Analysis and Engineering of Metabolic Pathway Fluxes in *Corynebacterium glutamicum*

Christoph Wittmann

Dedicated to Prof. Dr. Dietmar Hempel on the occasion of his 65th birthday

Abstract The Gram-positive soil bacterium *Corynebacterium glutamicum* was discovered as a natural overproducer of glutamate about 50 years ago. Linked to the steadily increasing economical importance of this microorganism for production of glutamate and other amino acids, the quest for efficient production strains has been an intense area of research during the past few decades. Efficient production strains were created by applying classical mutagenesis and selection and especially metabolic engineering strategies with the advent of recombinant DNA technology. Hereby experimental and computational approaches have provided fascinating insights into the metabolism of this microorganism and directed strain engineering. Today, *C. glutamicum* is applied to the industrial production of more than 2 million tons of amino acids per year. The huge achievements in recent years, including the sequencing of the complete genome and efficient post genomic approaches, now provide the basis for a new, fascinating era of research – analysis of metabolic and regulatory properties of *C. glutamicum* on a global scale towards novel and superior bioprocesses.

Keywords ^{13}C metabolic flux analysis, Anaplerosis, Genome-scale model, Glutamate, Lysine, Metabolic engineering, NADPH, Systems biology, Systems metabolic engineering, Tryptophan

Contents

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1   Introduction

C. glutamicum was discovered about 50 years ago. Stimulated by the increasing demand for L-glutamate as a flavor enhancer in human nutrition, a screening program in Japan led to the isolation of a soil microorganism, later classified as *C. glutamicum*, which was able to accumulate L-glutamate in the growth medium [1, 2]. Subsequent analysis revealed that glutamate secretion could be triggered by a limited supply of biotin, opening the possibility for industrial production. The application potential of *C. glutamicum* in biotechnology soon increased with successful manipulation of key regulatory properties that allowed accumulation of other amino acids such as lysine, threonine, arginine, or ornithine [3]. These findings initiated intensive research over the past decades. One of the key areas of research was the development of efficient production strains of *C. glutamicum* for which different strategies were pursued. This included classical mutagenesis and selection. With the advent of recombinant DNA technology, targeted genetic engineering of *C. glutamicum* became available and was successfully used to derive improved production properties. Today, *C. glutamicum* is applied to the industrial production of glutamate as flavor enhancer in human nutrition (1.5 million tons per year), [4], lysine (850,000 tons per year) [5] as well as tryptophan (10,000 tons per year) as additive in animal feed [6]. The improvement of existing production strains focuses on higher yield and productivity, better stress tolerance, or a broader substrate spectrum. In addition, current research also aims at the creation of efficient processes for biobased production of novel products involving other amino acids, organic acids, or biofuels. In this regard the present chapter highlights the major contributions dealing with investigation and optimization of the metabolic network of *C. glutamicum* for biotechnology. Hereby, concepts and approaches for systems biology analysis and engineering of metabolic pathways of *C. glutamicum* are presented and illustrated with actual examples.

2   Metabolism of *Corynebacterium glutamicum*

The extensive biochemical and physiological analysis of *C. glutamicum* during the past as reviewed in a recent handbook on *C. glutamicum* [7] provides a rich source of information on many of the enzymes and pathways present in this organism.

2.1   Nutritional Requirements and Assimilatory Pathways

C. glutamicum can use a multitude of organic compounds as sole carbon source or via coutilization. Sugars such as glucose, fructose, sucrose, or mannose are taken up by a phosphotransferase system [8, 9]. For glucose, an alternative uptake system involving intracellular phosphorylation of glucose by a glucokinase has been identified [10]. In addition, *C. glutamicum* can grow on different organic acids such as gluconate [11], lactate, [12], acetate [13], propionate [14], or citrate [15]. For growth and also for amino acid overproduction, cells further require nitrogen and sulfur as major elements in addition to carbon. Suitable nitrogen sources are ammonium or organic compounds such as urea or amino acids [16]. For the uptake of ammonium, two alternative systems are present. At high ammonium levels, assimilation is mainly catalyzed by the glutamate dehydrogenase (GDH), whereas a highly affine system comprising glutamine synthetase and GDH is active under low ammonium levels, although at higher energy expenditure [17]. Among suitable sulfur sources, inorganic sulfate is most common. Its assimilation, however, involves a high demand of redox power for reduction to the biologically compatible sulfide [18]. Other sulfur sources assimilated by *C. glutamicum* are cysteine, sulfonates, or sulfonate esters [18, 19]. With respect to industrial application the most important raw materials are based on molasses (Asia) and starch (America, Europe) applied together with inorganic salts [5]. Thus, the major nutrients utilized are glucose, sucrose and fructose (carbon), ammonia (nitrogen), and sulfate (sulfur).

2.2   Central Carbon Metabolism

Today the metabolic network of the central metabolism of *C. glutamicum* involving glycolysis, pentose phosphate pathway (PPP), TCA cycle as well as anaplerotic and gluconeogenetic reactions is well known (Fig. 1). Different enzymes are involved in the interconversion of carbon between TCA cycle (malate/oxaloacetate) and glycolysis (pyruvate/phosphoenolpyruvate). For anaplerotic replenishment of the TCA cycle, *C. glutamicum* exhibits pyruvate carboxylase [20] and phosphoenol-pyruvate (PEP) carboxylase as carboxylating enzymes. Malic enzyme [21] and PEP carboxykinase [22, 23] catalyze decarboxylation reactions from the TCA cycle
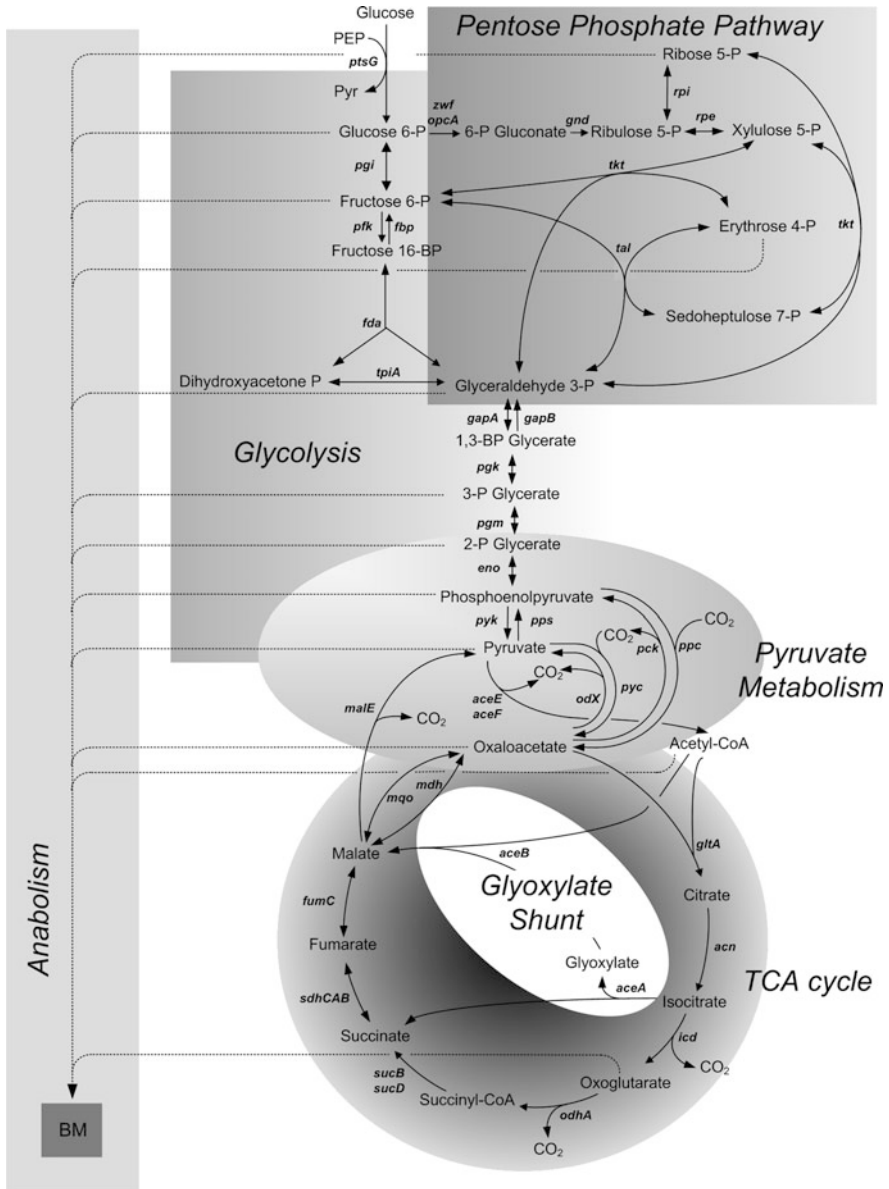
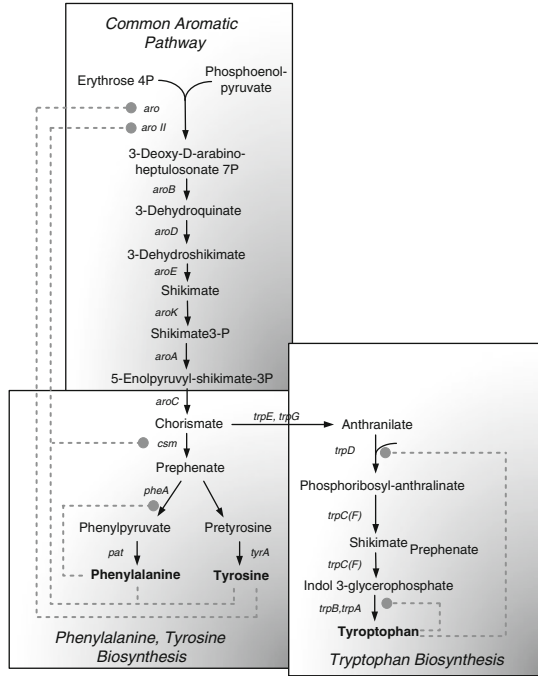**Fig. 1** Central metabolic pathways in *C. glutamicum*

