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Microbial production of amino acids and derived chemicals: Synthetic biology approaches to strain development

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Amino acids are produced at the multi-million-ton-scale with fermentative production of L-glutamate and L-lysine alone being estimated to amount to more than five million tons in the year 2013. Metabolic engineering constantly improves productivities of amino acid producing strains, mainly *Corynebacterium glutamicum* and *Escherichia coli* strains. Classical mutagenesis and screening have been accelerated by combination with intracellular metabolite sensing. Synthetic biology approaches have allowed access to new carbon sources to realize a flexible feedstock concept. Moreover, new pathways for amino acid production as well as fermentative production of non-native compounds derived from amino acids or their metabolic precursors were developed. These include dipeptides, α,ω-diamines, α,ω-diacids, keto acids, acetylated amino acids and ω-amino acids.

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Introduction

L-Amino acids find various applications in food and feed biotechnology as well as intermediates for the chemical industry [1]. Essential L-amino acids are used in human parenteral nutrition and L-glutamate and its salts are used as flavor enhancers in the food industry [1,2]. Fermentative production of L-amino acids in the million-ton-scale has shaped modern biotechnology [2]. The growth of the amino acid market, which is due a growing world population and a higher demand of animal products, drive strain optimization and amino acid process intensification.

Amino acid producing strains of *Corynebacterium glutamicum*, which has been used safely for more than 50 years in food biotechnology, and of *Escherichia coli* are continuously improved using metabolic engineering approaches [1,2]. This review on metabolic engineering of amino acid production highlights new methods, new amino acid biosynthesis pathways, biotin-prototroph recombinant *C. glutamicum* and access to alternative carbon sources. Moreover, products derived from amino acids or their precursors will be described. For production of non-amino acid derived products such as carotenoids [3,4], polymers [5], fuels [6] organic acids such as glycolate [7], the reader is referred to recent reviews, e.g. [8,9,10,11,12].

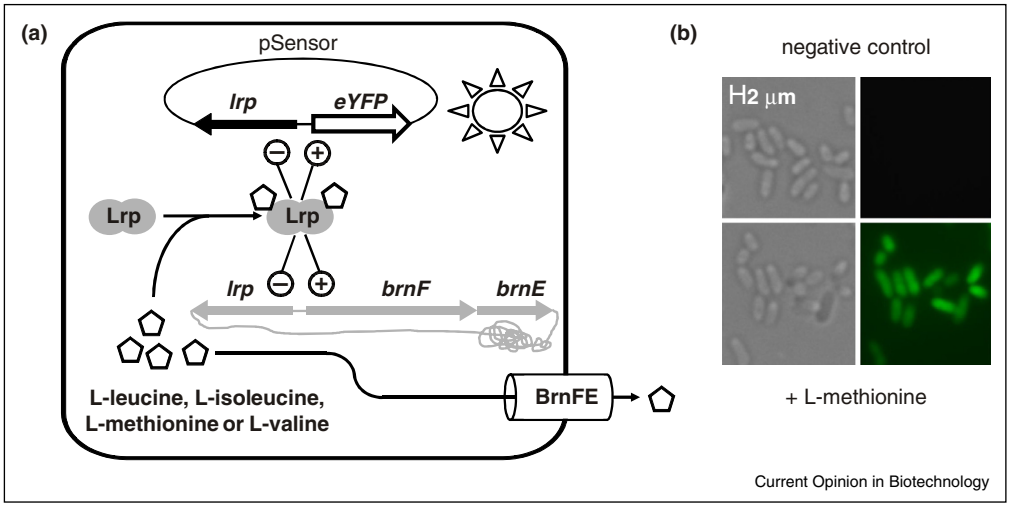
Strain improvement by classical mutagenesis combined with metabolite sensors

C. glutamicum, the workhorse of fermentative amino acid production, has been isolated as L-glutamate-secreting bacterium in auxanographic plate assays using L-glutamate requiring indicator strains [2]. Similarly, an acidophilic *Pantoea ananatis* strain resistant to high L-glutamate concentrations was isolated and shown to produce L-glutamate to saturating concentrations allowing product recovery by crystallization [13•]. On the basis of transcriptional regulatory proteins and their metabolite binding capacities sensor strains have been developed, e.g. high intracellular L-lysine concentrations are sensed by transcriptional activator LysG and the *lysE* promoter is activated and sensor cells fluoresce due to transcriptional coupling of the *lysE* promoter to a fluorescent protein gene [14•]. After chemical mutagenesis and FACS sorting mutants with high intracellular L-lysine levels could be isolated, some of which also excreted L-lysine [14•]. On the basis of the transcriptional regulator Lrp [15], this principle was used to isolate branched-chain amino acid producing mutants [16•] (Figure 1). Sensor strains for detection of O-acetyl serine and O-acetyl homoserine, of serine and of arginine have also been developed [14•]. Even indirect coupling to transcriptional regulation, e.g. activation of *E. coli* SoxR by increasing cellular NADPH demand, helped screening for variants of NADPH-dependent enzymes [17,18].

