et al. (2006)
	α-amylase from *S. bovis*	L-lysine	Tateno et al. (2007)
	α-amylase from *S. bovis*	L-glutamate	Yao et al. (2009)
CELLOBIOSE	β-glucosidase from *S. degradans*	L-lysine	Adachi et al. (2013)
CELLULOSE	Endoglucanases from *X. campestris* XCC3521 and XCC2387, β-glucosidase from *S. degradans*	L-lysine	Anusree et al. (2016)
B-GLUCAN	endoglucanase *from C. thermocellum* with *E. coli torA* signal sequence for protein secretion	L-glutamate	Tsuchidate et al. (2011)
GLUCOSAMINE	Overexpression of endogenous *nagB* (glucosamine-6-phosphate deaminase)	L-lysine	Uhde et al. (2013)
	Deletion of endogenous sialic acid repressor *nanR*	L-lysine	Uhde et al. (2016)
N-ACETYL-GLUCOSAMINE	*N*-acetyl-glucosamine-specific PTS from *C. glycinophilum;* Overexpression of endogenous *nagB* (glucosamine-6-phosphate deaminase) and *nagA* (*N*-acetylglucosamine-6-phosphate deacetylase)	L-lysine	(Matano et al., 2014, 2016)
	N-acetyl-glucosamine-specific PTS from *C. glycinophilum*; deletion of endogenous sialic acid repressor *nanR*	L-lysine	Uhde et al. (2016)
N-ACETYL-MURAMINIC ACID	MurNAc-specific PTS component MurP, general PTS component Crr and etherase MurQ	L-glutamate L-lysine	Sgobba et al. (2018a,b)
XYLOSE	xylose isomerase from *X. campestris*; Overexpression of endogenous *xylB* (xylulokinase)	L-glutamate; L-lysine; L-ornithine	(Meiswinkel et al., 2013a; Unthan et al., 2015a,b)
		sarcosine	Mindt et al. (2019)
		N-methyl-L-alanine	Mindt et al. (2018a,b)
		7-Cl-L-tryptophan	Veldmann et al. (2019)
		GABA	(Baritugo et al., 2018; Jorge et al., 2017a,b)
		ectoine	Perez-Garcia et al. (2017b)
		L-pipecolic acid	Perez-Garcia et al. (2017a)
		5AVA	(Jorge et al., 2017a,b; Shin et al., 2016)
		para-aminobenzoate	Kubota et al. (2016)
ARABINOSE	*araA* (arabinose isomerase, EC 5.3.1.3), *araB* (ribulokinase, EC 2.7.1.16), and *araD* (ribulose 5-phosphate 4-epimerase, EC 5.1.3.4) from *E. coli*	L-glutamate; L-lysine L-arginine; L-ornithine	Schneider et al. (2011)
LIGNOCELLULOSIC HYDROLYSATE	s. modifications for xylose and/or arabinose	L-glutamate L-lysine 5AVA	(Gopinath et al., 2011; Joo et al., 2017a,b; Meiswinkel et al., 2013a; Wang et al., 2018a,b,c,d,e,f)
SILAGE JUICE	overexpression of genes for D-lactate dehydrogenase (*dld*), pyruvate carboxylase (*pyc*), malic enzyme (*malE*), fructose 1,6-bisphosphatase (*fbp*) and glyceraldehyde 3-phosphate dehydrogenase (*gapX*)	L-lysine	Neuner et al. (2013)
LACTOSE	*lacY* (lactose permease), *lacZ* (beta-galactosidase) from *E. coli*	L-lysine	Barrett et al. (2004)
GLYCEROL	*glpD* (glycerol 3-phosphate dehydrogenase); *glpF* (aquaglyceroporin), *glpK* (glycerol kinase) from *E. coli*	L-glutamate; L-lysine	Rittmann et al. (2008)
	Overexpression of endogenous *glpD* (glycerol 3-phosphate dehydrogenase), *glpK* (glycerol kinase)	L-glutamate; L-lysine; L-ornithine; L-arginine	Meiswinkel et al. (2013a,b)
MANNITOL	Deletion of arabitol repressor gene *atlR*	L-lysine	Hoffmann et al. (2018)