towards glycolysis. As additional gluconeogenetic enzymes, oxaloacetate decarboxylase [24] and PEP synthetase [25] have been proposed. It has been proposed that cyclic cooperation of these carboxylating and decarboxylating enzymes around the pyruvate node is involved in the regeneration of excess ATP [22, 26, 27].

Different reactions are linked to the supply of NADPH. The major enzymes are glucose 6-phosphate dehydrogenase [28, 29] and 6-phosphogluconate dehydrogenase [28, 30] in the oxidative part of the PPP and the TCA cycle enzyme isocitrate dehydrogenase [31]. In selected cases, NADPH supply might involve malic enzyme [32, 33]. In addition to the central catabolism, anabolic routes have also been elucidated. Through rigorous analysis of the cellular composition, detailed information on the anabolic precursor demand was obtained [34]. These analyses were mainly driven by approaches for metabolic flux analysis in growing *C. glutamicum*, where anabolic reactions drain carbon, cofactors, and energy from central catabolism and thus have to be considered. In total, about (16.4 mmol NADPH) (g biomass)$^{-1}$ is required for anabolism. Considering a biomass yield of 0.5 (g dry biomass) (g glucose)$^{-1}$, which is achieved by *C. glutamicum* under aerobic conditions, this results in 1.7 mol NADPH (mol glucose)$^{-1}$ that have to be generated by the NADPH forming reactions in the PPP and the TCA cycle. This substantial anabolic NADPH requirement competes with demand for production pathways, e.g., towards lysine or methionine.
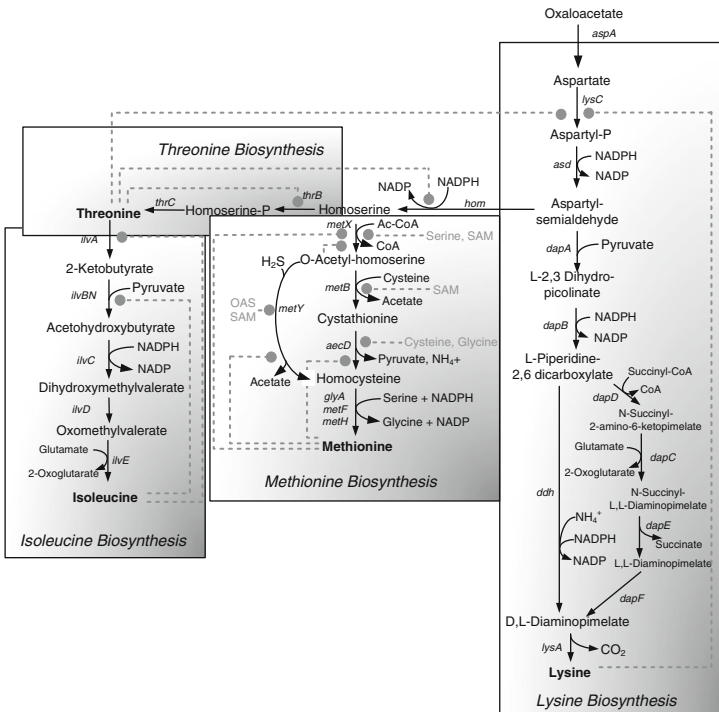
2.3 Biosynthetic Pathways Towards Biotechnology Products

Among the most important amino acids for industrial production are glutamate, the aromatic amino acids, and amino acids belonging to the aspartate family. The biosynthesis of these amino acids is closely linked to the central metabolism (Fig. 2). It is strictly controlled at several steps in *C. glutamicum* as is typically the case in microbial systems [35]. Obviously, the required precursor metabolites, cofactors and energy have to be supplied in appropriate amounts by the central catabolic routes, thus competing with the cellular requirements for growth. Particular problems with the synthesis of amino acids from the aspartate family are the long biosynthetic pathways and the highly connected network involving intermediates and reactions that are shared at the same time by different biosynthetic routes. Therefore, it is not surprising that multiple regulatory steps are required to ensure the balanced synthesis of all these metabolites for cellular demands (Fig. 2). An important branch point occurs at the level of aspartate semialdehyde, where the biosynthetic pathways separate. Upstream of this node is a key point for flux control of lysine biosynthesis. The responsible enzyme is aspartokinase, which catalyzes the formation of aspartylphosphate from aspartate. It is subjected to feedback inhibition by lysine and threonine [36, 37]. Downstream of this node, *C. glutamicum* exhibits a dual pathway for lysine biosynthesis, providing an increased flexibility in response to changing environmental conditions [38, 39]. The synthesis of methionine involves complex networks of regulatory interactions and metabolic pathways. Even with over-expressing of almost all genes involved in methionine biosynthesis via deletion of the central repressor McbR, *C. glutamicum* does not over-produce this amino acid as shown recently, pointing at other (regulatory) mechanisms that are limiting [40]. Due to extended research, complex and efficient

a

Common Aromatic Pathway

Erythrose 4P — Phosphoenol-pyruvate

● *aro*
● *aro II*

3-Deoxy-D-arabino-heptulosonate 7P
aroB
3-Dehydroquinate
aroD
3-Dehydroshikimate
aroE
Shikimate
aroK
Shikimate3-P
aroA
5-Enolpyruvyl-shikimate-3P
aroC
Chorismate → *trpE, trpG* → Anthranilate

● *csm*   *trpD* ●
Prephenate   Phosphoribosyl-anthranilate
pheA ●   *trpC(F)*
Phenylpyruvate   Pretyrosine   Shikimate Prephenate
pat   *tyrA*   *trpC(F)*
Phenylalanine **Tyrosine** Indol 3-glycerophosphate
                               trpB,trpA ●
                               Tryptophan

