Metabolic engineering of *Corynebacterium glutamicum* for production of glutamate derivatives

DISSERTATION

Submitted by

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The practical work of this thesis has been performed at the University of Bielefeld, Faculty of Biology, Genetics of Prokaryotes, from September 2012 till June 2015 under the supervision of Prof. Dr. Volker F. Wendisch.

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Erklärung

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Ich versichere, daß keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, sowie Zitate kenntlich gemacht habe. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

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Abstract

The identification of *Corynebacterium glutamicum* as a glutamate producer in the 1950's was the start of its career as an amino acid producer. *C. glutamicum* has now been employed as cell factory for industrial amino acid production for over five decades and has a market size to reach $20 billion by 2020. As *C. glutamicum* was isolated for its natural ability to produce glutamate it makes it an excellent chassis for engineering it to produce its derivatives ornithine, proline, putrescine, citrulline, and arginine. These products are becoming increasingly important as they have a wide range of applications and the demand for these products is rising. The construction of a platform strain can reduce the time and resources required for strain development. Another approach to reduce the time and resources required for strain development is by developing new tools for metabolic engineering. These strategies were both explored to ease the process of strain construction.

Proline, citrulline, and putrescine can be synthesized directly from ornithine, and three more reactions are required to synthesize arginine. Hence a strain engineered to produce ornithine can serve as a platform to produce the other four compounds. The first step was to establish proline and citrulline production by *C. glutamicum* as the rational engineering of *C. glutamicum* for production of these two amino acids had not previously been described.

Proline is synthesized from glutamate via the proline biosynthetic pathway in most microorganisms, but with the help of the enzyme ornithine cyclodeaminase (Ocd) it can be synthesized from ornithine. Overexpression of putative *ocd* from *C. glutamicum* in an ornithine overproducing strain did not result in detectable proline production. Plasmid-based expression of *ocd* from *Pseudomonas putida* (ocd_{Pp}) on the other hand allowed accumulation of proline with a yield of 0.06 g proline / g glucose (g pro/g glc). Interestingly replacing the stop codon TGA of ocd_{Pp} with TAA resulted in a remarkable decrease in glutamate accumulation while the yield of proline increased to 0.25 g pro/g glc. The byproduct accumulation was further reduced by medium optimization, which also entailed a 25% higher proline yield. Lastly, the yield was boosted by overexpression of $argB^{fbr}$ encoding feedback-alleviated *N*-acetylglutamate kinase to 0.36 ± 0.01 g pro/g glc.

A base strain suitable for the production of ornithine, proline, putrescine, citrulline, and arginine was constructed by deleting the repressor of the arginine pathway ArgR. Additionally the genes encoding enzymes of the arginine biosynthetic pathway were deleted, the deletion of *argF* allows ornithine to accumulate, and the deletion of *argG* allows citrulline to accumulate when *argF* is overexpressed. To add citrulline to the product spectrum of the platform strain we found it necessary to overexpress $argB^{fbr}$ along with *argF*. The strain accumulated citrulline with a yield of 0.38 ± 0.01 g citrulline / g glucose. The potential and versatility of the strain was demonstrated by producing citrulline from the alternative carbon sources starch, xylose, and glucosamine.

After the product range of the base strain had been extended to include citrulline, metabolic engineering was performed to increase the ornithine yield of the strain. The initial assumption was that an increased ornithine yield could be translated into increased yields of the other four

bioproducts. The ornithine yield was improved by 71% with a yield of 0.52 g ornithine / g glucose (g orn/g glc) compared to 0.31 g orn/g glc of the parent strain. This was achieved by feedback alleviation of *N*-acetylglutamate kinase, tuning of the promoter of *gdh* encoding glutamate dehydrogenase, lowering expression of *pgi* encoding phosphoglucoisomerase, along with the introduction of a second copy of the arginine biosynthetic operon *argCJB*^{fbr}*D* into the chromosome. Strains capable of efficiently producing citrulline, proline, arginine or putrescine were derived from ornithine producing strains by plasmid-based overexpression of appropriate pathway modules with one to three genes. It was found that optimizing the base strain for ornithine production did not increase citrulline and arginine yields any further, indicating that the reaction converting ornithine into to citrulline is a bottleneck in citrulline and arginine production.

Finally the popular CRISPR/dCas9 technology was adapted for the use of metabolic engineering in *C. glutamicum*. The system could be used to reversibly perturb gene expression. As proof of concept we targeted *pgi* in the lysine overproducing strain DM1729, where an almost complete repression of the transcription of the gene resulted in a 2-fold increase in the lysine titer. We also targeted the genes *pck* and *pyk* to increase glutamate production in wild-type *C. glutamicum* where we also observed nearly complete repression and increased glutamate titers.

With this work it was shown how valuable the concept of using a platform strain for production of several industrially relevant bioproducts is. Moreover an efficient way to screen for new targets for metabolic engineering in *C. glutamicum* was demonstrated. From the initial cloning in *Escherichia coli* the *C. glutamicum* clones could be obtained in as little as four days by adapting the CRISPR/dCas9 system.

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Chapter 1. Introduction

1.1 Motivation

Amino acids have been produced by microbial fermentation for over five decades. The central carbon metabolism and the amino acid pathways of several microorganisms including *Escherichia coli*, *Corynebacterium glutamicum*, and *Saccharomyces cerevisiae* are well known. Moreover a large number of publications on metabolism, characterization of pathways, and strain improvement for amino acid production are available.
Microbial amino acid production has been extensively explored in academia as well as in industry. From a scientific point of view this does not make amino acid overproduction any less interesting, in fact, this along with new technologies available makes it more interesting than ever. The vast information available on the microbes commonly used for microbial fermentation can now be used to predict phenotypes useful for production of a given compound [1], [2].
However, it remains a challenge to engineer strains that are genetically stable, robust, fit, and accumulating high product concentrations. There are still many unknowns, such as regulation of pathways and how the pathways are intertwined. Furthermore it is difficult to predict the effect of multiple deletions, insertions, and mutations performed for improved production.

Also from an industrial point of view amino acid production remains to be of major interest, not only because the compounds also can serve as precursors to high-value products, but mainly due to the growing amino acid market that is expected to reach an impressive size of US $20 billion in 2020 [3], keeping in mind that amino acids are bulk products.
Microbial amino acid production is a highlight of biotechnology; it is one of the few examples of production of bulk products that chemical synthesis cannot compete with. With only a few exceptions amino acids are nowadays produced by microbial fermentation, yielding the biologically active L-enantiomer [4]. Today over four million tons of amino acids are produced annually, many of them by coryneform bacteria [2], [4].

The industrial workhorse *C. glutamicum*, commonly used for amino acid production, has a strong flux towards glutamate synthesis and therefore can advantageously be employed as host for the production of glutamate derived bioproducts [2], [4]. These bioproducts include ornithine, citrulline, proline, putrescine, and arginine. They are industrial compounds with an increasing demand and importance due to their wide use in the chemical, bioplastic, cosmetic, food, feed, and pharmaceutical industries [5]–[8]. In this thesis the production of these compounds by *C. glutamicum* was explored.

1.2 Objectives of this study

Under the concept of reducing the time and resources spent on the construction of new strains for biotechnological production the aims of this study was to optimize the production of ornithine by *C. glutamicum* that can serve as precursor of several other valuable compounds, and to develop the CRISPR/Cas9 system for metabolic engineering of *C. glutamicum*. To meet these aims the following objectives were framed:

- Construct an ornithine producing base strain suitable for the production of proline, putrescine, citrulline, and arginine.
- Construct a citrulline producing *C. glutamicum* strain.
- During my master I found that proline could be produced by *C. glutamicum* Δ*argFR* with ornithine cyclodeaminase from *Pseudomonas putida*. In extension to this discovery, it was the aim to further explore proline production by *C. glutamicum*.
- Increase the ornithine yield of the base strain.
- Determine if an increase in ornithine production can be translated into increased production of citrulline, proline, putrescine, and arginine.
- Explore the use of the CRISPR/dCas9 system for metabolic engineering of *C. glutamicum*

Chapter 2. Background

Wendisch, V.F., Eberhardt D., Herbst M., **Jensen, J.V.K**. Chapter 3. Amino Acids and Nucleotides. Biotechnological production of natural ingredients for food industry. Edited by Bicas, J.L., Maróstica Jr., M.R., Pastore G.M. Bentham eBooks.

2.1 *Corynebacterium glutamicum* and its versatility

Corynebacterium glutamicum is an industrial workhorse, employed for the production of amino acids, nucleotides, and vitamins. Prior to the discovery of *C. glutamicum* and the production of glutamate by fermentation, amino acids were exclusively produced by chemical synthesis or extraction methods [4].
In the 1950s *C. glutamicum* was isolated from a soil sample in a zoo in Japan as part of a screening project to identify microorganisms capable of accumulating glutamate [9].
The screening project was initiated due to the increased demand for the seasoning glutamate responsible for the taste umami, the costly chemical decomposition of wheat and soybean to obtain glutamate, and the knowledge of the successful production of citric acid by fermentation [9].

An advantageous feature of *C. glutamicum* is its ability to co-utilize several different carbon sources. For instance acetate, fructose, lactate, or pyruvate can be co-utilized with glucose [7–9]. Moreover it can grow aerobically on a variety of compounds as sole carbon and energy sources such as ribose [13], ethanol [14], glutamate, [15] and propionate [16]. Glucose is widely used for industrial production of amino acids; however other cheap and renewable carbon sources for production are wanted. This could lower production costs and not compete with the use of carbon sources for the food industry. *C. glutamicum* has been engineered to grow on succinate [17], fumarate [17], malate [17], starch [18], lactose [19], galactose [20], cellobiose [21], xylose [22], arabinose [23], glucosamine [24], and glycerol [25].