for biotechnological processes (Clomburg et al., 2017). Based on the biochemical insight into methylotrophs (Müller et al., 2015; Ochsner et al., 2015) and methanol-dependent amino acid production by them (Brautaset et al., 2007; Ishikawa et al., 2008a,b), *E. coli* and *C. glutamicum* were engineered for methanol catabolism. The ribulose monophosphate cycle has first been installed in *E. coli* (Müller et al., 2015) and *C. glutamicum* (Lessmeier et al., 2015; Witthoff et al., 2015) and carbon-13-labeled L-lysine and cadaverine were produced by engineered *C. glutamicum* using carbon-13-labeled methanol as co-substrate (Lessmeier et al., 2015). Later, this was also shown for flavanone production by engineered *E. coli* (Whitaker et al., 2017). Following an approach first described for CO_2-essential growth, namely, decoupling of carbon fixation from provision of reducing power and energy that is supplied by an organic cosubstrate (Antonovsky et al., 2016), methanol-essential growth of *E. coli* was achieved (Meyer et al., 2018). Afterwards, methanol-dependent growth of engineered *C. glutamicum* was shown (Tuyishime et al., 2018) as well as *E. coli* or *C. glutamicum* strains engineered for methanol-dependent production of L-glutamate (Tuyishime et al., 2018) or other fine chemicals (Bennett et al., 2018; Chen et al., 2018; Wang et al., 2019; Zhang et al., 2018d). A modified serine cycle for formaldehyde assimilation was also used in *E. coli* for conversion of methanol and carbon dioxide to products (Yu and Liao, 2018). Formate is another C1 compound and its assimilation has been engineered in *E. coli* by reversing a reaction of amino acid metabolism: the glycine cleavage system (Bar-Even et al., 2013; Doring et al., 2018; Tashiro et al., 2018; Yishai et al., 2016, 2017, 2018), but formate dependent amino acid production has yet to be shown.

7. L-Serine and L-cysteine as examples of proteinogenic specialty amino acids

The biosynthesis of L-serine and L-cysteine is linked to C1 metabolism in bacteria. Thus, there overproduction has to take into account this central position in carbon metabolism.

7.1. Metabolic engineering advances for L-serine

L-serine production has been achieved in *C. glutamicum* and *E. coli*.

First, the serine pathway genes were overexpressed, production benefitted from the overexpression of a feedback-resistant variant of the initial enzyme (Peters-Wendisch et al., 2002). Second, degradation of L-serine to pyruvate was avoided by deletion of *sdaA* encoding serine deaminase. It was most important to down-regulate expression of *glyA* encoding serine hydroxylmethyl transferase, the enzyme that interlinks L-serine and glycine biosynthesis with C1 metabolism and the C1 carrier tetrahydrofolate (Peters-Wendisch et al., 2005). Since this strain is unstable, an alternative approach to decouple C1 metabolism from L-serine overproduction was developed (Stolz et al., 2007). When the para-aminobenzoate biosynthesis operon was deleted, L-serine production and growth responded to external folate supply in a dose-dependent manner. In a 20 L controlled bioreactor operated in fed-batch mode, a titer of 36 g/L-serine was reached and the strain found commercial application by Amino (Stolz et al., 2007). Replacing folate with cornsteep liquor led to an L-serine titer of 43 g/L by a different strain with a very similar yield (0.21 instead of 0.22 g/g) (Zhu et al., 2015). The yield could be increased when the full repertoire of metabolic engineering strategies was used (deletion genes coding for serine specific transaminases, the AceE subunit of pyruvate dehydrogenase, the repressor IolR and overexpression of the glycolytic genes *ppgK, pgi, pfkA,* and *gapA*) and a yield of 0.30 g/g was achieved, albeit only at a titer of 30 g/L (Zhang et al., 2019a). The L-serine yield achieved with metabolically engineered *E. coli* was higher (0.43 g/g) (Mundhada et al., 2016). Based on the *E. coli* wild-type strain MG1655, serine degradation to pyruvate and glycine was blocked by deletion of three L-serine deaminase genes (*sdaA, sdaB,* and *tdcG*) and the gene for serine hydroxyl methyl transferase (*glyA*). Native *serB* and *serC* were overexpressed in combination with a *serA* allele encoding a feedback insensitive version of 3-phosphoglycerate dehydrogenase with alanyl residues at positions 344, 346, and 364. However, *E. coli* suffered from L-serine toxicity. After adaptive laboratory evolution tolerance towards L-serine was increased. Even higher L-serine tolerance resulted from overexpression of cysteine/acetylserine exporter gene *eamA*. While the highest yield of a 0.43 g/g glucose was also achieved in fed-batch cultivation at the 1 L scale (Mundhada et al., 2016), the titer of about 12 g/L was low compared to the *C. glutamicum* strains (Stolz et al., 2007; Zhu et al., 2015). This may be attributed to toxicity of the side-products hydroxypyruvate (about 0.5 g/L) and 2-hydroxyglutarate (about 5 g/L) which likely arise by YeaB dephosphorylating the L-serine biosynthesis intermediate phosphohydroxypyruvate and by promiscuity of 3-phosphoglycerate dehydrogenase (accepting 2-oxoglutarate) (Mundhada et al., 2016). This limitation could be overcome by adaptive laboratory evolution and 37 g/L were achieved albeit at a reduced yield of 0.24 g/g (Mundhada et al., 2017). Taken together, *E. coli* and *C. glutamicum* strains with similar L-serine titers and yields have been obtained.