Phenylalanine, Tyrosine Biosynthesis

Tryptophan Biosynthesis

b

Oxaloacetate
aspA
Aspartate
lysC ●
Aspartyl-P
asd NADPH / NADP
Aspartyl-semialdehyde
dapA Pyruvate
L-2,3 Dihydropicolinate
dapB NADPH / NADP
L-Piperidine-2,6 dicarboxylate
dapD Succinyl-CoA / CoA
N-Succinyl-2-amino-6-ketopimelate
Glutamate *dapC*
2-Oxoglutarate N-Succinyl-L,L-Diaminopimelate
ddh *dapE* Succinate
NH₄⁺ / NADPH / NADP L,L-Diaminopimelate
dapF
D,L-Diaminopimelate
lysA → CO₂
Lysine

Lysine Biosynthesis

Threonine Biosynthesis

Threonine — *thrB* — Homoserine-P ● — Homoserine — *hom* —
NADP / NADPH
ilvA
2-Ketobutyrate
ilvBN Pyruvate
Acetohydroxybutyrate
ilvC NADPH / NADP
Dihydroxymethylvalerate
ilvD
Oxomethylvalerate
Glutamate *ilvE*
2-Oxoglutarate
Isoleucine

Isoleucine Biosynthesis

metX ● Ac-CoA / CoA
O-Acetyl-homoserine   Serine, SAM
H₂S Cysteine
metB Acetate   SAM
Cystathionine
OAS *metY* *aecD* ● Pyruvate, NH₄⁺   Cysteine, Glycine
SAM
Acetate Homocysteine
glyA Serine + NADPH
metF
metH Glycine + NADP
Methionine

Methionine Biosynthesis

feedback inhibition mechanisms have recently been identified in addition to extended transcriptional control [41, 42]. A similar complex picture is yielded for biosynthesis of the aromatic amino acids, where enzyme inhibition by feedback regulation and negative transcriptional control are involved [6, 43].

2.4   Genome Sequencing and Metabolic Network Reconstruction

These previous studies contributed significantly to our current understanding of the physiology of *C. glutamicum*. They each focused on only certain aspects of metabolism and investigated the corresponding reactions as isolated parts, so that an integrated view on metabolism of *C. glutamicum* as a functional network of highly interconnected reactions could not be provided. Such a holistic view, however, is highly desirable to understand the underlying complex system of metabolic and regulatory networks and derive ideas and concepts on how to engineer them in order to achieve superior production strains. As described above, the synthesis of industrial amino acids competes with cellular metabolism for building blocks and cofactors. As an example, pyruvate as precursor for lysine is potentially involved in more than 150 metabolic reactions of *C. glutamicum*. Keeping this in mind, we need a global picture of metabolism to understand the link between all these reactions and find the most efficient strategies to modify the flux to achieve optimum network performance. Metabolic flux analysis displays the first systems oriented approach to unravel the physiology of *C. glutamicum* since it combines experimental data with metabolic network models and allows determining absolute fluxes through larger networks comprising 100–200 reactions of central carbon metabolism [44]. A further milestone towards systems level understanding was the sequencing of the *C. glutamicum* ATCC 13032 genome by at least three different biotechnological companies (BASF, Degussa, and Kyowa Hakko). The circular genome comprises about 3000 genes with a total size of 3.3 kb [45–47]. Subsequent annotation has helped greatly in elucidating the genetic repertoire. Today, the genomic information of *C. glutamicum* is publicly available in bioinformatics data bases. Recommendable databases are provided by KEGG (www.genome.jp) or BioCyc (www.biocyc.com) and contain graphical information on genes, proteins, reactions, and pathways which is very useful when linking metabolites, enzymes and reactions to associate metabolism. The availability of genome information enabled new post genome technologies accelerating strain engineering by systems biology approaches. These comprise experimental and computational tools such as transcriptomics [48–51], proteomics [52–54], or in

Fig. 2 Metabolic pathways in *C. glutamicum* for biosynthesis of the aromatic amino acids tryptophan, tyrosine, and phenylalanine (**a**) and amino acids belonging to the aspartate family including lysine, methionine, threonine, and isoleucine (**b**). Metabolic regulation by feedback inhibition is indicated by *dotted lines*

silico pathway modeling [55, 56]. Meanwhile, sequence information is also available for other closely related species providing a detailed overview on the corynebacterial pan-genome and its metabolic pathways. Sequenced strains include the thermotolerant *C. efficiens* [57], *C. glutamicum* R [58], and the pathogens *C. diphtheriae* [59], *C. jeikeium* [60], and *C. kroppenstedtii* [61].

3 In Silico Metabolic Network Analysis

The engineering approach to analysis and design is using a mathematical or computer model. For target identification, modeling approaches are very useful to extract useful information on metabolic networks, their regulation and capacity. Models can be applied to predict phenotype behavior in response to different environmental or genetic perturbations, integrate complex data sets towards systems oriented understanding of network function, or design organisms with optimal network structure and activity. Overall, a wide variety of computational methods exploiting metabolic models have been developed and applied to *C. glutamicum*, yielding valuable insights into its metabolism and providing a sound basis for computer-assisted design in metabolic engineering. The type and complexity of the model hereby depends on the aim of the study. Although the ultimate goal of modeling is the development of dynamic models for the complete simulation of cellular systems, the success of such approaches has been severely hampered by the lack of kinetic information [62]. However, it is possible to assess accurately the theoretical capabilities and operative modes of metabolic systems using stoichiometric models.

3.1 Genome-Scale Reconstruction of the Metabolic Network

With the availability of annotated genome sequences, it has become possible to reconstruct genome-scale biochemical reaction networks for microorganisms. Genome-scale models have been reconstructed for almost 20 bacterial species so far [63–65]. Such models bridge the gap between genome-derived biochemical information and metabolic phenotypes and enable straightforward in silico experiments with whole-cell metabolism. Also for *C. glutamicum*, a genome-scale stoichiometric model was recently created [55]. As for other microorganisms, the construction was carried out in several steps. First a crude model consisting of mass balances for catabolic reactions was assembled from information on the present metabolic pathways and their stoichiometry available in KEGG and BioCyc. Subsequently, the anabolic reactions involved in polymer and biomass synthesis were defined whereby the information was collected from different literature sources. Finally the model was cured and completed by adding missing reactions and integrating information on reaction reversibility. The *C. glutamicum* model was validated against data found in the literature under different conditions such as different biomass production burdens

Table 1 Statistical data on the genome-scale stoichiometric model of *Corynebacterium glutamicum* ATCC 13032 constructed on basis of the genome with 3002 open reading frames [55]

Metabolic reactions	446
Total	213
Biochemical evidence available	209
Clear functional annotation	22
Tentative functional annotation	2
Putative functional annotation	
Metabolites	411
Total	356
Internal metabolites	55
External metabolites (substrates or products)	

and growth on different carbon sources. The model comprises 446 reactions and 411 metabolites (Table 1). Overall, it displays a systematic verification and compilation of data from various sources concerning the metabolic network of *C. glutamicum* and provides a useful basis for in silico studies as well a first step for future integration of complex data sets in systems biology approaches.

3.2   Stoichiometric Modeling

New genome wide stoichiometry based modeling of metabolic pathways is now possible. The stoichiometric data and models available provide the basis for various in silico applications differing in the methodology and algorithms used. Major contributions for *C. glutamicum* have been obtained from flux balancing [66] and elementary (flux) mode analysis [67]. In short, flux balance analysis is a constraint based method to analyze stoichiometric networks. Through introduction of specific biological constraints the set of various possible solutions for the typically under-determined metabolic system can be limited to a certain solution space. Within this space, optimal steady-state solutions can be calculated by minimizing or maximizing for objective functions such as growth or overproduction of a metabolite using linear programming techniques [68]. Accordingly, using flux balance analysis, a single solution is found to the optimization problem [69]. Hereby, flux balance analysis can highlight the most efficient pathway through the network in order to achieve the particular objective function. Elementary flux mode analysis systematically enumerates all independent minimal pathways through a network, each a unique elementary mode, that are stoichiometrically and thermodynamically feasible [70]. All possible steady-state flux distributions through the metabolic network are nonnegative linear combinations of the set of elementary modes [71]. This can be exploited to extract key properties from metabolic networks such as maximum

network capacity, optimal pathways, network robustness, or phenotype prediction in response to environmental or genetic perturbations [56, 72, 73].