Not only is *C. glutamicum* able to utilize a vast number of carbon sources, over recent years it has been engineered to produce a wide range of bioproducts including poly-3-hydroxybutyrate [26], ethanol [27], lactate [28], succinate [29], isobutanol [30], 1,2-propanediol [31], cadaverine [32], putrescine [33], xylitol [34], and proteins [35]. The whole genome sequencing of *C. glutamicum* in 2003 [36], [37], the development of genetic tools [38], and the development of molecular technologies helped this development along the way.

2.2 The arginine pathway

The arginine pathway is fascinating both from a biochemical and evolutionary point of view. There is a great range of information available on arginine biosynthesis and regulation for prokaryotes and eukaryotes. Covering only a part of this information would extend this thesis beyond reason. Therefore I here mainly present information specific for *C. glutamicum*.

2.2.1 Arginine biosynthesis in *Corynebacterium glutamicum*

2-oxoglutarate of the tricarboxylic acid (TCA) cycle is a branch point from where the metabolic flux can continue through the TCA cycle for energy production or glutamate can be synthesized. Once glutamate has been synthesized it is the acetylation of the amino group of glutamate that initiates de novo arginine biosynthesis. It is this acetylation that distinguishes the arginine pathway from the proline pathway, as it prevents the spontaneous cyclization of the semi aldehyde into the direct precursor of proline, pyrroline carboxylate (**Figure 1**).

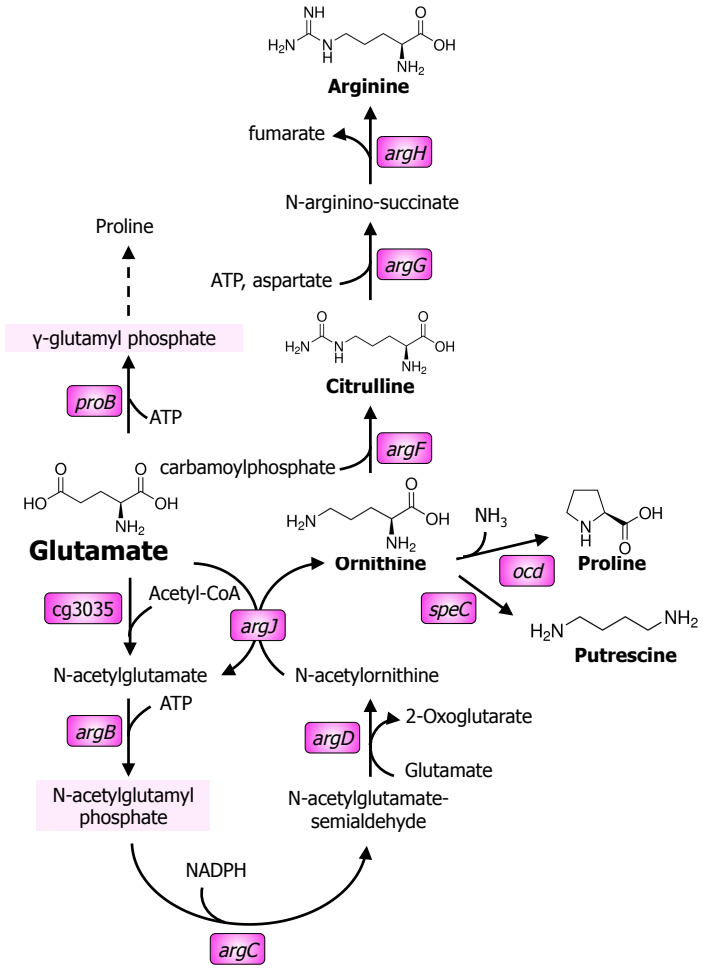

Figure 1. The arginine pathway of *Corynebacterium glutamicum* extended to include heterologous synthesis of putrescine and proline. *ocd* from *Pseudomonas putida* and *speC* from *Escherichia coli*. See text for enzyme names. Shaded pink boxes denote that the only difference between the compounds of the proline and arginine pathway is the acetyl group.

The acetylation reaction is catalyzed by *N*-acetylglutamate synthase (NAGS, EC 2.3.1.1). Not until recently was an enzyme with NAGS activity in *C. glutamicum* identified [39]. The gene cg3035 was shown to encode an enzyme that catalyzes the acetylation with acetyl-CoA as the acetyl donor [39]. This enzyme belongs to a novel class of NAGS genes, which thus far only have been found in bacteria of the suborder *Corynebacterineae* [39]. The function of the enzyme is anaplerotic i.e. it serves to drive the flux into the arginine pathway. It is ornithine acetyltransferase (OAT, EC 2.3.1.35, *argJ* gene product) that is responsible for the main supply of NAG as it recycles it via the transfer of the acetyl group of acetylornithine to glutamate, leaving ornithine as the product.
Three more reactions are required to synthesize ornithine from *N*-acetylornithine; the second step of the pathway is catalyzed by *N*-acetylglutamate kinase (NAGK, EC 2.7.2.8, *argB* gene product) where *N*-acetylglutamate is converted into *N*-acetylglutamyl phosphate. This intermediate is then converted into *N*-acetylglutamate semialdehyde by *N*-acetyl-γ-glutamyl phosphate reductase (EC 1.2.1.38, *argC* gene product) and lastly into *N*-acetylornithine by acetylornithine aminotransferase (EC 2.6.1.11, *argD* gene product). Biosynthesis of arginine from ornithine further requires three more reactions: Ornithine carbamoyltransferase (OTC, EC 2.1.3.3, *argF* gene product) catalyzes the carbamoylation of ornithine into citrulline. Argininosuccinate synthase (EC 6.3.4.5, *argG* gene product) is responsible for the conversion of citrulline into argininosuccinate, which is following split into arginine and fumarate catalyzed by argininosuccinase (EC 4.3.2.1, *argH* gene product).

Interestingly some microorganisms including *Bacillus subtilis* have bifunctional OATs that catalyze both the first and fifth reactions of the pathway [40]. Microorganisms with bifunctional OAT that have no NAGS have been reported [40]. Some groups of Proteobacteria including *E. coli*, and possibly Archaea possess acetylornithinase (AO, EC 3.5.1.16, ArgE) instead of OAT, which catalyzes the conversion of acetylornithine to ornithine with the release of acetate [41]. This is referred to as the linear pathway whereas *C. glutamicum* has the cyclic pathway. The cyclic pathway is so named because the acetyl group of *N*-acetylornithine is reused by transferring it to glutamate. The cyclic pathway is more economical since the acetyl group is recycled and not released as acetate [40].
A variety of enzymes can carry out the synthesis of acetylglutamate, for instance ArgO of *Campylobacter jejuni* that is a short NAGS homologous to the C-terminal domain of NAGS. A fusion of short NAGS and ArgH are found in some marine Bacteria [40].

2.2.2 Regulation of the arginine pathway in *Corynebacterium glutamicum*

In microorganisms like *E. coli* with the linear pathway NAGS catalyzes the committed step and is feedback inhibited by arginine. In microorganisms with the cyclic pathway NAGK catalyzes the committed step and is feedback inhibited by arginine [40]. In *C. glutamicum* the acetyl-group is recycled in the OAT reaction and therefore NAGK is feedback regulated to maintain the regulation of arginine synthesis [42]. In *C. glutamicum* crude cell-free extracts, arginine was shown to inhibit NAGK activity with a half-inhibitory concentration of 2 mM and NAGS activity with 40 mM, but the enzymes were not inhibited by ornithine [42].

In some organisms OAT is also a target of feedback inhibition. In *Geobacillus stearothermophilus* the activities of the bifunctional OAT are both inhibited by ornithine, wheras NAGK is not regulated by inhibition [40]. OAT of *C. glutamicum* is feedback inhibited by ornithine where the half-inhibitory concentration was determined to be 5 mM [42] and very recently it was also shown to be inhibited by citrulline with a half-inhibitory concentration of 30 mM [43].

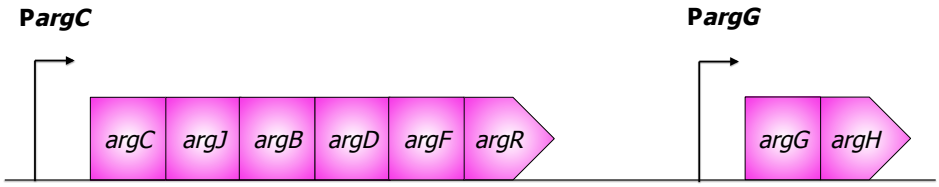

Figure 2. The arginine operons of *Corynebacterium glutamicum.* Arrows denote promoters, and pink boxes genes. The figure was not drawn to scale.

The arginine repressor ArgR exerts regulation at the transcriptional level in *C. glutamicum*. ArgR binds to ARG boxes that overlap promoter elements, and thereby the binding of RNA polymerase is repressed by steric exclusion [44]. The genes encoding the enzymes of the arginine pathway are arranged in two operons; *argCJBDFR* and *argGH* (**Figure 2**). In *C. glutamicum* ArgR represses the transcription of the arginine operon *argCJBDFR*, *gdh* (encoding glutamate dehydrogenase), and *gltB* (encoding the large subunit of glutamine 2-oxoglutarate aminotransferase) [44], [45]. Besides binding to the ARG boxes, located in the promoter region of the arginine operon, ArgR also binds within the operon upstream *argB* [45]. In a study it was shown that the addition of $FeSO_4$ decreases the binding affinity of ArgR to upstream regions of *gdh* and *gltB*, which was beneficial for the biotransformation of phenol to glutamate and proline [45]. Later it was shown that when arginine was added to the growth medium the binding affinity of ArgR upstream *argB* was increased, resulting in reduced ornithine production, whereas the addition of proline or ornithine had the opposite effect, and ornithine production increased [46].

Another transcriptional regulator FarR was found to bind upstream *argC* and *argG* [47]. Transcriptomics performed on *C. glutamicum* Δ*farR* showed an up-regulation of the *arg* genes compared to the wildtype [47]. Hence the function of the regulator was assumed to be repression. The authors also showed that FarR bound upstream *gdh*, but were not conclusive on the type of regulation exerted [47].
The transcriptional control of *gdh* is still not fully comprehended. It has been shown that FarR, AmtR, GlxR, WhiH, and OxyR bind upstream *gdh.* Moreover *gdh* transcription is dependent on growth conditions, and it was reported to have two transcriptional start sites [48].