7.2. Metabolic engineering advances for L-cysteine

C. glutamicum has recently been engineered for production of L-cysteine using the full repertoire of metabolic engineering strategies (Joo et al., 2017a,b; Kondoh and Hirasawa, 2019; Wei et al., 2019). The serine biosynthesis pathway genes were overexpressed and conversion of L-serine to glycine and pyruvate was blocked by deletion of serine deaminase and serine dehydratase genes (*glyA* and *serA*) as shown previously for L-serine production (Peters-Wendisch et al., 2005; Stolz et al., 2007). In addition, the L-cysteine desulfhydrases gene was deleted to avoid L-cysteine degradation to pyruvate and the genes coding for feedback-resistant serine acetyltransferase ($cysE^{FBR}$) and L-cysteine synthase (*cysK*) were overexpressed. When thiosulfate was used as sulfur source, a titer of about 1 g/L L-cysteine was achieved (Wei et al., 2019). Higher titers have been achieved with *Pantoea annanatis* (2 g/L) (Takumi et al., 2017) and *E. coli* (5.1 g/L) (Liu et al., 2018b) using similar metabolic engineering approaches. Noteworthy, the *E. coli* L-cysteine process developed by Wacker (Maier, 2003) has been diversified to provide a whole spectrum of sulfur-containing unnatural L-α-amino acids when overproduction of O-acetylserine and the broad substrate specificity of O-acetylserine sulfhydrylase was exploited for in vivo incorporation of an unnatural side chain (Maier, 2003). Interestingly, product titers of these unnatural L-α-amino acids exceeded that of L-cysteine (up to 13 g/L as compared to 3.8 g/L L-cysteine). It is not known whether *C. glutamicum* that supports lower L-cysteine titers (about 1 g/L) would be suitable to produce unnatural L-α-amino acids following the Wacker approach.

8. Specialized amino acids: non-proteinogenic cyclic amino acids and omega amino acids as examples

The cyclic amino acids L-pipecolic acid (L-PA) and ectoine as well as the omega-amino acids gamma-aminobutyric acid (GABA) and 5-aminovalerate (5AVA) may serve as examples of specialized non-proteinogenic amino acids produced by engineered *E. coli* and *C. glutamicum* strains.

8.1. Metabolic engineering advances for cyclic amino acids

L-PA is related to the cyclic proteinogenic amino acid L-proline and also called L-homoproline. L-PA serves as an osmoprotectant for *E. coli* (Gouesbet et al., 1994) and *C. glutamicum* (Pérez-García et al., 2019). It finds applications as precursor of immunosuppressants (Maddess et al., 2008), peptide antibiotics and piperidine alkaloids (Clevenstine et al., 1979). L-PA and *N*-hydroxy-L-PA are critical elements of plant systemic immunity (Hartmann et al., 2018; Navarova et al., 2012). L-PA can be generated from L-lysine by L-lysine cyclodeaminase (LCD) e.g. in *Streptomyces pristinaespiralis* (Gatto et al., 2006; Tsotsou and Barbirato, 2007) or by L-lysine 6-aminotransferase (LAT) and pyrroline-5-carboxylate reductase (Fujii et al., 2002) or by L-lysine α-oxidase from *Scomber japonicus* and Δ(1)-piperideine-2-carboxylae reductase (Tani et al., 2015). Finally, L-lysine can be oxidized by L-lysine 6-dehydrogenase (LysDH) from *Silicibacter pomeroyi*, followed by spontaneous cyclization and reduction by pyrroline-5-carboxylate reductase (Perez-Garcia et al., 2016). *E. coli* strains produced about 5 g/L L-pipecolic acid with a yield of 0.13 g/g of glucose in fed-batch cultivation when the LCD pathway was used (Ying et al., 2017) and about 47 g/L with a yield of 0.89 g/g when the LAT pathway was used (Cheng et al., 2018b). A *C. glutamicum* process based on the LysDH pathway yielded about 14 g/L L-PA at a yield of 0.2 g/g (Perez-Garcia et al., 2017a,b). The latter process was extended for L-PA production from alternative carbon sources (Perez-Garcia et al., 2017a,b; Sgobba et al., 2018a,b) and was used in a synthetic consortium (Sgobba et al., 2018a,b).

Ectoine, as L-PA, is a cyclic non-proteinogenic amino acid with a function as osmoprotectant in *E. coli* and *C. glutamicum* (Czech et al., 2018; Pastor et al., 2010). As its biosynthesis shares aspartate semialdehyde as a common intermediate with L-lysine biosynthesis *Pseudomonas stutzeri* ectoine gene cluster *ectABCD* was expressed in a lysine producing *C. glutamicum* strain (Becker et al., 2013). In a fed-batch process, 4.5 g/L ectoine was produced with a yield of 0.24 g/g (Becker et al., 2013). Using a genome-reduced, more advanced *C. glutamicum* strain expressing *ectABC* gene cluster of *Chromohalobacter salexigens* allowed for ectoine production to a titer of 22 g/L, with a productivity of 7.7 g/L/d (Perez-Garcia et al., 2017a,b). In a similar approach, recombinant *E. coli* produced about 25 g/L ectoine by fed-batch fermentation with a yield of 0.11 g/g (Ning et al., 2016).