3.2.1 Capacity of the Network of *C. glutamicum* for Lysine Production

For *C. glutamicum* stoichiometric network modeling has been utilized to assess the capacity for the production of lysine as one of the major industrial products [5, 11, 55, 74, 75]. The theoretical maximum molar yield of *C. glutamicum* for lysine production obtained by elementary flux mode analysis is 0.82 mol mol^{-1} [5]. Under these conditions of zero growth, PPP and malic enzyme supply the required NADPH, whereas the TCA cycle is shut off and lysine is exclusively formed via the dehydrogenase branch (Fig. 3a). This mode would require a metabolic cycle of pyruvate carboxylase, malate dehydrogenase, and malic enzyme acting as a transhydrogenase to convert NADH into NADPH. Biochemical evidence for such a cycle has not been obtained so far, but a similar cyclic pathway involving pyruvate carboxylase, malate dehydrogenase, and PEP carboxykinase has recently been shown to operate in vivo in *C. glutamicum* [22, 27]. Omitting the transhydrogenase-like cycle from the network results in a slightly lower maximum lysine yield of 0.75 mol mol^{-1} [77]. Admittedly, all these scenarios imply zero growth which can hardly be realized in a real fermentation. Due to this, the achievable optimum can be expected to be somewhat lower than the value calculated here, but should still be significantly higher than the yield achieved in practice, which is in the range of about 40–50%. Further insight into lysine production has been obtained from flux balance analysis characterizing different in silico mutants and metabolic scenarios [55]. The model described experimental observations from flux analysis of *C. glutamicum* fairly well, but failed in certain rather important aspects. The reason is that biochemical regulation affecting the fluxes cannot be accounted for and reactions with identical overall stoichiometry cannot be resolved using such stoichiometric approaches.

3.2.2 Rational Design of *C. glutamicum* for Methionine Production

Elementary flux mode analysis has been further applied to study possible routes for the production of methionine in *C. glutamicum* [56]. The maximum theoretical methionine yield on glucose was calculated as 0.59 mol mol^{-1} (Fig. 3b). Also here, a detailed insight into the metabolic network is obtained. The PPP flux supporting the high yield is even higher than that for optimal lysine production, which reflects the enormous amount of 8 NADPH required per methionine. Additional simulations identified promising genetic targets for improved production such as heterologous expression of a transhydrogenase or of a glycine cleavage system. Moreover, it could be shown that the supply of reduced sulfur is beneficial for high carbon yield. The most effective sulfur source was methanethiol, allowing an almost complete conversion of glucose into methionine with a yield of 0.91 C-mol
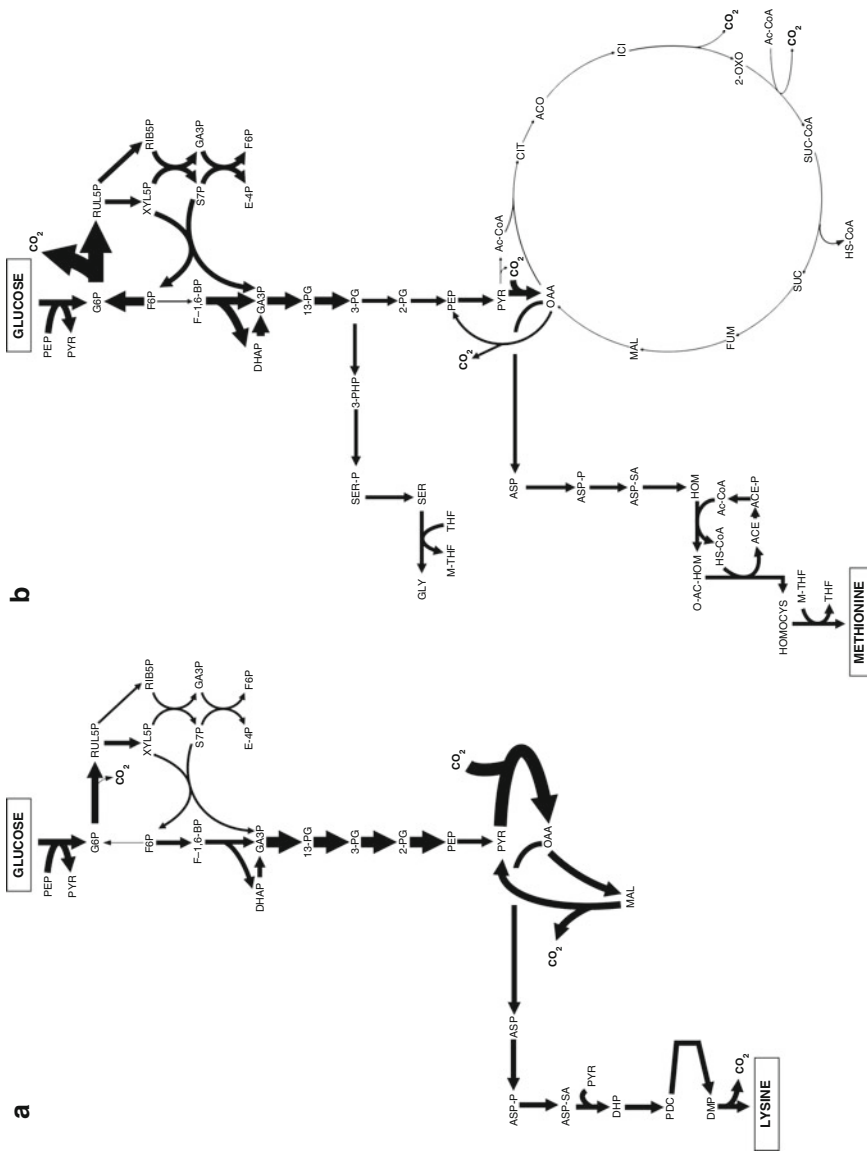
Fig. 3 Metabolic flux distribution of *C. glutamicum* reflecting maximal theoretical carbon yield of lysine (**a**) and methionine (**b**) from glucose. All fluxes are given as relative molar fluxes to the glucose uptake, whereby the relative flux of a reaction is reflected by the thickness of the corresponding arrow. The data are taken from recent simulation studies [5, 76]

C-mol^{-1}. Summarizing, stoichiometric modeling studies for *C. glutamicum* provide a sound basis for strain or process optimization. By calculating the theoretical capacity of an organism for a novel product they also allow useful estimates on the general economical feasibility [56].

3.3 Dynamic Modeling Approaches

Dynamic metabolic network models theoretically allow a most comprehensive and directed optimization of a pathway or of a whole cell. Therefore the ultimate goal of systems biology is the development of dynamic models for the complete simulation of cellular systems. Such dynamic models, however, require the understanding of all interactions influencing the reaction rates in the network. We are still far from that. For *C. glutamicum* detailed dynamic models, based on mechanistic equations for the participating enzymes and concentration measurements of the pathway intermediates involved, have been developed at least for the biosynthesis of lysine [78] and valine [79]. Through metabolic control analysis, such models allow the prediction of bottlenecks and of optimal factors, e.g., enzyme concentrations to achieve increased flux. Admittedly, for most pathways of *C. glutamicum* such kinetic information is still not available. This can be partly overcome by the application of power law or lin-log kinetics in the underlying equations [79, 80]. Dynamic models are also needed for dynamic labeling experiments for flux analysis as has been shown for *E. coli* [81]. The current progress in method development for metabolomics of *C. glutamicum* will surely stimulate the future generation of dynamic models for such organisms by providing extended data sets on intracellular metabolite concentrations required to derive in vivo kinetics [82–85]. For many years, dynamic macroscopic models have been used to describe dynamic phenomena of growth and production and derive control or feeding strategies for optimized bioprocesses. A recent example deals with fed-batch production of valine [79]. Such overall phenomenological models are surely useful but do not provide any detailed description of network activities [86].