2.3 Production of glutamate derivatives

The arginine pathway encompasses reactions responsible for ornithine and citrulline production (**Figure 1**). Moreover the pathway can be extended from ornithine to include putrescine and proline (**Figure 1**). Hence several engineering targets for production of ornithine, proline, putrescine, citrulline, and arginine are similar. Here the use and specific modifications for the production of each of these products is described. In the next section strain construction strategies that might be beneficial for all products are covered. Focus was put on the metabolic engineering strategies rather than the obtained yields as comparing published yields can be difficult; not only are different strains used, but also the components of the media, the production conditions, and the presentation of the results vary greatly.

2.3.1 Ornithine

Ornithine has a use in the pharmaceutical industry where for instance the stable salt L-ornithine-L-aspartate is used for the treatment of hepatic encephalopathy [49] or for wound healing as ornithine 2-oxoglutarate [50].
Shortly after the isolation of *C. glutamicum* the overproduction of ornithine by an arginine or citrulline requiring *C. glutamicum* mutant capable of accumulating 26.2 g/L ornithine from 10% (w/v) glucose was reported [51]. Since then several approaches have been taken to increase the yield of ornithine, which will be explored in the following section.

2.3.2 Proline

Proline, the only proteinogenic amino acid with a secondary amine, is commonly used by the chemical industry as an organocatalyst [5]. Additionally it serves as a feed additive and as a precursor in the pharmaceutical and cosmetic industry [52], [53]. Initially, proline was extracted from protein hydrolysates, but has now been produced by fermentation for more than five decades [54], [55]. The natural functions of proline, besides in protein synthesis, have been shown to be as carbon, nitrogen, and energy source, as a virulence factor of some pathogenic bacteria [56]–[58], and as a protectant against osmotic stress [59], [60].
In *C. glutamicum* proline is synthesized from glutamate by phosphorylation by γ-glutamyl kinase encoded by *proB*. Then a reduction to glutamate-γ-semialdehyde occurs, consuming NADPH by the *proA* gene product γ-glutamyl phosphate reductase. A spontaneous reaction occurs whereby glutamate-γ-semialdehyde cyclizes into Δ1-pyrroline-5-carboxylate and lastly the *proC* gene product catalyzes the formation of proline [61]. Some plants and bacteria are able to convert ornithine of the arginine pathway to proline with the enzyme ornithine cyclodeaminase as shown in **Figure 1** [62].
Published or patented proline producers have mainly been obtained by mutagenesis and selection, where the most common traits of these strains are a disrupted proline degradation system and a feedback resistant γ-glutamyl kinase [63]. *C. glutamicum* does however not appear to posses an

active proline degradation system as proline cannot be used as carbon source and is a poor source of nitrogen [64].
B. flavum was the first L-proline producing strain reported, with a yield of 0.12 g proline / g glucose [54]. Another example of a proline producing strain is a *Thermus thermophiles* mutant resistant to 3,4-dehydroproline that was constructed by site-directed mutagenesis of *proB*. To disrupt proline degradation the strain was mutated by UV irradiation and one of the isolated mutants unable to utilize proline for growth produced 2 mg/L in 12h [65].

2.3.3 Citrulline

Citrulline is a precursor of arginine (**Figure 1**) and a key intermediate for urea formation in humans [66]. Most ingested free arginine does not reach the bloodstream because it is cleared by the liver. Citrulline is however not cleared from portal circulation and is converted to arginine in the liver where it is distributed to other organs in the body. Hence citrulline can be used as an alternative way to supplement arginine and has potential to be applied for short bowel syndrome, immunostimulation, and blood pressure control [8]. The non-proteinogenic amino acid citrulline has been produced by extraction from watermelon juice, by chemical and biochemical methods, and by microbial fermentation [67], [68]. In an early report on microbial citrulline production, a *B. subtilis*, strain obtained by irradiation, auxotrophic for arginine produced 19 g/L citrulline [69]. Until recently, rationally engineered citrulline producing microbes had not been described. For the production of citrulline by metabolically engineered *C. glutamicum* ATCC 13032 *argR* and *argG* were deleted and with plasmid-based overexpression of *argJ* the strain accumulated 8.51 g/L [43]. In another study it was shown that the overexpression of *argB* encoding a feedback resistant NAGK was required for citrulline accumulation [68].

2.3.4 Arginine

Arginine has applications in the food, pharmaceutical, and cosmetic industries [70], [71]. It is regarded a nutraceutical as it is a conditionally essential amino acid for humans. Arginine has several functions in the body, while humans produce arginine, a supplement can be required for growth or tissue repair [72]. Moreover arginine is a precursor of the neurotransmitter nitric oxide that is an important component of the endothelium-derived relaxing factor [6].
Biosynthesis of arginine is energetically demanding; from glutamate it requires the donation of three nitrogen atoms. The nitrogen donors in these reactions, glutamate, glutamine, and aspartate need to be regenerated at the expense of ATP and NADPH. Furthermore there is a high demand for bicarbonate, which is assimilated during the formation of carbamoyl phosphate [73]. Therefore, sparging with carbon dioxide during the fermentation is important and might be advantageous for the arginine yield [74]. In arginine production by *C. glutamicum* citrulline is a by-product [75]. By replacing the promoter of the *argGH* operon with the constitutive promoter of the elongation factor Tu the accumulation of citrulline could be prevented [76]. Plasmid-borne heterologous overexpression of *carAB* in *C. glutamicum* led only to a small increase of the arginine titer in the fermentation supernatant [77]. In *C. glutamicum* the lysine exporter LysE exports excess arginine,

as it cannot catabolize it [79–81]. Heterologous as well as homologous overexpression of *lysE* and *argO* in *C. crenatum* had a positive impact on arginine accumulation in the medium [81]. Arginine import in *C. glutamicum* seems to be absent [82]. The gene *cg3045*, however, was proposed to function as a permease of an uptake system for glutamine or arginine [82].

2.3.5 Putrescine

Putrescine is a diamine belonging to the group of biogenic amines and can be used as monomer in polyamide-4,6 production, including nylon-4,6 (bioplastic) [7]. Putrescine is primarily produced chemically by hydrogenation of acrylonitrile. The process requires high temperature and pressure, highly toxic and flammable petrochemical products as raw materials, and expensive catalyst systems. Therefore effort has been put into the development of microbial strains for production of putrescine by fermentation as a sustainable and more environmental friendly alternative [7].

Putrescine can be synthesized via ornithine decarboxylation or arginine decarboxylation. Although the metabolism of polyamines remains unknown in *C. glutamicum*, a putrescine producing strain was constructed by overexpression of ornithine decarboxylase encoded by *speC* from *E. coli*. Efforts made to increase putrescine production include deleting *cg1722* that encodes an enzyme with acetyltransferase activity. Upon the deletion cells no longer accumulated the byproduct N-acetylputrescine and the putrescine concentration was increased [83]. A *C. glutamicum* ATCC 13032 strain with deletions of genes *argF* and *yggB* (encoding a protein involved in glutamate export), substitution of the promoter of the *argCJBD* operon with a synthetic promoter, thereby also disrupting repression by ArgR, and *speC* from *E. coli* was inserted on the chromosome. On 2% (w/v) glucose 8.1 g/L putrescine was accumulated [84]. Moreover a protein possibly involved in putrescine export encoded by *cgmA* was identified. The gene of the *cgmAR* operon is repressed by the CgmR regulator [85]. Interestingly it was shown that putrescine and other diamines perturb the binding of CgmR to its operator upstream *cgmAR*. The deletion of *cgmR* or overexpression of *cgmA* could increase putrescine accumulation by 19 and 24%, respectively [85].

2.4 Metabolic engineering of the ornithine/arginine pathway

A quick search on Pubmed reveals that publications on the topic ornithine or arginine production by *C. glutamicum* has been increasing over the past ten years. In this section some of the findings of these publications are presented and have been ordered into the subsections regulation, tricarboxylic acid cycle, increasing glutamate supply, directing the flux into the arginine pathway, energetics, and other strategies.

2.4.1 Regulation

To create either an ornithine or arginine producing strain it is crucial to prevent the negative regulator ArgR from binding the promoter region of the *argCJBDFR* operon (**Figure 3, 1**). Once the arginine concentration within the cell passes a certain threshold, ArgR binds to the promoter region and physically prevents the RNA polymerase from binding the promoter; as a result transcription cannot be initiated [44]. In one study the deletion of *argR* in wild type *C. glutamicum* did not result in any apparent L-arginine accumulation, however the simultaneous overexpression of *argB* gene variants encoding feedback resistant NAGKs increased the arginine titer to between 15 and 46 mM (**Figure 3, 2**) [41]. As the regulator FarR also binds upstream *argCJBDFR, argGH*, and *gdh* the effect of the deletion of *farR* for the production of arginine was tested. In *C. glutamicum* RES167 the deletion did not result in a difference in the intracellular arginine concentration [47]. It cannot be excluded that the knock out of *farR* does not have an effect on arginine production as the strain tested only excreted very low concentrations of arginine. The deletion of *farR* had also been implemented in another study for increased arginine production, the effect of the deletion appeared to be beneficial although it was not completely clear due to the experimental setup [76]. Hence the quantitative benefit the deletion of *farR* poses on arginine production requires further research.

2.4.2 Tricarboxylic acid cycle

2-oxoglutarate is a branch point where the flux can continue trough the TCA cycle to be converted to succinyl coenzyme A by the 2-oxoglutarate dehydrogenase complex (ODHC) or it can be converted to glutamate by glutamate dehydrogenase (GDH) (**Figure 3**). Three subunits encoded by *odhA* (2-oxoglutarate dehydrogenase E1 component), *aceF* (dihydrolipoamide acetyltransferase), and *lpd* (dihydrolipoamide dehydrogenase) make up ODHC, and deleting one of these subunits has been a target for increasing the flux towards glutamate production [86].
As an example *odhA* was knocked out in the *C. glutamicum* Δ*argF*Δ*proB* double deletion mutant resulting in an ornithine titer of 4.78 g/L compared to 2.68 g/L of the parent strain (**Figure 3, 3**) [87]. This strategy was based on the observation that ODHC activity is decreased during overproduction of glutamate by *C. glutamicum* with biotin limitation, addition of penicillin, or addition of surfactants [88].