8.2. Metabolic engineering advances for omega-amino acids

The omega-amino acid GABA finds application mainly as a building block for pharmaceuticals, foods and biodegradable plastics (Jorge et al., 2016). By contrast, the alpha-amino acid L-2-aminobutyrate that recently has been produced by an engineered *E. coli* strain (after conversion of an L-threonine producing strain by deletion of L-threonine export genes and expression of the leucine dehydrogenase gene from

Thermoactinomyces intermedius) is a precursor of many chiral drugs (Xu et al., 2019). The most straightforward pathway leading to GABA is decarboxylation of the proteinogenic amino acid L-glutamate by glutamate decarboxylase. As L-glutamate is abundantly available from the *C. glutamicum* MSG process, its biocatalytic conversion to GABA has been attempted with isolated glutamate decarboxylase or in whole-cell biotransformation e.g. using lactobacilli or recombinant *E. coli* (Fan et al., 2018; Lyu et al., 2018). *E. coli* has been engineered for GABA production via glutamate decarboxylase using CRISPR genome editing (Cho et al., 2017), scaffolding *Neurospora crassa* glutamate decarboxylase with *E. coli* GABA transporter (Somasundaram et al., 2017), and systems metabolic engineering (Soma et al., 2017). GABA production from xylose via the Weimberg pathway was achieved as well (Zhao et al., 2017). Since *C. glutamicum* is known for its capacity to overproduce L-glutamate, the basis of a million-ton-scale business, it was obvious to engineer this bacterium for GABA production. To this end, the glutamate decarboxylase genes from either *Lactobacillus brevis* (Shi and Li, 2011) or *E. coli* (Okai et al., 2014; Takahashi et al., 2012) have been used. The process was improved by coexpression of two glutamate decarboxylase alleles (Shi et al., 2013) and by deregulation of the TCA cycle by deleting the serine/threonine protein kinase G gene (Okai et al., 2014) or by deletion of the gene for the 2-oxoglutarate decarboxylase subunit gene OdhA (Wang et al., 2015). A completely independent approach was chosen by developing a synthetic metabolic route involving conversion of L-ornithine via putrescine and γ-aminobutyraldehyde to GABA (Jorge et al., 2016). To this end, an L-ornithine producing strain was endowed with L-ornithine decarboxylase SpeC, putrescine transaminase PatA and γ-aminobutyraldehyde dehydrogenase PatD genes. GABA degradation was avoided by deletion of the γ-aminobutyrate aminotransferase, succinate-semialdehyde dehydrogenase, and GABA-specific importer genes (Jorge et al., 2016). The metabolic dead-end conversion to *N*-acetyl-diaminobutane was abolished and TCA cycle flux was redirected in a similar manner as described above for *E. coli* allowing GABA production to a titer of 63 g/L (Jorge et al., 2017a,b). Besides glucose, also xylose, glucosamine and *N*-acetyl-glucosamine were used as substrates for GABA production (Jorge et al., 2017a,b). Approaches to lactamization of GABA are described below.

The interest in 5-aminovalerate (5AVA) is mainly due to its use as building block for bio-plastics. 5AVA arises from amino acid degradation, e.g. from L-proline via a two-step pathway with proline racemase and D-proline dehydrogenase (Kenklies et al., 1999) or from L-lysine by action of L-lysine monooxygenase and 5-aminovaleramidase (Revelles et al., 2007). *E. coli* was engineered for conversion of L-lysine, which is abundantly available from the amino acid producing industry, by heterologous expression of the δ-aminovaleramidase and lysine 2-monooxygenase encoding genes from *P. putida* (Li et al., 2016b; Park et al., 2013, 2014a,b). Using this pathway, fermentative production of 5AVA without externally added L-lysine was also achieved in L-lysine overproducing *E. coli* (Adkins et al., 2013) and *C. glutamicum* (Joo et al., 2017a,b; Rohles et al., 2016; Shin et al., 2016) strains. A completely independent metabolic route to 5AVA was established in *C. glutamicum* (Jorge et al., 2017a,b). This strategy was inspired by the synthetic pathway leading to GABA via putrescine (Jorge et al., 2016) since it was hypothesized that cadaverine, the C5 equivalent of putrescine, would also be converted to 5AVA, the C5 equivalent of GABA (Jorge et al., 2017a,b). Therefore, an L-lysine producing strain was endowed with lysine decarboxylase, cadaverine/putrescine transaminase, and γ-aminobutyraldehyde dehydrogenase from *E. coli* and 5AVA was produced to a titer of 5.1 g/L after formation of the by-products cadaverine, *N*-acetyl-cadaverine and glutarate was abolished (Jorge et al., 2017a,b). 5AVA could also be produced from *Miscanthus* hydrolysates (Joo et al., 2017a,b), xylose, arabinose, glucosamine and starch (Jorge et al., 2017a,b).

8.3. Lactamization of omega-amino acids

GABA and 5AVA and omega-amino acids with longer chain lengths are polycondensated to bioplastics after being converted to lactams (e.g. γ-butyrolactam, δ-valerolactam and ε-caprolactam). Thus, enzymes supporting ring closure of the omega amino acids to the lactams were sought and found: acyl-CoA ligase either from *Clostridium propionicum* or *Streptomyces aizunensis* (Chae et al., 2017; Zhang et al., 2017a,b). These enzymes activate the omega-amino acid to its CoA ester in an ATP dependent manner, but it is currently unclear if ring closure of the omega-amino acyl-thioester is a spontaneous reaction or catalyzed by a hitherto unknown enzyme. A lactam biosensor was developed based on the transcriptional activator of the cyclohexanone degradation pathway in *Acinetobacter* sp. (Zhang et al., 2017a,b). A fermentative glucose-based fed-batch led to production of γ-butyrolactam to a titer of about 54 g/L by recombinant *E. coli* (Chae et al., 2017).