4 Analysis of Metabolic Fluxes

In recent years, powerful approaches were developed which allow the quantification of small molecule fluxes through metabolic networks, i.e., in vivo reaction rates. Among the achievements obtained from flux analysis are the identification of novel pathways, the elucidation of metabolic control, identification of targets for rational strain engineering in biotechnology, and first insights into design principles of metabolic networks in systems biology studies. Concerning *C. glutamicum*, metabolic flux analysis provided a fascinating view of its metabolic pathways and offered new possibilities for rational strain engineering as recently reviewed [34, 87].

4.1 Tools and Concepts

4.1.1 Conventional Metabolic Flux Analysis by Stoichiometric Balancing

First flux estimates through larger parts of its metabolism were based on constraining assumed reaction networks with measurement of uptake and production rates [74]. A number of studies utilized stoichiometric balancing to assess the flexibility of the metabolic network [11, 75] and to investigate the influence of environmental conditions such as dissolved oxygen level [88], salt content [89], or nutrient status [12, 90, 91]. However, this conventional approach cannot yield reliable information about parallel or bidirectional reactions and has to rely on balances for NADH or NADPH, which may not be accurate [34]. Moreover, it is limited to derive new conclusions since the results are strongly based on the taken assumptions and not on data [92].

4.1.2 State-of-the-Art ^{13}C Metabolic Flux Analysis and Current Developments

Recent developments combining stoichiometric networks with additional intracellular information from ^{13}C-isotopomer analysis have overcome these limitations and display the state-of-the-art in metabolic flux analysis. Hereby, the feeding of ^{13}C labeled substrates to the growing cells leads to a distribution of the ^{13}C label throughout the metabolic network. The resulting labeling pattern in the intermediary metabolites and other cellular constituents depends on the well defined and well known carbon transfer of the involved reactions and their particular fluxes. As such, the labeling patterns contain the key information about the fluxes and can be used for their estimation. For label quantification, MS or NMR techniques are available, whereby MS has evolved to the preferred method due to its high accuracy, sensitivity, speed, and robustness [93, 94]. The most common method hereby exploits the ^{13}C pattern in proteinogenic amino acids as a rich source of labeling information for the determination of steady-state fluxes in growing cells [95, 96]. Respirometric flux analysis, conceptually based on sole labeling measurement of CO_2 extended the application of flux analysis to nongrowing cells [97–99]. Additionally, labeling measurement of intracellular metabolites has also received increasing attraction since it offers the possibility to resolve fluxes under dynamic conditions [81, 100]. After a decade of intense research and development, such ^{13}C-based flux methods can routinely track steady-state fluxes in microbes [92]. Convenient software tools can be used to calculate the intracellular fluxes from the labeling data utilizing either global parameter fitting from the entire labeling data set [101] or the estimation of local flux ratios from selected labeling patterns [102].

4.2   Metabolic Fluxes in C. glutamicum

With respect to metabolic fluxes, *C. glutamicum* is probably the most extensively studied organism so far [34]. Metabolic flux analysis in this organism has become a fundamental tool of strain engineering, since it provides otherwise not accessible key data on cellular function and regulation. The first pioneering studies of *C. glutamicum* using stable isotopes to elucidate metabolic properties were initiated soon after its isolation [103]. Focusing each on selected reaction, these labeling studies identified the dual lysine pathway in *C. glutamicum* [104] or provided first estimates on central pathways, i.e., citric acid cycle, anaplerosis, and glyoxylate shunt [105]. The major findings on metabolic fluxes and their regulation in *C. glutamicum* result from the fully integrated approaches comprising stoichiometric and isotopomer balancing with ^{13}C labeling and thus are quite recent (Table 2).

4.2.1   Metabolic Fluxes of Precursor Metabolism

The synthesis of relevant amino acids such as lysine, threonine, methionine, or glutamate demands for precursor compounds stemming from the TCA cycle. This has stimulated intensive research on fluxes involved in replenishment of the TCA cycle, involving pyruvate carboxylase, PEP carboxylase as carboxylating enzymes, as well as malic enzyme and PEP carboxykinase that catalyze decarboxylation reactions from the TCA cycle towards glycolysis. Metabolic flux analysis unraveled the metabolism around the pyruvate node in great detail (Fig. 4a). It could be shown that carboxylating and decarboxylating enzymes are active at the same time, forming a cyclic pathway for interconversion of C_4 metabolites of the TCA cycle and C_3 metabolites of glycolysis [27]. This cyclic pathway is highly flexible, as cells can redistribute the flux depending on the metabolic burden (Fig. 4b–d). The role of this cycle has been attributed to regeneration of excess ATP under certain conditions [121, 122] or equilibration of metabolite levels around the pyruvate node [123]. Additionally, a contribution to NADPH metabolism has been suggested [12, 124]. Moreover, flux studies identified the enzymes around the pyruvate node as key targets for metabolic engineering of the precursor supply in *C. glutamicum*. An increase of flux through the lysine pathway is linked to an increase of flux through the carboxylating enzymes, providing the lysine precursor oxaloacetate (Fig. 5a). In contrast, the flux through the decarboxylating enzymes, withdrawing oxaloacetate, is negatively correlated with lysine production (Fig. 5b). In light of these findings, amplification of the carboxylating and deletion of the decarboxylating enzymes became a promising strategy to enhance the anaplerotic net flux for improved production.

Beyond lysine, the close link of anaplerotic replenishment of the TCA cycle and production could be also observed for the formation of glutamate, demanding for the TCA cycle precursor α-ketoglutarate (Fig. 5c). Regarding the supply of α-ketoglutarate as product precursor, flux analysis revealed that the specific activity of isocitrate dehydrogenase (ICDH) and GDH did not show large changes

Table 2 Recent key contributions of ^{13}C metabolic flux analysis to *Corynebacterium glutamicum*. The compilation of studies is partly adapted from an actual review on metabolic flux analysis in bioprocess development [87]

Product	Substrate	Scope of the study	References
Lysine	Glucose	Production process monitoring using a ^{13}C-sensor reactor coupled to the production process	[106]
Lysine	Glucose	Conceptual work on systematic quantification of metabolic fluxes using MS	[107]
Lysine	Glucose	Investigation of metabolic fluxes in phosphoglucose isomerase mutants	[108]
Lysine	Glucose Fructose	Serial flux mapping of fed-batch cultures using the sensor reactor approach	[109]
Lysine	Glucose	Comparative metabolic flux analysis on glucose and fructose as carbon source	[110]
Lysine	Glucose	Systems biology profiling of a production process with transcriptome, metabolome, and fluxome analysis	[76]
Lysine	Sucrose	Metabolic fluxes in sucrose-grown cells	[111]
Lysine	Glucose	Metabolic flux analysis at a miniaturized scale enabling high-throughput flux analysis	[112]
Lysine	Glucose	Investigation of the effects of amplified FBPase expression on metabolic fluxes	[113]
Lysine	Glucose	Investigation of the effects of a novel *gnd* mutation on lysine production	[30]
Lysine	Glucose	In vivo flux estimation using respirometric metabolic flux analysis with sole labeling analysis of CO_2	[114]
Lysine	Glucose	Flux profiling in response to feedback deregulation of lysine biosynthesis	[115]
Lysine	Glucose	Investigation of the effects of overexpression and modification of G6P dehydrogenase	[116]
Glutamate	Glucose	Metabolic flux analysis of glutamate production	[117]
Glutamate	Glucose	Investigation of the roles of anaplerotic pathways in glutamate overproduction	[119]
Methionine	Glucose	Identification and flux analysis through of a novel pathway for isoleucine biosynthesis	[40]
Methionine	Glucose	Flux analysis under conditions of oxidative stress resulting from deletion of the global repressor McbR	[119]
Lysine	Glucose	Flux response to deletion of pyruvate kinase	[120]

throughout the fermentation, while that of the α-ketoglutarate dehydrogenase complex (ODHC) significantly decreased upon induction of glutamate production, clearly leading to flux redistribution (Shirai et al. 2005). The results suggest that ODHC plays the largest role in controlling flux at the key branch point of α-ketoglutarate from the view of metabolic flux.