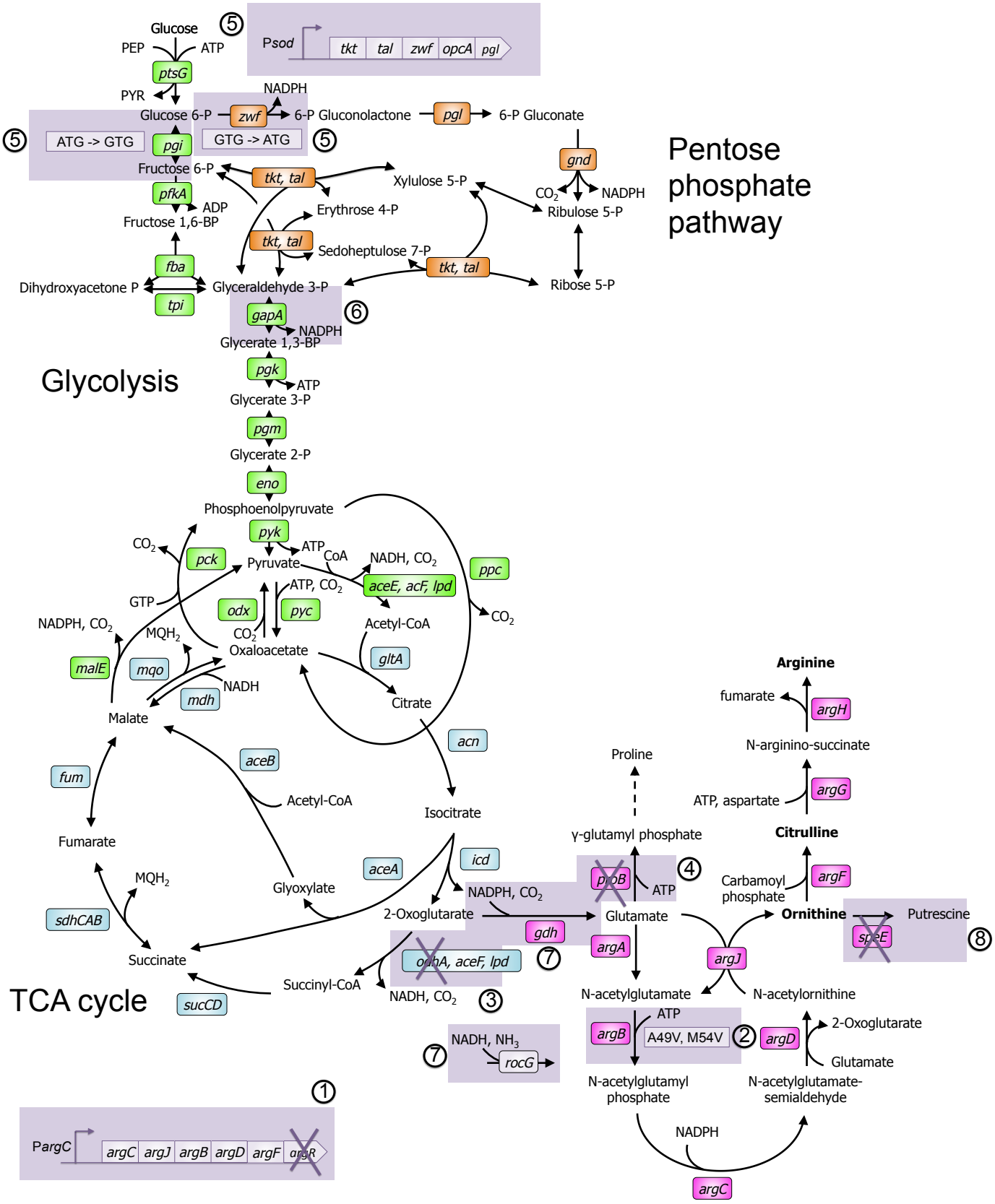

Figure 1. Selected metabolic engineering targets potentially beneficial for the production of glutamate derivatives. Genes in green boxes are part of glycolysis/gluconeogenesis, genes in orange boxes are part of the pentose phosphate pathway, genes in blue boxes are part of the tricarboxylic acid cycle, genes in pink are part of the glutamate, proline, or arginine biosynthetic pathways. 1. Deletion of Δ*argR*. 2. Feedback resistant NAGK 3. Deletion of *odhA* 4. Deletion of *proB* 5. Exhange of *pgi* start codon, exchange of *zwf* start codon, exchange of native promoter with P*sod* 6. Overexpression of *gapA* 7. Overexpression of *gdh* and *rocG* from *Bacillus subtilis* 8. Deletion of *speE*.

A different strategy employed to reduce the level of ODHC for increased putrescine production was to exchange the start codon of the subunit encoded by *odhA*. An exchange of the start codon from GTG to TTG resulted in an increased putrescine yield. OdhI, the inhibitor of OdhA, inhibits ODHC activity in its unphosphorylated state. Introduction of a mutation in *odhI* results in partial or complete loss of phosphorylation of OdhI. This means that the inhibition of ODHC will be maintained and the complex therefore will have a lower activity. This modification also resulted in increased putrescine production. When the two modifications were combined, an even further increase in production was observed, namely 28% more than the parent strain [89].

2.4.3 Increasing the glutamate supply

Increasing the availability of the precursor glutamate for ornithine production was tested by deleting *pck* encoding phosphoenolpyruvate carboxykinase (catalyzing the first step of gluconeogenesis) and overexpressing pyruvate carboxylase. This strategy was previously shown to be beneficial for glutamate production by *C. glutamicum* [92, 93] however when tested in the Δ*argFR*Δ*proB* background no increase in ornithine production was observed [92]. Moreover supplementing glutamate to the growth medium also had no effect on ornithine production [92]. Glutamate does not readily diffuse through the cell membrane and relies on transport proteins. However with the addition of up to 50 mM glutamate the authors did expect some glutamate to be taken up by the cells [92]. On this account it has to be noted that glutamate uptake is repressed by glucose that was the carbon source used in the study [15].

2.4.4 Directing the flux into the arginine pathway

Disrupting the first gene of a competing pathway is a common way to direct the flux into the desired pathway. As glutamate is a precursor of both the proline and arginine pathways the flux can be directed into the arginine pathway by disrupting *proB*, encoding the enzyme catalyzing the first step of the proline pathway (**Figure 3, 4**). This strategy has been applied for both arginine and ornithine production [76], [93]. In the Δ*argFR* background a disruption of *proB* resulted in an increase in ornithine production from 9.65 mg ornithine / g dry cell weight (mg/g) to 12.65 mg/g [92]. Depending on the production process a disruption of *proB* may not be desired as it results in a proline auxotrophic strain that requires proline supplementation. Tuning the level of transcription/translation of *proB* could be used as strategy to avoid auxotrophy, while still benefitting from the increased flux into the arginine pathway. A lowered translation of *proB* mRNA was achieved by exchanging the start codon with the less commonly used TTG codon, this was shown to be beneficial for putrescine production as the exchange not only abolished formation of the byproduct acetylputrescine but also increased the putrescine titer [89]. It remains to be determined if the exchange of the start codon also is beneficial for the production of the other compounds of the glutamate family.

2.4.5 Energetics

Both ornithine and arginine biosynthesis are NADPH requiring processes with a demand of three or four NADPH molecules, respectively. This high demand for NADPH could pose a limiting factor for overproduction of the amino acids. Hence increasing the intracellular NADPH level of the cell has been addressed by several approaches.
One strategy relies on redirecting the carbon flux into the pentose phosphate pathway (PPP). For lysine production this was successfully implemented by a knock out of *pgi* encoding phosphoglucoisomerase resulting in a significantly higher yield. While the disruption of *pgi* results in a higher NADPH availability it is however associated with poor growth on glucose as sole carbon source [94]. Although the NADPH availability in *C. glutamicum* Δ*argFR*Δ*pgi* doubled compared to the Δ*argFR* parent strain, ornithine production had decreased from 8.78 to 1.37 g/L [95]. Redirecting the carbon flux into the PPP was also applied in other studies with arginine and ornithine producing strains. Instead of disrupting *pgi* the start codon of *pgi* ATG was exchanged with GTG thereby lowering the translation of the mRNA. Moreover the start codon of *zwf* encoding glucose-6-phosphate dehydrogenase was changed from GTG to ATG, and the promoter of the *tkt* operon, that *zwf* is part of, was exchanged with the strong constitutive *sod* promoter (**Figure 3, 5**). For both the arginine and ornithine producing strains this resulted in significantly higher yields [77, 96].

Jiang *et al.* took a different approach. They compared plasmid-based overexpression of the genes *gapA* from *C. glutamicum* (**Figure 3, 6**) and *gapC* from *Clostridium acetobutylicum* both encoding the NADPH generating enzyme glyceraldehyde-3-phosphate dehydrogenase of glycolysis [97]. In both cases a significant increase in ornithine production was observed. In the same paper they furthermore showed that reducing the NADPH requirement of the ornithine biosynthetic pathway by overexpressing *rocG* from *B. subtilis* encoding a NAD-dependent GDH resulted in a higher ornithine titer (**Figure 3, 7**) [97]. It should be noted that they also showed that plasmid-based overexpression of endogenous *gdh*, encoding NADPH requiring GDH, resulted in a similar titer (**Figure 3, 7**). Integration of both *gapC* and *rocG* on the chromosome did however not increase the ornithine production any further [97].