9. Fermentative production of amino acids functionalized by hydroxylation, halogenation or methylation

Besides non-proteinogenic cyclic amino acids and omega-amino acids also hydroxylated, halogenated and *N*-alkylated amino acids (Fig. 2) have been target of metabolic engineering.

9.1. Hydroxylated amino acids

The hydroxylated amino acids hydroxy-L-lysine, trans-4-hydroxy-L-proline, hydroxy-L-isoleucine, and hydroxy-L-tryptophan are pharmaceutically relevant, but hydroxylation via C—H bond activation in the absence of any harmful oxidizing reagents is technically difficult in chemical synthesis. Therefore, biocatalytic processes have been developed and transferred to *C. glutamicum* and/or *E. coli* fermentation processes in the case of hydroxy-L-proline, hydroxy-L-isoleucine, and hydroxy-L-tryptophan.

Trans-4-hydroxy-L-proline is an abundant component of mammalian collagen and can be used as chiral synthon in the pharmaceutical industry e.g. for the syntheses of anti-inflammatory drugs. Proline 4-hydroxylase, an enzyme belonging the Fe(II)/2-oxoglutarate-dependent dioxygenases, from *P. stutzeri, Bordetella bronchiseptica* and *Dactylosporangium* sp. have been tested, the latter of which supported the highest titers (Falcioni et al., 2015; Theodosiou et al., 2017; Wang et al., 2018a,b,c,d,e,f; Yi et al., 2014; Zhang et al., 2019b). Efficient fermentative production of trans-4-hydroxy-L-proline has been described when the proline 4-hydroxylase gene from *Dactylosporangium* sp. was expressed in L-proline producing *C. glutamicum* strains (Falcioni et al., 2015; Zhang et al., 2019b). Production of L-proline resulted from use of a feedback resistant glutamate 5-kinase (ProB ^{G446A}) and deletion of *sucCD* to increase supply of 2-oxoglutarate (Zhang et al., 2019a,b) or from bradytrophy for isoleucine in *C. glutamicum* (Falcioni et al., 2015) or from abrogation of proline degradation combined with flux enforcement by multiple deletions *ΔsucAΔaceAΔput*A in *E. coli* (Theodosiou et al., 2017; Zhang et al., 2018c). In each case, the canonical L-proline biosynthesis pathway was used in *E. coli* and *C. glutamicum*. An alternative L-proline biosynthesis pathway that operates via ornithine cyclodeaminase with high volumetric productivity and has been engineered in *C. glutamicum* (Jensen et al., 2015; Jensen and Wendisch, 2013) may be relevant to accelerate production of trans-4-hydroxy-L-proline.

(2S, 3R, 4S)-4-Hydroxyisoleucine (4-HIL) is a promising candidate for the treatment of diabetes due to its glucose-dependent insulinotropic activity. The stereo-specific L-isoleucine-4-hydroxylase, also named L-isoleucine dioxygenase IDO, was used to convert L-isoleucine to 4-HIL (Smirnov et al., 2010). Flux enforcement of an *E. coli* strain lacking 2-oxoglutarate dehydrogenase, isocitrate lyase, and isocitrate dehydrogenase kinase/phosphatase necessitating IDO activity to