4.2.2   Metabolic Fluxes of NADPH Metabolism

The synthesis of most amino acids demands NADPH so that efficient supply of this cofactor is highly relevant in production processes. In *C. glutamicum*, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, isocitrate
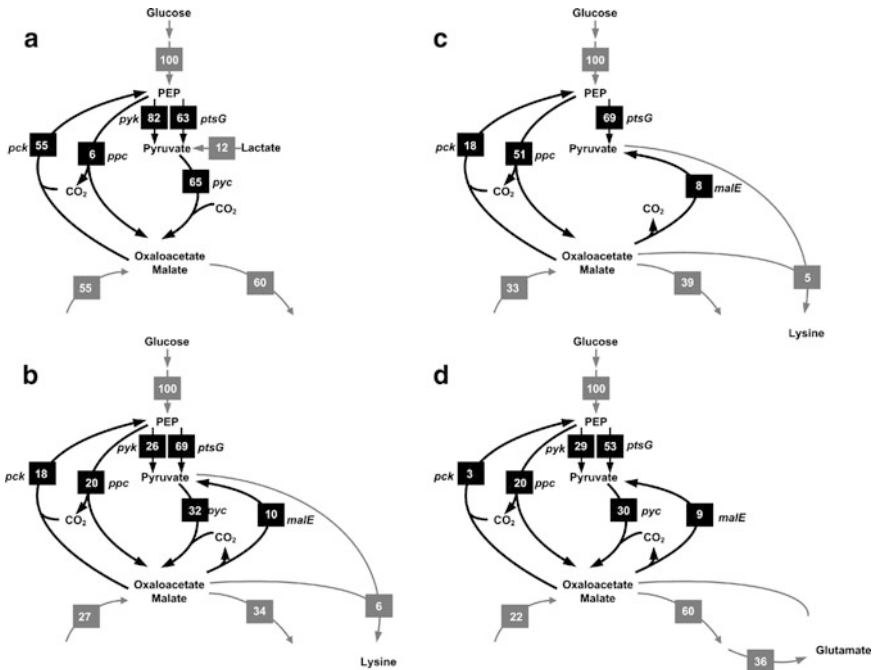
Fig. 4 Metabolic fluxes around the pyruvate node of *C. glutamicum* under different physiological conditions: Growth of the wild type *C. glutamicum* ATCC 13032 in continuous culture on glucose [27] (**a**), batch cultivation of the lysine producing strain *C. glutamicum* ATCC 13032 lysC^fbr (**b**), its pyruvate kinase deficient variant *C. glutamicum* ATCC 13032 lysC^fbr Δpyk [120] (**c**), and glutamate production in batch culture by *C. glutamicum* AJ-1511 (ATCC13869) on glucose [118] (**d**). All fluxes are given as relative molar fluxes to entry flux into the PEP pool
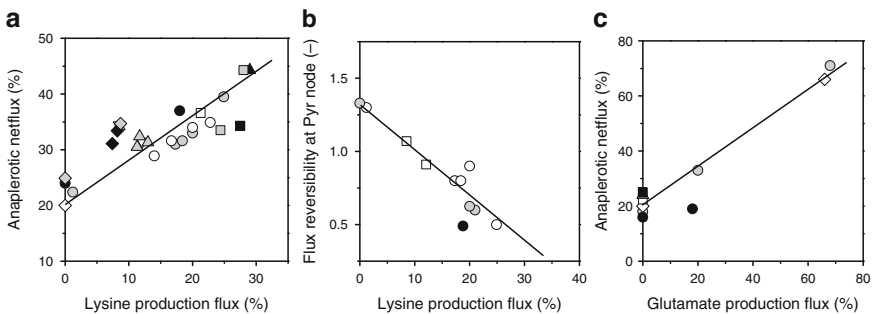
Fig. 5 Metabolic flux analysis of precursor supply and production of lysine (**a**, **b**) and glutamate (**c**) in different *C. glutamicum* strains investigated under various cultivation conditions by ^{13}C flux analysis: [112] (*white circles*), [125] (*gray circles*), [126] (*black circles*), [127] (*white squares*), [111] (*gray squares*), [111] (*black squares*), [128] (*white triangles*), [116] (*gray triangles*), [26] (*black triangles*), [129] (*white diamonds*), [115] (*gray diamonds*), [120] (*black diamonds*), [76] (*white hexagons*), and [118] (*gray hexagons*). The flux reversibility at the pyruvate node is defined as the ratio of the back flux to the anaplerotic net flux [130]

dehydrogenase, and malic enzyme catalyze NADPH generating reactions, whereas NADPH consuming reactions comprise growth with a stoichiometric demand of 16.4 mmol NADPH (g biomass)$^{-1}$ [34] and overproduction. From ^{13}C metabolic flux studies of lysine producing strains under different physiological conditions, remarkable insights into function and regulation of the NADPH metabolism of *C. glutamicum* could be obtained. The NADPH metabolism of *C. glutamicum* is highly flexible, adjusting to the physiological growth state [126], the nutrient status [13, 32, 111], or the genetic background [108, 131]. In most cases this results in an apparent NADPH excess, pointing at so far unassigned reactions which consume NADPH. Possible, but not validated, candidates are NADPH oxidase [132] or cyclic fluxes around the pyruvate node involving malic enzyme [27, 124]. Towards higher production, this apparent excess diminishes and turns into an apparent NADPH limitation [5]. Extrapolating these findings to industrial producers with much higher yields makes a limitation of production by NADPH very likely and strongly suggests the pathways involved in NADPH supply as promising targets for metabolic engineering of *C. glutamicum*. Hereby, stoichiometric investigation of the lysine network almost 20 years ago predicted that an increased lysine yield is linked to an increased flux through the PPP [133]. The importance of the PPP for efficient lysine production was later shown by metabolic flux analysis. Studies, investigating different strains under different conditions, revealed a close link of lysine production flux with the flux through the PPP (Fig. 6a). In contrast to the PPP, the contribution of isocitrate dehydrogenase decreases with increasing lysine production (Fig. 6b). The role of malic enzyme is still not completely clear. Its contribution to NADPH supply has been demonstrated for growth on fructose [32] as well as in a pyruvate kinase deficient lysine production strain [120].
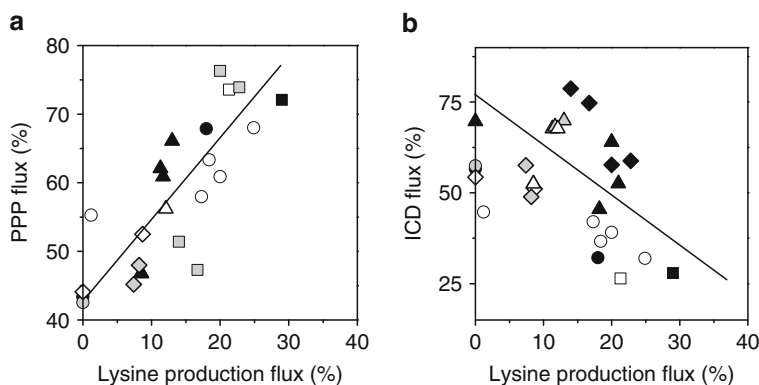

Fig. 6 Metabolic flux analysis linking lysine production with NADPH metabolism involving the pentose phosphate pathway (**a**) and isocitrate dehydrogenase (**b**) in different *C. glutamicum* strains investigated under various cultivation conditions by ^{13}C flux analysis: [125] (*white circles*), [129] (*gray circles*), [126] (*black circles*), [127] (*white squares*), [112] (*gray squares*), [126] (*black squares*), [128] (*white triangles*), [116] (*gray triangles*), [26, 76] (*black triangles*), [115] (*white diamonds*), and [120] (*gray diamonds*)

However, overexpression of the malic enzyme gene did not result in improved lysine production independent from the carbon source tested [134].