Based on the assumption that 6-phosphogluconate can be synthesized through the gluconate bypass in *C. glutamicum*, genes *NCgl2399* and *NCgl2905* assumed to encode gluconate kinases were disrupted in *C. glutamicum* Δ*argFR*. The double deletion strain exhibited increased 6-phosphogluconate dehydrogenase (6PGD, encoded by *gnd*) and glucose dehydrogenase activities, an increased NADPH level and an increase in ornithine production from 0.71 g ornithine/ g dry cell weight (g/g) to 13.16 g/g [95].
The genes NCgl0281, NCgl2582, and NCgl2053 were speculated to encode $NADP^+$-dependent isoenzymes of glucose dehydrogenase catalyzing the oxidation of glucose to gluconate. So far it has however not been demonstrated that *C. glutamicum* should posses such an activity [98]. The deletions entailed an increased production of the *C. glutamicum* strain with the genetic background Δ*argFR*ΔNCgl0281ΔNCgl2582ΔNCgl2053 from 14 g/L compared to 8 g/L of the parent strain with the genetic background Δ*argFR*.

Glucose dehydrogenase and gluconate kinase appear to provide an alternative path for glucose to enter the PPP. Both the inactivation of three putative oxidoreductases and two gluconate kinases were shown to induce activity of glucose-6-phosphate dehydrogenase (encoded by *zwf* and *opcA*) and 6PGD of the oxidative PPP and increased NADPH levels, by what appears to be a loss of operation of the gluconate bypass system, although the mechanism of activation appears to be different for the gluconate kinase and the glucose dehydrogenase deficiencies [98].

2.4.6 Other strategies

speE encodes putative spermidine synthase catalyzing the conversion of putrescine to spermidine (**Figure 3, 8**). The specific function of the enzyme in *C. glutamicum* has not been proven, nor have the other polyamine synthetic enzymes, however *C. glutamicum* does contain putrescine and polyamines [101, 102]. It was proposed that the deletion might prevent ornithine degradation, although no biochemical/biological evidence for this exists. The deletion of *speE* resulted in an increased ornithine titer from 10.2 ± 0.2 g/L to 11.3 ± 0.3 g/L in the Δ*argF*Δ*proB* background [100].

Plasmid based overexpression of *argCJBD* to increase the enzymatic activity of the enzymes of the ornithine pathway has also been explored. Such overexpression led to a 30% increase in ornithine production in the Δ*argFR* Δ*proB* background, from 12.73 mg ornithine/g dry cell weight to 16.49 mg/g [92].
Similarly, overexpression of the *argCJBDFRGH* operon in the arginine producing *Corynebacterium crenatum* strain SYPA 5-5, insensitive to ArgR regulation, resulted in increased arginine production by about 25% [101].

A combination of adaptive evolution and metabolic engineering was used to obtain the strain *C. glutamicum* ΔAPE6937R42 producing 24.1 g/L ornithine in a 5L fermenter [100]. The strain was constructed by deleting the genes *speE*, *argF*, and *proB*. Then the strain was subjected to 70 passages of adaptive evolution. First by adding glucose and ornithine to the medium, after 30 passages ornithine addition was omitted and an increased glucose concentration was added to the medium and after 20 more passages the glucose concentration was increased further. One clone that produced 20% more ornithine (from 11.3 g/L to 13.6 g/L) than the parent strain was selected and *argR* was disrupted to result in the strain ΔAPE6937R42 with a titer of 17.3 g/L [100].

Although the strategies above have all had an impact on increasing yields of ornithine and arginine there is still much room for improvement. Taking the step to the next level requires a combination of several engineering strategies to obtain superior producers. Engineering strains for industrial production of a compound is no trivial task; building a new strain can cost from millions to hundreds of millions of US dollars [102].

2.5 Metabolic engineering and the CRISPR/dCas9 system

Traditionally, strains for production of amino acids were improved by chemical or UV-mutagenesis and selection based on resistance to antimetabolites or other analogues. Amino acid biosynthesis is commonly regulated by end-product inhibition, and by this approach mutants with feedback alleviated enzymes could be isolated. Moreover mutants with a higher tolerance to the desired product could be isolated [93].
Although mutagenesis and selection is a powerful approach for obtaining high producing strains, they often have growth defects, are genetically unstable, have low tolerance to stress, or have a demand for specific nutrients [103]. A further disadvantage is that it can be difficult to identify the individual effects of the mutations introduced.
Targeted changes to cells could be performed already back in the 1970s with the introduction of recombinant DNA technology, and in 1991 Bailey defined the term metabolic engineering as "The improvement of cellular activities by manipulations of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology" [103]. The constantly expanding information available on enzyme kinetics, genetics, and regulation along with genome sequencing projects, advances in the fields of DNA synthesis, sequencing, fluxomics, metabolomics, transcriptomics, and proteomics all facilitate the construction of rationally engineered high producing strains [104], [105]. Moreover computational methods for metabolic engineering have been successfully employed [106][107].

A time-limiting factor in strain construction can be the modifications performed on the genome. The widely used two-step homologous recombination approach with a suicide plasmid can at times be difficult, and therefore time consuming [108].
Here the widely used Clusters of Regularly Interspaced Short Palindromic Repeats (CRISPR)/dCas9 system could potentially be applied for fast strain construction. The system has already been successfully employed in a variety of organisms including bacteria [109][110], yeast [111], plants [112], and mammals [113].

2.5.1 Using the CRISPR/dCas9 system as a tool for metabolic engineering

The CRISPR/Cas system is found in a wide range of bacteria and archaea and functions as an adaptive immune system [114]. In short the system works by cleaving foreign DNA in a sequence specific manner using small base-pairing RNA to target the sequence with [115].
Several different CRISPR systems exist, one of the simplest, a type II CRISPR system, was recently used for targeted genome editing [110]. This system from *Streptococcus pyogenes* employs the double-stranded DNA endonuclease Cas9, mature CRISPR RNA (crRNA), and *trans*-acting RNA (tracrRNA) to silence foreign DNA [116].

CRISPR loci consist of short multiple repeats separated by spacers as the name CRISPR implies [117]. These spacer sequences are short stretches of DNA that match mobile genetic elements and genomes of bacteriophages [117]. The stretch of repeats and spacers are transcribed as one precursor mRNA. This precursor mRNA is then processed within the repeats to generate the

crRNA. The target sequence that is cleaved by the CRISPR systems is specified by the crRNA [118]. The tracrRNA along with RNase III are required to mature the crRNA [119], these elements are not needed if instead engineered small guide RNA (sgRNA) with a hairpin mimicking the tracrRNA-crRNA complex is used [116]. The sgRNA should have a 20 bp region complementary to the target sequence. Cas9 can then introduce double-strand breaks once the sgRNA and the target DNA base pair. For the binding to occur the promoter adjacent sequence NGG juxtaposed the complementary region is required [120].

The CRISPR/Cas9 system was recently modified to enable its use for regulation of transcriptional expression. With this system binding of the RNA polymerase to a promoter, binding of transcription factors, and transcriptional elongation can be prevented [110]. To achieve this, a catalytically inactive Cas9 (dCas9) was constructed by introducing two mutations in the RuvC1 and HNH nuclease domains of Cas9. The gene expression could thereby be reversibly repressed in *E. coli* with no off-target effects [110].

What makes this technology particularly useful for metabolic engineering is that it can be used to target several targets at once. This allows the researcher to assess the combined effect of multiple deletions, without going through the taunting, slow, and laborious process the deletion of one gene after another can be.

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Chapter 3. Ornithine cyclodeaminase-based proline production by *Corynebacterium glutamicum*

Jensen, J.V.K., & V.F. Wendisch (2013). Ornithine cyclodeaminase-based proline production by *Corynebacterium glutamicum. Microb Cell Fact*, *12*, 63.

3.1 Abstract

The soil bacterium *Corynebacterium glutamicum*, best known for its glutamate producing ability, is suitable as a producer of a variety of bioproducts. Glutamate is the precursor of the amino acid proline. Proline biosynthesis typically involves three enzymes and a spontaneous cyclization reaction. Alternatively, proline can be synthesized from ornithine, an intermediate of arginine biosynthesis. The direct conversion of ornithine to proline is catalysed by ornithine cyclodeaminase. An ornithine overproducing platform strain with deletions of *argR* and *argF* (ORN1) has been employed for production of derived compounds such as putrescine. By heterologous expression of *ocd* this platform strain can be engineered further for proline production.

Plasmid-based expression of *ocd* encoding the putative ornithine cyclodeaminase of *C. glutamicum* did not result in detectable proline accumulation in the culture medium. However, plasmid-based expression of *ocd* from *Pseudomonas putida* resulted in proline production with yields up to 0.31 ± 0.01 g proline/g glucose. Overexpression of the gene encoding a feedback-alleviated N-acetylglutamate kinase further increased proline production to 0.36 ± 0.01 g/g. In addition, feedback-alleviation of *N*-acetylglutamate kinase entailed growth-coupled production of proline and reduced the accumulation of by-products in the culture medium.

The product spectrum of the platform strain *C. glutamicum* ORN1 was expanded to include the amino acid L-proline. Upon further development of the ornithine overproducing platform strain, industrial production of amino acids of the glutamate family and derived bioproducts such as diamines might become within reach.

3.2 Background

The workhorse *Corynebacterium glutamicum* has for decades been used as an amino acid producer. Although, in terms of quantity, the main contributors to the amino acid market are L-lysine and L-glutamate, minor amino acids such as L-proline are also of importance. Proline is predominantly used as an organocatalyst by the chemical industry, as a precursor for compounds with pharmaceutical and cosmetic applications, and as a feed additive [1-3]. The natural functions of proline in prokaryotic and eukaryotic cells have been reported to be as an osmolyte, a potential virulence factor for some pathogenic bacteria, and a source of carbon, nitrogen, and energy [4]. The amino acid functions as a compatible solute of *C. glutamicum* and in this respect the organism has been shown to grow at intracellular concentrations of up to 94 g/L proline with no determined upper limit [5]. In *Escherichia coli* the bifunctional enzyme PutA catalyses the two-step oxidation of proline to glutamate with proline dehydrogenase and Δ1-pyrroline-5-carboxylate dehydrogenase activities: reactions that occur at high proline concentrations [6]. *C. glutamicum* contains a putative *putA* gene, but the activity of the encoded enzyme has thus far not been confirmed. It is not clear whether *C. glutamicum* can utilise proline as a carbon or nitrogen as contradictory statements about proline utilisation by this bacterium have been published [7,8].