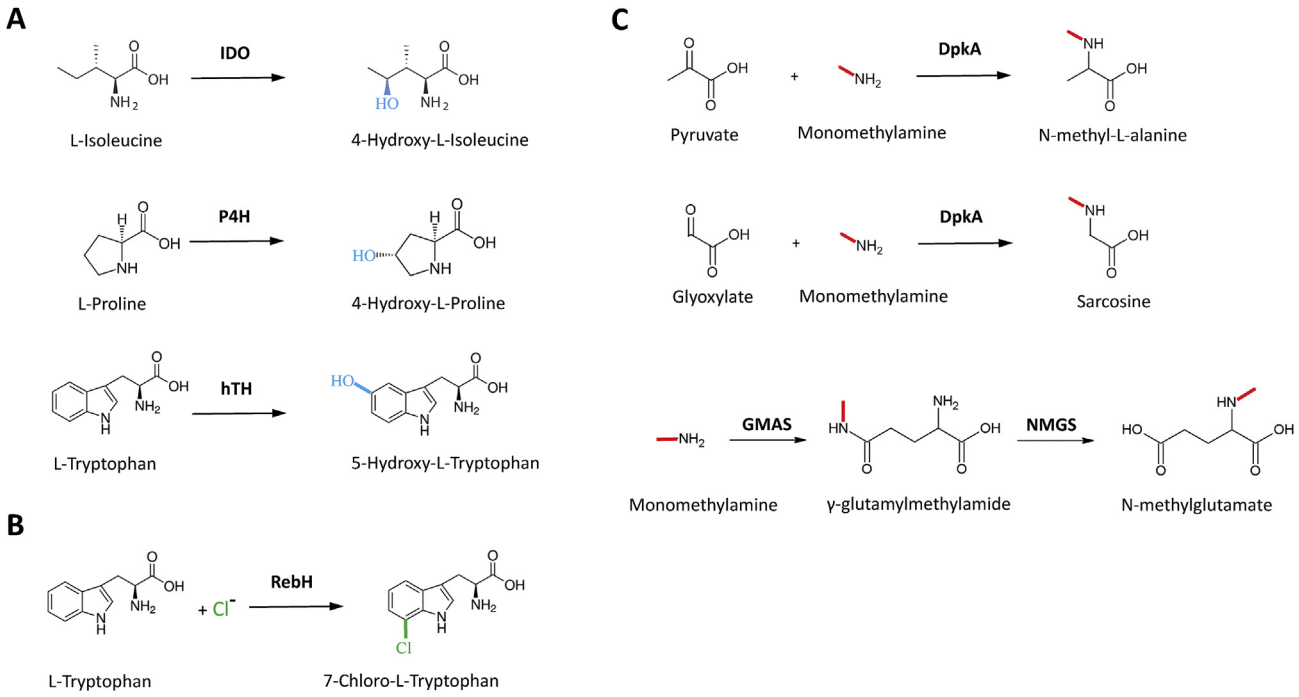

Fig. 2. Key enzyme reactions for hydroxylation (A), halogenation (B) and *N*-methylation (C) of amino acids used in metabolic engineering of amino acid overproducing *E. coli* and *C. glutamicum* strains. IDO, 2-oxoglutarate depended L-isoleucine hydroxylase from *Bacillus thuringiensis*; P4H, 2-oxoglutarate depended L-phenylalanine 4-hydroxylase from *Dactylosporangium* sp.; hTH, human 2-oxoglutarate depended L-tryptophan hydroxylase; RebH, flavin-dependent tryptophan halogenase from *Lechevalieria aerocolonigenes*; DpkA, imine reductase DpkA from *Pseudomonas putida*; GMAS, γ-glutamylmethylamide synthase from *Methylobacterium extorquens*; NMGS, *N*-methylglutamate synthase from *M. extorquens*.

complement the disrupted TCA cycle enabled biotransformation of L-isoleucine to 4-HIL with 82% yield (Smirnov et al., 2010), which later was improved by mutant IDO variants (Zhang et al., 2018a). Production of 4-HIL by *C. glutamicum* was also based on IDO and various mutations of central carbon metabolism have been tested (Shi et al., 2016, 2018). The best titer (about 34 g/L) and yield (0.15 g/g) were obtained when six genes involved in oxaloacetate and α-ketoglutarate conversion were overexpressed (*ppc,* and *pyc*) or deleted (*pck, pyk, gdh1*, and *gdh2*) and when OdhI^{T14A} was conditionally produced under the control of a biosensor (Mahr et al., 2015; Mustafi et al., 2012, 2014) based on the L-leucine, L-methionine, L-isoleucine, and L-valine responsive transcriptional activator Lrp (Lange et al., 2012) to fine-tune 2-oxoglutarate provision for 4-HIL production according to the biosynthetic demand (Zhang et al., 2018a).

5-hydroxy-L-tryptophan can be used against depression, insomnia, obesity, or chronic headaches. Variants of phenylalanine 4-hydroxylase obtained by sequence- and structure-based protein engineering enabled conversion of tryptophan to 5-hydroxy-L-tryptophan. Tetrahydromonapterin present in *E. coli* supported an exogenous recycling pathway and whole-cell bioconversion yielded about 1 g/L 5-hydroxy-L-tryptophan from tryptophan in shake flasks (Lin et al., 2014). This approach could be coupled to conversion of 5-hydroxy-L-tryptophan to serotonin based on L-tryptophan decarboxylase from *Catharanthus roseus* and about 0.15 g/L of serotonin were obtained (Mora-Villalobos and Zeng, 2018). In an independent approach, *E. coli* produced human tryptophan hydroxylase I along with the human tetrahydrobiopterin biosynthesis and regeneration pathways. Whole-cell bioconversion yielded about 1 g/L 5-hydroxy-L-tryptophan from 2 g/L L-tryptophan in shake flasks. After further improvements including use of a variant of human tryptophan hydroxylase 5.1 g/L 5-hydroxy-L-tryptophan were produced in a fed-batch bioreactor culture with glycerol as the carbon source (Wang et al., 2018a,b,c,d,e,f).