Metabolic engineering to improve L-lysine production and to access alternative carbon sources

New variants of aspartokinase beneficial for L-lysine production were identified either based on screening mutant libraries [19] or by rational deregulation of allosteric inhibition guided by co-evolutionary analysis [20•].

Figure 1



LRP-based fluorescent metabolite sensor. **(a)** *C. glutamicum* WT transformed with a sensor plasmid encoding transcriptional regulator Lrp and a *eYFP* gene driven by the promoter of the brnFE operon [16•]. Plasmid encoded genes are given in black, chromosomal genes are given in gray. Lrp is activated by binding to L-methionine or branched-chain amino acids (depicted as pentagons) and represses transcription of its own gene and while activating transcription of the chromosomal *brnFE* operon [15] or the *eYFP* gene on the sensor plasmid. L-Methionine and branched-chain amino acids are exported by two-component transport system BrnFE. **(b)** Phase contrast and fluorescence microscopy images of *C. glutamicum* WT cells transformed with the sensor plasmid during feeding with L-lysyl-L-alanine dipeptide (upper panel) or with L-alanyl-L-methionine dipeptide (lower panel). Figure (b) is modified from [16•] and the photograph was kindly provided by Julia Frunzke, Jülich.

The latter approach was also applied to dihydrodipicolinate synthase and phosphoenolpyruvate carboxykinase, which is subject to complex genetic regulation [21,22,23].

Improving *C. glutamicum* strains mainly focused L-lysine production from glucose, however, L-lysine production, e.g. from crude glycerol [24], starch [25,26], glucans [27], lignocellulosics [28•,29,30], grass juice [31] or components of chitin [32,33] has also been described. Unlike *E. coli* and *S. cerevisiae*, *C. glutamicum* typically does not show sequential carbon source utilization [34], but simultaneous utilization of carbon sources present in blends [35]. This advantage of *C. glutamicum* prevails even when exogenous carbon utilization pathways were introduced, e.g. for co-utilization of glucose with glycerol [36], xylose and arabinose [28•], or of cellobiose with xylose and glucose [37].

Metabolic engineering of L-ornithine cyclodeaminase-based L-proline production

L-Proline, the only proteinogenic amino acid with a secondary amine, is used as feed additive, organocatalyst and chemical synthon [1,2]. Besides extraction from protein hydrolysates, fermentative L-proline production is known. L-Proline is synthesized from L-glutamate by γ-glutamyl kinase, γ-glutamyl phosphate reductase, spontaneous cyclization and pyrroline-5-carboxylate reductase (Figure 2), but can also be synthesized from L-ornithine in plants, animals, and some bacteria (Figure 2). Classical strain development targeted proline degradation and/or feedback-deregulation of γ-glutamyl kinase [1,2]. Recently, production of about 13 g/L L-proline with a yield of 0.36 g L-proline per g glucose via the L-ornithine pathway was achieved [38•]. This required plasmid-borne expression of the ornithine cyclodeaminase gene from *Pseudomonas putida* for conversion of L-ornithine to L-proline and ammonia. Moreover, conversion of L-ornithine toward L-arginine was blocked by deletion of *argF*, the *arg* operon was derepressed by deletion of L-arginine repressor gene *argR* and a feedback-deregulated N-acetylglutamate kinase was produced [38•].

Overcoming biotin auxotrophy of *C. glutamicum*

C. glutamicum is auxotrophic for biotin. Biotin limitation elicits L-glutamate production while production of L-lysine requires sufficient biotin [1]. *C. glutamicum* takes up biotin by the energy-coupling factor transporter BioYMN [39] and deletion of *bioY* caused biotin hyperauxotrophy [40••] (Figure 3). *C. glutamicum* possesses only two biotinylated proteins, pyruvate carboxylase and acetyl-CoA carboxylase, which are biotinylated by biotin-protein ligase BirA [41] (Figure 3). Surprisingly, biotin auxotrophic *C. glutamicum* possesses functional biotin transcriptional regulator BioQ [42] and BioA, BioD, and BioB, the enzymes for the final reactions of biotin synthesis [2]. *C. glutamicum* lacks *bioF* (Figure 3) and heterologous expression of *bioF* from *E. coli* allowed for biotin synthesis only from externally added pimelic acid suggesting its inability for *de novo* synthesis of pimelate thioester as biotin precursor [43••]. In the *E. coli* BioC–BioH pathway, BioC methylates malonyl-CoA followed by two elongation cycles in fatty acid