Proline is synthesized from glutamate via three enzymatic and one spontaneous reaction, in most investigated microorganisms [4,9,10]. The enzymes of the proline pathway encoded by *proB, proA,* and *proC* catalyse the phosphorylation of glutamate followed by reduction to glutamate-γ-semialdehyde, a spontaneous cyclisation, and finally the reduction to proline. An alternative route to proline biosynthesis involves ornithine cyclodeaminase (OCD) which catalyses the conversion of ornithine to proline and ammonia with deamination of the α-amino group prior to cyclization (Figure 1). However, only a few organisms such as *Clostridium sporogenes*, *Treponema denticola*, *Agrobacterium tumefaciens*, and *Pseudomonas putida* have been reported to contain OCD [11-15]. The genome of *C. glutamicum* contains a putative *ocd* gene, however, evidence for its function as ornithine cyclodeaminase has not been reported [16].
In *C. glutamicum* ornithine is an intermediate of arginine biosynthesis and is synthesized from glutamate through five enzymatic steps where the second enzyme, *N*-acetylglutamate kinase (NAGK), is feedback-inhibited by arginine [17]. It is known that the arginine biosynthetic pathway of *C. glutamicum* is regulated at the transcriptional level by the repressor ArgR that has been shown to bind upstream regions of *argC*, *argB*, *argF*, and *argG* [18]. Further genetic regulation, although not fully comprehended, is concerted by the acyl-responsive transcriptional regulator FarR and by the potential allosteric inhibition of ornithine acetyltransferase by ornithine [18,19].

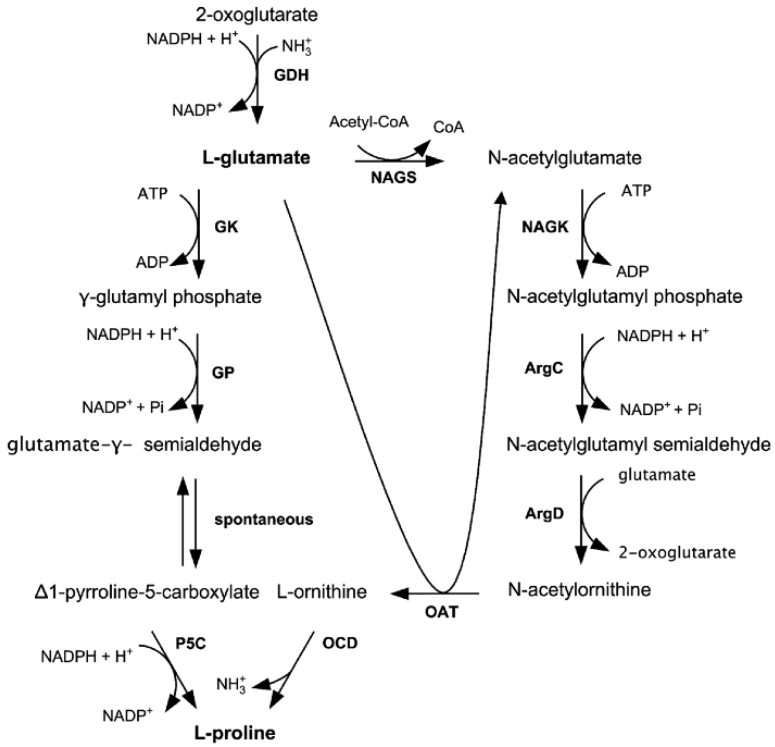

Figure 1 Proline biosynthesis in *Corynebacterium glutamicum*. Reactions and enzymes of the proline and ornithine biosynthetic pathways of *Corynebacterium glutamicum* are depicted together with the reaction catalysed by ornithine cyclodeaminase (OCD): glutamate dehydrogenase (GDH) encoded by *gdh*, γ-glutamyl kinase (GK) encoded by *proB*, γ-glutamyl phosphate reductase (GP) encoded by *proA*, pyrroline-5-carboxylate reductase (P5C) encoded by *proC*, N-acetylglutamate synthase (NAGS) activity, N-acetylglutamate kinase (NAGK) encoded by *argB*, N-acetyl-γ-glutamyl-phosphate reductase (ArgC) encoded by *argC*, acetylornithine aminotransferase (ArgD) encoded by *argD*, ornithine acetyltransferase (OAT) encoded by *argJ*, and OCD encoded by *ocd*.

An ornithine overproducing *C. glutamicum* strain [20] [21] with deletions of the genes *argR* and of *argF*, the gene encoding ornithine carbamoyl-transferase, which converts ornithine to citrulline in the arginine biosynthetic pathway, has been constructed. This strain, called ORN1 has the potential of serving as a platform for the production of several industrially relevant bioproducts of the glutamate family; namely ornithine, citrulline, arginine, putrescine, and spermidine. Based on *C. glutamicum* ORN1, strains PUT1 [22] and PUT21 [23] have been developed for production of 1,4-diaminobutane (putrescine), and ARG1 [20] is an arginine-producing ORN1 derivative. Here, the production of proline based on ORN1 is reported. Heterologously produced ornithine cyclodeaminase from *P. putida* led to a conversion of ornithine to proline and thereby constitutes an expansion of the product palette of the platform strain ORN1.

3.3 Results

3.3.1 Proline utilisation

To establish if the putative proline degradation system of *C. glutamicum* had an effect on extracellular accumulation of proline, we first investigated the utilisation of proline as carbon and nitrogen source. Wild-type *C. glutamicum* was inoculated to an optical density (OD) of 1 in CGXII minimal medium with 20 g/L glucose or a C-equimolar concentration of proline as carbon source and 20 g/L ammonium sulfate and 5 g/L urea or a N-equimolar concentration of proline as nitrogen source. For cells cultured in medium with proline as carbon source no biomass formation was observed during 48 h of incubation. An OD of ~30 could be reached within 10 h by cultivation in CGXII medium with glucose, ammonium sulfate, and urea. When an N-equimolar proline concentration was used as the sole nitrogen source, the cells were able to duplicate twice in 24 h within the 48 h of incubation resulting in a maximum OD of ~3.5. For comparison an OD of ~1.5 could be reached when no nitrogen source was added to the medium.

3.3.2 Plasmid-based overexpression of *ocd*

The gene encoding the biochemically characterized OCD from *P. putida* [24] and the putative *ocd* from *C. glutamicum* were cloned into the IPTG-inducible expression plasmid pVWEx1 [25]. The resulting plasmids pVWEx1-ocd_{Cg} and pVWEx1-ocd_{Pp} were transformed into the ornithine producer ORN1 to yield strains JJ002 (ORN1 carrying pVWEx1-ocd_{Cg}) and JJ003 (ORN1 carrying pVWEx1-ocd_{Pp1}). For a presumed more efficient translational termination, the original stop codon TGA of ocd_{Pp} was replaced by the more frequently used TAA (JJ004).

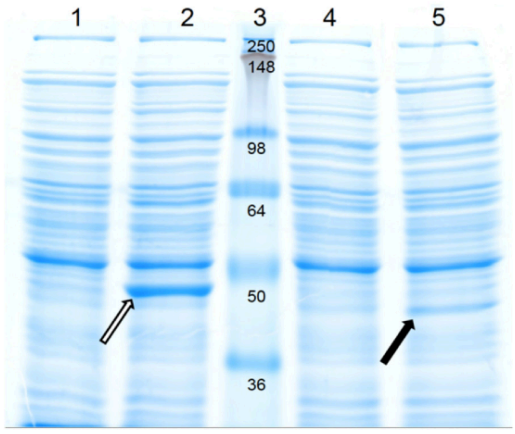

Figure 2 SDS-PAGE analysis of crude extracts of *C. glutamicum* strains JJ001, JJ002, JJ003, and JJ004. JJ001 (lane1), JJ002 (lane 2), protein standard (lane 3), JJ003 (lane 4), and JJ004 (lane 5). The deduced molecular mass of putative OCD from *Corynebacterium glutamicum* in JJ002 is 41 kDa (open arrow) and OCD from *Pseudomonas putida* in JJ003 and JJ004 is 38 kDa (filled arrow). The units of the protein standard are in kDa. Crude extracts were prepared from cells grown in BHI with 1 mM IPTG and 25 μg/mL kanamycin.

Table 1 Ornithine cyclodeaminase activity

Crude extract	Specific activity (μmol/min/mg protein)
JJ001	$< 4{\cdot}10^{-4}$
JJ002	$< 4{\cdot}10^{-4}$
JJ003	0.06 ± 0.02
JJ004	0.71 ± 0.09

Crude extracts were obtained by sonication of cells cultured in BHI medium supplemented with 1 mM IPTG and 25 μg/mL kanamycin.

The specific cyclodeaminase activities in crude extracts of these strains and the control strain JJ001 (ORN1 carrying pVWEx1) were determined and the presence of the overproduced proteins visualized by SDS-PAGE (Figure 2, Table 1). No activity could be observed for crude extracts of JJ001 and JJ002, although the crude extract of JJ002 exhibited a band on an SDS-gel corresponding to the weight of the putative *C. glutamicum* OCD of 40.96 kDa, calculated based on the amino acid sequence (Figure 2). Substitution of the translational stop codon caused an almost 12-fold increased specific activity of OCD_{Pp}.