9.2. Halogenated amino acids

Halogenated amino acids are among the more than 5000 naturally occurring halogenated compounds (Schnepel and Sewald, 2017) including *i. a.* the antibiotics chloramphenicol and pyrroindomycin, the plant growth-regulating thienodolin, and the antifungal pyrrolnitrin (Hammer et al., 1997; van Pee and Holzer, 1999). Since halogenated compounds show metabolic stability and can be modified efficiently, e.g., by metal-catalyzed cross-coupling or nucleophilic substitution reactions, halogenated amino acids find applications in the pharmaceutical, chemical and agrochemical industries (Diederich and Stang, 2008). Chemical methods for halogenation are established, however, they require hazardous conditions, lead to unwanted byproducts and show poor regioselectivity. By contrast, metabolic pathways leading to halogenated compounds such as the rebeccamycin precursor 7-chloro-L-tryptophan (7-Cl-Trp) use halide ions and molecular oxygen. Biosynthesis of rebeccamycin, an indolocarbazole alkaloid antibiotic from *Lechevalieria aerocolonigenes* inhibiting DNA topoisomerase I (Nishizawa et al., 2005; Onaka et al., 2003) involves two enzymes for halogenation of L-tryptophan: FADH-dependent halogenase RebH and NADH-dependent flavin reductase RebF (Nishizawa et al., 2005). NADH-dependent flavin reductase recycles the reduced cofactor $FADH_2$. Isolated enzymes and crude extracts of *E. coli* cells producing these enzymes have been used for chlorination of L-tryptophan and using cross-linked enzyme aggregates including alcohol dehydrogenase for NADH recycling with isobutanol led to production of 7-Cl-Trp to the gram scale (Frese and Sewald, 2015; Schnepel et al., 2016). Recently, an L-tryptophan producing *C glutamicum* strain has been engineered for production of 7-Cl-Trp by overexpression of *rebH* and *rebF* from *L. aerocolonigenes* (Veldmann et al., 2019). Production of 7-Cl-Trp based on the alternative carbon sources arabinose, xylose and glucosamine that do not have competing uses for human and animal nutrition was shown as well. Since the regioselectivity of the related halogenase *Tha*I from *Streptomyces albogriseolus* (Moritzer et al., 2019) could be changed

based on determination of its 3D structure and since halogenases preferring bromination over chlorination are known (Neubauer et al., 2018), it is foreseeable that fermentative production not only of 7-Cl-Trp, but also of 5-Cl-Trp, 6-Cl-Trp, 5-Br-Trp, 6-Br-Trp, and 7-Br-Trp is possible.

9.3. *N*-alkylated amino acids

N-alkylated amino acids occur in nature and have physiological functions in bacteria, archaea and eukaryotes. *N*-alkylated amino acids are, for example, present in the mycotoxin destruxin B and the immunosuppressant cyclosporine A (Wu et al., 2013). As consequence of *N*-alkylation amino acids are more lipophilic and peptides containing them are less prone to proteolysis. Therefore, *N*-alkylated amino acids are sought after in order to stabilize peptide-based drugs and to increase their membrane permeability (Di Gioia et al., 2016). As an example for improved pharmacokinetics, *N*-methylation of tubulysin increased its bioavailability and antimitotic activity as compared to tubulysin (Patterson et al., 2008). Since chemical syntheses of *N*-methylated amino acids suffers from incomplete stereoselectivity and low yields, two independent fermentative routes have been developed.

The first fermentative route makes use of the fact that *N*-methylglutamate is an intermediate of monomethylamine assimilation by *Methylobacterium extorquens*, a methylotrophic bacterium able to use several carbon sources without C—C bonds. Metabolic engineering of *P. putida* for heterologous expression of the genes for two enzymes of this monomethylamine assimilation pathway, namely *N*-methylglutamate synthase (NMGS) and γ-glutamylmethylamide synthetase (GMAS), enabled *N*-methylglutamate production from glucose and monomethylamine (Mindt et al., 2018b). Oxoglutarate formed during growth with glucose is methylaminated at its C2 position by NMGS to yield *N*-methylglutamate in a reaction that resembles reductive amination of oxoglutarate by glutamate dehydrogenase. Alternatively or in parallel, a two-step pathway resembling the reaction sequence for conversion of glutamine and oxoglutarate to two molecules of glutamate catalyzed by glutamine synthetase (GS) and glutamate synthase (GOGAT) in many bacteria, GMAS first amidates glutamate at its C5 position before NMGS catalyses the transfer of the *N*-methyl group of γ-glutamylmethylamide to 2-oxoglutarate (Mindt et al., 2018b). The base strain was then improved by derepression of glycerol catabolism (deletion of *glpR*) and overexpression of glutamate dehydrogenase GdhA. After medium optimization, a bioreactor culture produced about 18 g/L N-methylglutamate from glycerol and monomethylamine in fed-batch mode (Mindt et al., 2018b).