5 Metabolic Pathway Engineering

In *C. glutamicum* the biosynthesis of amino acids is strictly regulated through feedback inhibition. Therefore, overproduction requires, first of all, the removal of all metabolic control mechanisms. Moreover, the amplification of biosynthetic pathway genes is a logical step of strain improvement, especially in the presence of transcriptional repression. Moreover, balanced supply of precursor compounds and cofactors has to be addressed to achieve efficient production of the amino acid. For amino acids, these supporting pathways are part of the highly interconnected network of central carbon metabolism, so that modifications will likely interfere with growth related metabolic reactions. Due to these various hurdles, classical mutagenesis and selection did not yield high levels of production, although efforts have been continuing now for almost four decades [6]. The development of recombinant DNA technologies, allowing targeted genetic modification, has initiated intensive research towards rational optimization of *C. glutamicum*, resulting in remarkable progress in production efficiency [30, 135, 136]. In the following, the advances in metabolic pathway engineering of *C. glutamicum* are highlighted. Due to the strong impact of raw material costs, optimization of the conversion yield was at the focus of many studies in order to improve the economy of the production process [137].

5.1 Metabolic Engineering of Lysine Production

5.1.1 Metabolic Engineering of Lysine Biosynthesis

A number of studies have addressed the optimization of lysine production by direct modification of enzymes of the biosynthetic pathway (Fig. 7). The high relevance of engineering these enzymes is underlined by the fact that today every single gene of the lysine biosynthetic pathway is covered with patents by the major industrial players in the field [137]. The release of aspartate kinase, controlling the pathway flux, from feedback inhibition is one of the most important targets. Different point mutations in the *lysC* gene, i.e., its regulatory β-subunit, result in feedback resistant enzyme variants and increase lysine production [36, 138, 140]. Also, overexpression of aspartate kinase is beneficial for production [141]. Another relevant target is the *dapA* gene, encoding dihydrodipicolinate synthase. Amplified expression, increasing lysine was realized using plasmids [138, 142–144] as well as mutation of the promoter sequence [145]. Also, overproduction of diaminopimelate epimerase (DapF) and succinyl-aminoketopimelate transaminase (DapC), two enzymes of the succinylase branch, was beneficial for lysine formation [137]. Following the
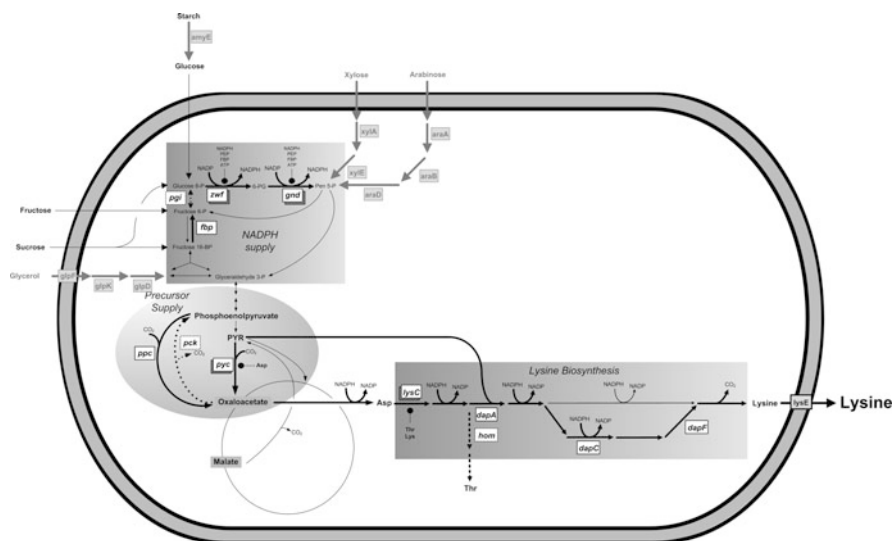
Fig. 7 Metabolic engineering strategies for optimization of lysine production in *C. glutamicum*. Highlighted are modifications for improved supply of precursors and NADPH, for increased flux through lysine biosynthesis and secretion and for extension of the substrate spectrum. The optimization comprises decrease of flux (*thick dotted arrow*), via deletion or attenuation of genes (*white box with dotted line*), increase of flux (*thick solid arrow*) via amplification of genes (*white box with solid line*), modification of metabolic control for feedback regulated enzymes (*dark grey box with solid line*) and the introduction of new reactions (*grey arrows*) via heterologous expression of foreign genes (*white box with grey solid line*)

discovery of the lysine exporter (LysE), the subsequent overexpression of the *lysE* gene resulted in an increased lysine secretion rate [146–148]. The recently performed expression of *lysE* from *C. glutamicum* in a *Methylophilus methylotrophus* lysine producing strain was also shown to improve lysine production from methanol by this organism [149].

5.1.2 Metabolic Engineering of Precursor Supply

The importance of anaplerotic enzymes for supply of the lysine precursor oxaloacetate stimulated intensive metabolic engineering activities (Fig. 7). Among the most striking findings was the identification of pyruvate carboxylase as the major anaplerotic enzyme in *C. glutamicum* [27]. Subsequently, overexpression of its gene has been shown to improve lysine production [150]. Knowing the importance of pyruvate carboxylase for lysine production, the point mutation P458S, identified in a classically derived producer, was introduced into the *pyc* gene and also resulted in strong increase of lysine production [136]. Moreover, also overexpression of PEP carboxylase is beneficial for the formation of amino acids of the aspartate family [151]. In order to reduce the back flux from the TCA cycle and the withdrawal of

oxaloacetate to glycolysis, deletion of PEP carboxykinase resulted in a significant improvement of lysine production [22]. For malic enzyme no clear effects could be observed so far. Neither deletion nor overexpression of the corresponding gene influenced the metabolism of *C. glutamicum* on sugars markedly [21, 152].

5.1.3 Metabolic Engineering of NADPH Supply

As revealed by metabolic flux analysis, the PPP is the major pathway for supply of NADPH required in high amounts for lysine biosynthesis. Due to this, different approaches have aimed at increasing flux through the PPP (Fig. 7). Metabolic flux studies on glucose, fructose, and sucrose identified fructose 1,6-bisphosphatase as a non-obvious target [110, 111]. Subsequent amplified expression of the *fbp* gene indeed increased lysine yield on glucose, fructose, and sucrose up to about 40% [128]. Hereby, the mutant with overexpression of fructose 1,6-bisphosphatase exhibited 10% enhanced PPP flux. A second major target approached was glucose 6-phosphate dehydrogenase. Overexpression of the encoding *zwf* gene in the feed-back-deregulated lysine producing strain *C. glutamicum* ATCC13032 lysC^{fbr} resulted in increased lysine production on different carbon sources including the two major industrial sugars, glucose and sucrose [116]. In further successful examples, an increased PPP flux was achieved by modifying the regulatory properties of enzymes in the PPP, i.e., partially releasing glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from allosteric inhibition. The substitution A243T in the *zwf* gene encoding for glucose 6-phosphate dehydrogenase [116, 153] and the substitution S361F in the *gnd* gene encoding for 6-phosphogluconate dehydrogenase [30] both led to significantly increased lysine titer. The modifications cause an increase in the PPP flux which probably resulted from positively changed kinetics of the enzymes. For glucose based processes, disruption of glycolysis via deletion of phosphoglucose isomerase forces the cell to metabolize the substrate completely via the PPP, and indeed results in increased lysine production [108]. This strategy, however, is only applicable for glucose based processes, since other substrates such as sucrose, glycerol, fructose, xylose, or arabinose require an active phosphoglucose isomerase for channeling carbon into the PPP [111, 128].

5.2 Metabolic Engineering of Aromatic Amino Acid Production

Remarkable progress was also achieved for the synthesis of aromatic amino acids in *C. glutamicum* [6]. Effective approaches comprised the modification of terminal pathways leading to removal of undesired control mechanisms, engineering of the PPP for increased supply of precursors, and transport engineering leading to reduced intracellular pools. Among the most striking findings was the overexpression of the *tkt* gene in the PPP encoding transketolase which strongly enhanced tryptophan production by improved supply of the precursor E4P [154, 155]. To avoid accumulation of indole, the last intermediate in the tryptophan pathway, due

to a limiting supply of L-serine, the availability of L-serine was improved by amplification of the *serA* gene for 3-phosphoglycerate dehydrogenase, the key enzyme in the L-serine biosynthesis [156]. A key to increased production was also the deletion of transport systems for aromatic amino acids impairing their uptake from the medium [6].