Shake flask fermentations in glucose minimal medium with IPTG were performed with the aforementioned strains. Samples were withdrawn for product quantification by HPLC. Upon glucose depletion, proline could not be detected in the supernatants of strains JJ001 and JJ002, whereas ornithine was produced by both strains (Table 2). By contrast, JJ003 and JJ004 accumulated proline in the supernatant (Figure 3, Table 2). Owing to the feedback inhibition of NAGK by arginine, production of proline was growth decoupled. Strain JJ004 accumulated about three folds more proline than JJ003 indicating that the improved translational termination of ocd_{Pp} entailed not only increased OCD activity, but also increased proline production. All strains accumulated ornithine and the by-products threonine, alanine, and valine. Furthermore trace amounts of glutamate (up to 5 µM) could be detected, except for strain JJ003 where 0.24 ± 0.1 g/L was accumulated.

Table 2 Accumulation of proline and by-products

	Proline (g/L)	Ornithine (g/L)	Threonine (g/L)	Alanine (g/L)	Valine (g/L)
JJ001	$< 10^{-4}$	14.8 ± 0.2	0.3 ± 0.01	0.7 ± 0.03	0.7 ± 0.01
JJ002	$< 10^{-4}$	12.1 ± 0.3	0.3 ± 0.01	0.5 ± 0.01	0.5 ± 0.01
JJ003	2.4 ± 0.1	5.5 ± 0.3	0.3 ± 0.01	0.6 ± 0.01	0.4 ± 0.01
JJ004	10.0 ± 0.1	2.8 ± 0.2	0.2 ± 0.01	0.3 ± 0.01	0.3 ± 0.01
JJ004*	10.9 ± 0.3	0.2 ± 0.1	0.2 ± 0.01	0.3 ± 0.01	0.6 ± 0.01

Titers were obtained from cells grown in CGXII medium with 40 g/L glucose, 0.75 mM arginine, 1 mM IPTG, and 25 µg/mL kanamycin. * Titers were obtained from cells grown in CGXIIm medium with 35 g/L glucose and supplements as stated above.

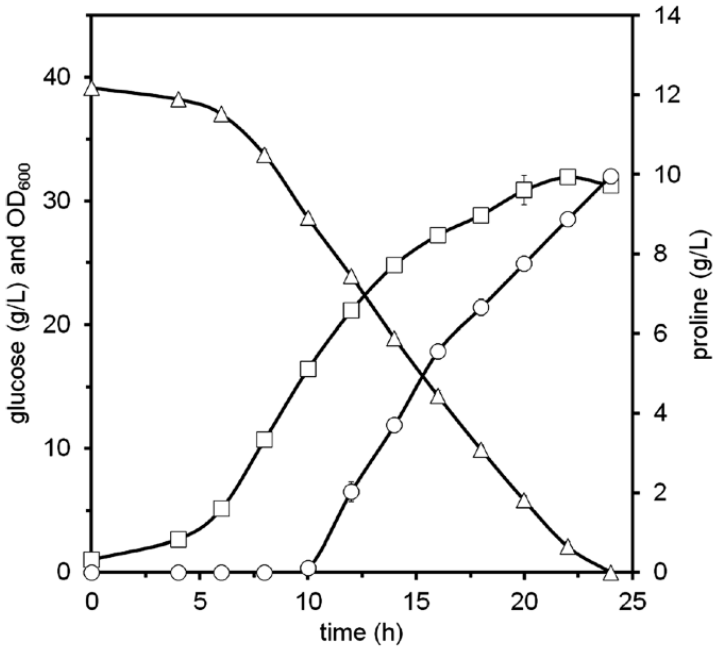

Figure 3 Growth, substrate consumption, and proline formation by *C. glutamicum* JJ004 in CGXII minimal medium containing 40 g/L glucose, 0.75 mM arginine, 1 mM IPTG, and 25 µg/mL kanamycin. Open square, biomass; open triangle, glucose; open circle, proline. Data are means and standard deviations of at least three cultivations.

3.3.3 Identification of key medium components for proline production

As deamination of ornithine by OCD yields ammonia besides proline, and as CGXII contains a high nitrogen concentration, it was tested if the nitrogen content of the medium had an effect on proline formation and if other medium components influenced production. First the effect of the nature of the nitrogen source was tested; here urea, and/or ammonium sulphate or ammonium chloride were selected (Table 3). Urea as sole nitrogen source at the concentration tested appeared to be superior for proline overproduction.

Table 3 Proline production with different nitrogen sources

Nitrogen source	Proline (g/L)
Urea, $(NH_4)_2SO_4$	8.0 ± 0.2
Urea	11.5 ± 0.4
$(NH_4)_2SO_4$	6.2 ± 0.1
NH_4Cl	6.0 ± 0.1

Titers were obtained from cells grown in CGXII medium with 40 g/L glucose, 0.75 mM arginine, 1 mM IPTG, and 25 µg/mL kanamycin.

After the initial screen a Plackett-Burman design was used to identify key components in the medium affecting proline production and to verify that the nitrogen source significantly affects proline production. The design made it possible to determine the relevant factors with a small number of trials. Twelve factors were screened; all components of CGXII medium and in addition IPTG and arginine. The experimental design and responses are shown in Table 4. Significant effects on proline production were observed when concentrations of glucose, urea, and monopotassium phosphate were varied (Figure 4). A positive effect on proline production was observed for high concentrations of glucose, while low concentrations of urea and monopotassium phosphate improved proline production. As the *t*-values of the effects of glucose, urea, and monopotassium phosphate lie above not only the *t*-value threshold, but also the conservative Bonferroni threshold, the components are more likely to be key medium components and their effect not stochastic.

Table 4 Plackett-Burman design represented by coded values, and proline concentration as the response

Run	A	B	C	D	E	F	G	H	I	J	K	L	D1	D2	D3	D4	D5	D6	D7	Response Proline (g/L)
1	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	+	+	+	4.3 ± 0.01
2	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	3.4 ± 0.05
3	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	+	-	+	4.1 ± 0.4
4	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	-	+	+	5.8 ± 0.2
5	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	7.9 ± 0.3
6	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	+	+	-	8.2 ± 0.08
7	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	-	-	+	5.3 ± 0.1
8	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	-	-	-	4.9 ± 0.01
9	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	6.7 ± 0.1
10	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	5.9 ± 0.05
11	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	6.8 ± 0.08
12	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	-	+	-	7.7 ± 0.4
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.2 ± 0
14	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	7.8 ± 0.4
15	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	+	-	3.0 ± 0.2
16	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	6.5 ± 0.2
17	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	+	-	-	2.5 ± 0.2
18	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	3.0 ± 0.2
19	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	2.7 ± 0.07
20	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	+	+	-	5.1 ± 0.09

+ indicates that the medium component was used at its high level concentration.
– indicates that the medium component was used at its low level concentration.

Thus, three different concentrations of urea (2.5, 5, and 7.5 g/L), glucose (20, 35, and 50 g/L) and monopotassium phosphate (0.5, 1.25, and 2 g/L) were tested (data not shown) and an improved medium was derived (5 g/L urea as nitrogen source, 35 g/L glucose as carbon source and 2 g/L potassium phosphate) and was used for further proline production experiments. Fermentations of JJ004 in the modified medium increased proline production by 25% compared to production in CGXII medium (0.31 ± 0.01 as compared to 0.25 ± 0.003 g proline/ g glucose, Table 2) and ornithine accumulation was reduced.

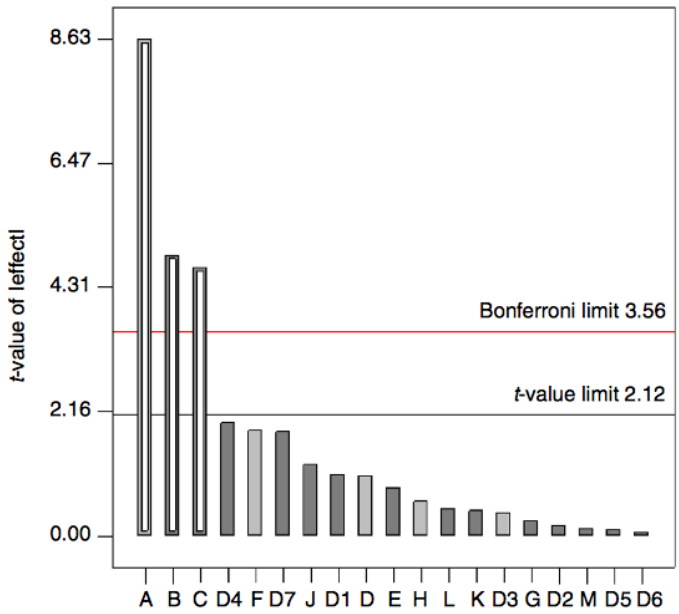

Figure 4 Pareto chart showing the standardised effects of medium components on proline production. Light grey, the influence of the tested factor upon proline production is greater at the high concentration; dark grey, the influence of the tested factor upon proline production is greater at the low concentration. Statistically significant effects (open columns) are differentiated from insignificant effects (closed columns). A-L and D1-D7 are defined in Table 6.

3.3.4 Coupling growth to proline production

In *C. glutamicum* the second enzyme of the ornithine pathway NAGK is feedback inhibited by arginine. The feedback inhibition therefore constitutes a rate-limiting step at high arginine concentrations. Hence, both the feedback alleviation of NAGK and leaky expression of *argF* have been employed as means to increase/ couple growth to production [20,23]. Here we show that the overexpression and feedback alleviation of *argB* (strain JJ006) resulted not only in increased and growth-coupled proline production, but also in a reduction of by-products when cultured in the modified CGXII medium (Figure 5, Table 5).