Fermentative production of *N*-methylated amino acids via the potentially more versatile second route has been developed for *C. glutamicum* and the two *N*-methylated amino acids sarcosine and *N*-methyl-L-alanine (Mindt et al., 2018a, 2019). Of central importance is the imine reductase (IRED) DpkA from *P. putida*, an enzyme actively reducing imine bonds in cyclic and aliphatic compounds. In *P. putida*, DpkA is part of the catabolic D-lysine degradation pathway reducing piperideine-2-carboxylate to L-pipecolic acid, however, this IRED also catalyses the conversion of some 2-oxo acids such as pyruvate and monomethylamine to yield the respective *N*-methyl-L-amino acids, possibly because monomethylamine and 2-oxo acids spontaneously form imines that in turn are substrates of reduction by DpkA (Mihara et al., 2005; Muramatsu et al., 2005). As first demonstration, *N*-methyl-L-alanine was efficiently produced by fermentation in mineral salts medium containing monomethylamine by engineered *C. glutamicum* overproducing pyruvate and expressing *dpkA* (Mindt et al., 2018a). *C. glutamicum* strain ELB-P was used as base strain since it overproduced pyruvate due to systems metabolic engineering (Wieschalka et al., 2012). In this strain conversion of pyruvate to acetyl-CoA (deletion of *aceE* encoding the E1p subunit pyruvate dehydrogenase), acetate (deletion of *pqo* encoding pyruvate-quinone oxidoreductase), L-lactate (deletion of *ldhA* encoding fermentative NAD-dependent L-lactic acid dehydrogenase), branched-chain amino acids (deletion of *ilvN* encoding the C-terminal regulatory domain of acetohydroxyacid synthase), and to L-alanine (deletion of *alaT* and *avtA* encoding alanine aminotransferase and valine-pyruvate aminotransferase, respectively) has been blocked (Wieschalka et al., 2012). Growth of *C. glutamicum* ELB-P requires acetate due to the *aceE* deletion, which allows to decouple growth from pyruvate production from the second carbon source added (e.g. glucose) (Wieschalka et al., 2012). After the ratios of the medium components ammonium sulfate and urea as nitrogen sources and monomethylamine as substrate for *N*-alkylation as well as acetate and glucose as carbon and energy sources were optimized, the process was transferred from shake flasks to 4 L bioreactor cultivation (Mindt et al., 2018a). A fed-batch process enabled *N*-methyl-L-alanine production to a titer of about 31.7 g/L with a product yield on glucose of 0.71 g/g and a volumetric productivity of 0.35 g/L/h. Also blends of acetate with starch or the lignocellulosic pentose sugars xylose and arabinose allowed for efficient *N*-methyl-L-alanine production (Mindt et al., 2018a).

A metabolic pathway involving DpkA and leading to sarcosine, an *N*-methylated amino acid with potential as antipsychotic drug and building block for peptide-based drugs or detergents, has recently been developed (Mindt et al., 2019). It could be demonstrated that DpkA also accepts glyoxylate as 2-oxo acid for *N*-methylamination with good catalytic activity (Mindt et al., 2019). Based on this finding, *dpkA* was expressed in a *C. glutamicum* strain engineered for glyoxylate overproduction (Zahoor et al., 2014) and, when monomethylamine was added to mineral salts media efficient fermentative production of sarcosine resulted. The glyoxylate overproducing strain was derived from a glycolic acid overproducing strain and it lacks glyoxylate converting malate synthase (Δ*aceB*) and has less than 20% isocitrate dehydrogenase enzyme activity (initiation codon changed from ATG to GTG) (Zahoor et al., 2014). Fortunately, sarcosine production based on the second generation feedstocks xylose and arabinose led to higher product titers than glucose-based production. Shake flask cultivation allowed for sarcosine production from xylose with a product titer of 8.7 ± 0.2 g/L within 72 h (Mindt et al., 2019).

Of the two fermentative pathways leading to *N*-alkylated amino acids, the catabolic monomethylammonium pathway from *M. extorquens* is characterized by high substrate specificity (Gruffaz et al., 2014), but the IRED pathway is more versatile. DpkA from *P. putida* accepts several 2-oxo acids (glyoxylate, pyruvate next to the native substrate piperidei*N*-2-carboxylate) as well as several the alkyl amines monomethylamine and monoethylamine (Mihara et al., 2005; Muramatsu et al., 2005). Since DpkA converts glyoxylate with monomethylamine as well as with monoethylamine (Mindt et al., 2019), fermentative routes to *N*-ethylated amino acids will likely be developed in the future. Similarly, the substrate promiscuity of DpkA with respect to the 2-oxo acids will likely be used in the development of new processes to new *N*-alkylated amino acids. The approach of *N*-alkylating 2-oxo acids may be diversified in a similar manner as the conversion of 2-oxo acids to alcohols by decarboxylation using ketoisovalerate decarboxylase KivD from *Lactococcus lactis* followed by reduction using an aldehyde dehydrogenase (Liao et al., 2016). *E. coli, C. thermocullum, Synechococcus elongatus* and *C. glutamicum* strains developed for biofuel production (Atsumi et al., 2008, 2009; Blombach et al., 2011; Liao et al., 2016; Smith et al., 2010) may be reengineered to production of *N*-alkylated amino acids by exchanging the *kivD-adhA* module with DpkA and feeding mono alkylamines. Alternatively, *C. glutamicum* strains engineered for overproduction of 2-oxo acids (Bückle-Vallant et al., 2014; Jo et al., 2012; Kataoka et al., 2019; Krause et al., 2010; Li et al., 2016a; Lubitz and Wendisch, 2016; Vogt et al., 2015) as well as other strains engineered or selected for this purpose (Coban et al., 2014; Song et al., 2016) may be used as basis.

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