5.3 Metabolic Engineering of Glutamate Production

Concerning synthesis of glutamate in *C. glutamicum* which demands efficient replenishment of the TCA cycle, pyruvate carboxylase was identified as a major anaplerotic enzyme [118, 150]. The flux through pyruvate carboxylase strongly increased under glutamate production induced by Tween 40 addition, while PEP carboxylase and PEP carboxykinase fluxes remained constant. An impressive set of studies investigated the metabolism within the TCA cycle linked to glutamate production in great detail [4, 117, 157, 158]. Successful strategies to increase glutamate production involved deletion of *odhA*, encoding a key protein of the complex [159] or attenuation of *odhA* expression via the use of antisense RNA [160].

5.4 Utilization of Alternative Substrates

The major industrial carbon sources for fermentation processes with *C. glutamicum* are cane molasses, beet molasses, sucrose, and dextrose [3]. Since the carbon source is the major cost factor in industrial lysine production [137] and prices for molasses and raw sugar have increased significantly in recent years, many attempts have been aimed at extending the substrate spectrum of *C. glutamicum* to exploit alternative raw materials. Today, *C. glutamicum* has been engineered to utilize a large number of different substrates (Fig. 7). The future use of the corresponding raw materials in industrial fermentations of *C. glutamicum*, however, requires further intensive efforts in metabolic and process engineering. So far, key contributions enable the utilization of the pentose sugars which *C. glutamicum* naturally cannot metabolize. These sugars, namely xylose and arabinose, display significant fractions in agricultural residues and other lignocellulosic biomass recently receiving increasing interest as a cheap and most abundant raw material for biobased production [161]. Through heterologous expression of the *E. coli* genes *xylA* and *xylB* *C. glutamicum* was able to consume xylose as sole source of carbon [162]. In substrate mixtures, glucose-mediated regulation still exerts a measurable influence on xylose consumption kinetics. Similarly, arabinose utilization was also achieved, whereby the genes *araA*, *araB*, and *araD* were also derived from *E. coli* [163]. An interesting study recently investigated the tolerance of *C. glutamicum* to toxic compounds such as furfurals or phenols, typically present in lignocellulosic raw materials [164]. These compounds cause significant inhibition of growth. For

growth-arrested production processes this is not a critical point, but open questions remain to be elucidated for the majority of bioprocesses with *C. glutamicum* which typically are fed-batch processes with important phases of growth of biomass to achieve efficient production. Introducing the *S. griseus amy* gene on an expression vector into a lysine producing strain of *C. glutamicum* allowed synthesizing and secreting of α-amylase into the culture broth [165]. Although some high-molecular-weight degradation products remained in the culture broth, the recombinant strain effectively used soluble starch as carbon and energy substrate for growth and also for lysine production. This could allow the direct conversion of starch into desired products and the avoidance of cost intensive hydrolysation pretreatment currently required when feeding starch hydrolysates from corn, wheat, or cassava for production [166]. *C. glutamicum* cannot utilize glycerol, a stoichiometric by-product of biodiesel production. By heterologous expression of *Escherichia coli* glycerol utilization genes, *C. glutamicum* was engineered to grow on glycerol [167]. The engineered strains were able to produce glutamate efficiently as well as lysine. Recent attempts to enable utilization of lactose by heterologous coexpression of genes from *Lactococci* have shown the basic feasibility, but are still linked to suboptimal performance of the obtained strains [168]. At present, metabolic engineering has significantly broadened the substrate spectrum of *C. glutamicum*.

5.5 *Global Strain Engineering Through Applied Systems Biology*

The experience of the past clearly shows that detailed quantitative knowledge of metabolic physiology is required for rational design of superior production strains [169]. Especially for the optimization of amino acid production by *C. glutamicum*, characterized by a close connection between central metabolism and product biosynthetic pathways, understanding of global metabolic regulation has turned out to be crucial [5]. In this light, systems biology approaches elucidating cell physiology on a global level, displaying powerful strategies. Metabolic flux analysis may be regarded as a first systems approach to *C. glutamicum* since it combines measurement data with metabolic network models of biosynthetic and central metabolic pathways and predicts metabolic engineering strategies based on such systems oriented insights. Hence, these systems biology approaches per se are not new concepts to design and improve *C. glutamicum*. However, the availability of the genome sequence and the rapid progress in postgenomics methods such as transcriptomics, proteomics, fluxomics, and metabolomics today allow one to study its metabolic and regulatory properties on a truly global level, opening a new era of industrial strain improvement [44]. Rational strain improvement was initially done on a gene-by-gene basis but was recently put on the genome level by comparing the genome sequence of producer and wild-type to identify relevant mutations. The success of this strategy, named "genome breeding" was successfully demonstrated for a *C. glutamicum* L-lysine producer [136]. From an engineering perspective this strategy worked, but it is still based on trial and error, so that a large number of

targets identified from the genome sequence have to be tried. Moreover, it requires an already efficient production strain for target identification, which in the case of lysine took decades of development and is not available for other processes. Here, new omics tools from functional genomics can help to identify key regulators and guide rational strain engineering. Impressive progress has been made toward this goal, especially in the frame of systems biological study of *C. glutamicum* [44]. Transcriptome analysis through DNA microarrays [44, 170] allows global expression profiling of *C. glutamicum*. The proof of value of this technique for rational strain engineering has recently been shown, when novel targets for improved lysine production could be identified from selected transcriptome studies [171, 172]. Similarly, the analysis of the proteome, based on 2-D gel electrophoresis [52–54, 173] has also provided some insights into metabolic processes such as nitrogen starvation [174] or the utilization of citrate [15]. Among all systems oriented approaches, ^{13}C metabolic flux analysis has clearly contributed the most to our current detailed picture of the *C. glutamicum* metabolism. Among the achievements obtained from flux analysis are the identification of novel pathways, the elucidation of metabolic control, identification of targets for rational strain engineering in biotechnology, and first insights into design principles of the metabolic network. Hereby, metabolic flux analysis provided quantitative data which directly reflect the phenotype of the investigated *C. glutamicum* strain, whereas other omics approaches often do not allow a direct conclusion on the active pathways determining the phenotypic behavior [76, 175]. Recent developments provide the next level of systems biology studies, the parallel investigation of *C. glutamicum* on levels of gene expression, proteins, metabolites, and fluxes providing important links between the different functional components of cellular physiology. First examples of such systems-oriented studies already reveal a great potential [76, 119, 176]. Such approaches are especially promising for the targeted multidimensional alteration of complex regulatory networks towards better tolerance of production strains to high temperature or salt levels, or extreme pH values [137], but also reveal a great potential for efficient design of novel bioprocesses. Similarly, systems based metabolic engineering approaches have also created impressive progress in developing superior strains of *Escherichia coli*, the second major industrial amino acid producer [177]. As an example, production of valine [178] or threonine [179] using *E. coli* could be substantially optimized.

5.6 Towards Novel Products

In light of the established fermentation on cheap substrates [5], its known genome sequence [47] and the availability of genetic engineering tools *C. glutamicum* is regarded as key candidate to develop production strain for various other products. In addition to the above-mentioned traditional products, *C. glutamicum* has been recently engineered to accumulate a great variety of different compounds (Table 3) Due to impressive work in recent years, *C. glutamicum* is applicable to produce

Table 3 Metabolic engineering of *Corynebacterium glutamicum* for biotechnological applications. The papers listed comprise either review papers summarizing the progress achieved or recent key contributions.

Product	References
Amino acids	
Glutamate	[180]
Isoleucine	[181, 182]
Lysine	[5]
Methionine	[56, 184]
Serine	[184, 185]
Threonine	[186]
Tryptophan	[6]
Tyrosine	[43]
Valine	[187]
Panthothenate	[188]
Diamines	
Cadaverine	[189]
Organic acids	
Succinate	[190]
D-Lactate	[191]
Biofuels	
Ethanol	[192]

efficiently amino acids, organic acids, diamines, or biofuels, and the coming years will surely see a further broadening of its potential.

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