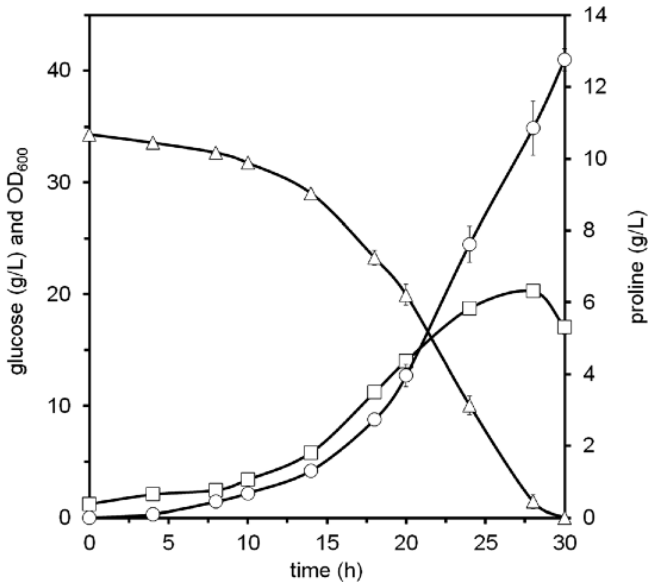

Figure 5 Growth, substrate consumption, and proline formation by *C. glutamicum* JJ006 in modified CGXII medium containing 35 g/L glucose, 0.75 mM arginine, 1 mM IPTG, and 25 µg/mL kanamycin. Open square, biomass; open triangle, glucose; open circle, proline. Data are means and standard deviations of at least three cultivations.

Table 5 Accumulation of proline and byproducts

	Proline (g/L)	Ornithine (g/L)	Threonine (g/L)	Alanine (g/L)	Valine (g/L)
JJ005	11.7 ± 0.1	$< 10^{-4}$	0.2 ± 0.005	0.09 ± 0.005	0.28 ± 0.004
JJ006	12.7 ± 0.3	$< 10^{-4}$	0.09 ± 0.003	0.09 ± 0.002	0.16 ± 0.001

Titers were obtained from cells grown in CGXIIm with 35 g/L glucose, 0.75 mM arginine, 1 mM IPTG, 25 µg/mL kanamycin, and 50 µg/mL spectinomycin.

3.4 Discussion

C. glutamicum is, especially with regards to carbon and amino acid metabolism, a well-studied bacterium. Nevertheless, details on the proline degradation pathway and regulation of the proline and arginine biosynthetic pathways remain to be elucidated. *C. glutamicum* can utilise several amino acids as sole carbon and/or nitrogen source [7] e.g. glutamine has been demonstrated to be an excellent nitrogen source and also allows growth when used as sole carbon and nitrogen source [26]. In the case of proline contradictory observations of its utilisation as nitrogen source have been made [7]. Bott & Niebisch reported that *C. glutamicum* could utilize proline as carbon and nitrogen source, however very slowly. In this study, it was shown that proline did not serve as sole source of carbon for growth of *C. glutamicum*, and that it is a very poor source of nitrogen.

When comparing proline synthesis starting from 2-oxoglutarate via the proline pathway to the conversion via the ornithine pathway, there is a difference in the requirement for ammonia. In the proline biosynthetic pathway one molecule of ammonium is assimilated in the reductive amination of 2-oxoglutarate to glutamate by glutamate dehydrogenase (GDH) per molecule of proline produced (Figure 1). In the OCD-based pathway, a second ammonium molecule is required to be assimilated by GDH, however, it is released during the final ornithine cyclodeaminase reaction. Therefore the conversion of 2-oxoglutarate to glutamate via GDH must be twice as high in the OCD-based pathway as compared to the proline pathway. By medium optimization employing the Plackett-Burman design it could be shown that a low urea concentration had a positive effect on proline accumulation. While urea utilization needs to be induced, ammonium assimilation via GDH only shows a weak dependency on nitrogen availability [27]. Besides the low affinity assimilation to ammonium via GDH, *C. glutamicum* also possesses the high affinity GS-GOGAT system for ammonium assimilation, which is induced upon nitrogen starvation [27]. The *in vivo* fluxes of ammonia assimilation via GDH and GS-GOGAT could be determined in *C. glutamicum* ATCC 13032 and a direct dependency of flux via the GS-GOGAT system on ammonium availability was observed [27]. Under C-limited conditions in a continuous culture GS and GOGAT activities in crude extracts were significantly reduced [27]. Proline production was performed with sufficient ammonium, therefore it is assumed that GDH primarily contributes to ammonium assimilation under these conditions.

The genome of *C. glutamicum* contains a gene annotated to encode ornithine cyclodeaminase [16] within the nitrogen-regulated putative *amt-ocd-sox* operon [28]. Overexpression of this operon was beneficial for lysine production, but the molecular mechanism remained unknown [29]. The deletion of *ocd* in an *argF*$^-$*, argR*$^-$ strain of *C. glutamicum* increased ornithine production by this strain. Supplementing 5 mM proline improved ornithine production further, which was hypothesized to indicate a possible role of OCD in the conversion of proline to ornithine [30]. As shown here, overexpression of *ocd* from *C. glutamicum* neither entailed proline production nor detectable OCD activity, although a SDS-PAGE of crude extracts revealed overproduction of the protein. Thus, the protein encoded by ocd_{Cg} either does not possess OCD activity or its activity was too low to be detected. It is interesting to note that multiple protein sequence alignments of biochemically characterized OCDs with putative OCDs [32, 24] revealed that some conserved

active site residues of OCDs are not conserved in OCD_{Cg}. Instead of Arg45 (numbering according to OCD from *P. putida*), one of three residues whose side chains interact with the ornithine carboxyl group, OCD_{Cg} contains a glutamate residue and Asp228, whose side chain forms a hydrogen bond with the leaving ammonia group, is a glycine residue in OCD_{Cg}. The lack of conservation of these and further amino acids might explain why no OCD activity could be detected in *C. glutamicum*. Physiologically, *C. glutamicum* differs from pseudomonads that typically are able to catabolize arginine and ornithine as sole carbon and nitrogen source. In most *Pseudomonas* species utilization of ornithine as carbon source involves succinylation of ornithine, however *P. putida*, which is devoid of such activity, catabolizes ornithine via OCD and subsequently via proline degradation [32]. OCD is also involved in opine degradation by *Agrobacterium tumefaciens* with e.g. the nopaline catabolism region of Ti plasmid C58 encoding OCD for degradation of nopaline via arginine and ornithine to proline [33]. The observation that *C. glutamicum* does not appear to be able to utilise proline, as shown in this study, or ornithine (unpublished observation) as sole nitrogen or carbon source is commensurate with the lack of OCD activity. It remains to be shown if the protein annotated as putative OCD is active as ornithine cyclodeaminase, or whether it catalyses another reaction.

Heterologous expression of ocd_{Pp} by the ORN1 strain resulted in proline accumulation, and a significant increase in production could be achieved by changing the stop codon from TGA to TAA. While examples of modulating translation initiation by changing the start codon or by changing the sequence or spacing of the ribosome binding site exists for *C. glutamicum* [23], modulating translation termination by altering the stop codon has to the best of our knowledge not yet been reported. Increased OCD levels and activities as consequence of changing the stop codon from TGA to TAA is in line with a bioinformatic study on codon usage of *C. glutamicum*. Putative highly expressed genes exhibited a strong bias for the UAA stop codon, while such a preference was not observed in lowly expressed genes [34]. It is likely that optimization of sense codons of ocd_{Pp} to fit the sense codon preference of *C. glutamicum* better, could contribute to a further increase in proline production.

As previously demonstrated, glutamate is not limiting the flux through the ornithine pathway, rather it is the feedback inhibition of NAGK by arginine and potentially the feedback inhibition of OAT by ornithine [18, 21]. Accordingly, overproduction of feedback-alleviated NAGK not only led to growth-coupled production of proline, but improved proline production. In addition, growth was affected as less biomass formed and as the growth rate was reduced. It is noteworthy that proline production already started early during growth which may be beneficial for the overall space-time yield of the process. Moreover, formation of the by-products valine and threonine that are not amino acids of the glutamate family was reduced. As trace amounts of glutamate could be detected in the samples taken for all strains constructed (JJ001-JJ006), this is an indication of that the bottleneck in proline production is located between glutamate and proline. A further improvement of the conversion of glutamate to ornithine can be envisioned by alleviating a potential feedback inhibition of OAT and/or employing a bifunctional enzyme with OAT and NAGS activities.

3.5 Conclusions

Heterologous overexpression of *ocd* in *C. glutamicum* ORN1 resulted in the overproduction of proline through the ornithine pathway. *C. glutamicum* JJ004 had a yield of 0.31 ± 0.01 g proline / g glucose. Alleviating feedback inhibition of *N*-acetylglutamate kinase entailed growth-coupled and improved proline production with a yield of 0.36 ± 0.01 g/g. The addition of proline to the product palette of the ornithine producing strain ORN1 emphasises that this strain might be exploited as platform strain for industrially relevant bioproducts such as ornithine, proline, putrescine, spermidine, citrulline, and arginine. Moreover, engineering strategies of the platform strain can easily be transferred and applied to improve derived producer strains.

3.6 Methods

3.6.1 Strains, plasmids, and media

C. glutamicum strain ATCC 13032 [35], ORN1 [21], and its derivatives have been used in this study. As ornithine is a precursor to several interesting products, such as amino acids, di- and polyamines of the glutamate family, ORN1 has the potential to serve as a platform strain. *E. coli* DH5α [36] was used for the cloning procedures and cultured at 37°C in Lysogeny Broth (LB) [37] or on LB-agar. Competent *E. coli* cells and molecular techniques were performed according to standard procedures [37]. Chromosomal DNA from *C. glutamicum* and *Pseudomonas putida* KT2440 was isolated by resuspending overnight cultures in 360 µL 50 mM Tris-HCl (pH 8) followed by the addition of a spatula tip of lysozyme, and incubation at 37°C for two hours. Thereafter the procedure "DNA purification from tissues" with the QiaAmp DNA mini kit (Qiagen, Hilden, Germany) was followed. Preparation and transformation of *C. glutamicum* competent cells was performed according to published methods [38]. Plasmids pVWEx1-ocd_{Pp2} and pVWEx1-ocd_{Pp1} were constructed by amplifying *ocd* from *P. putida* [NCBI-GeneID: 1046312] with primers *ocd2*-FW (CTTctgcagAAGGAGATATAGATATGACGTATTTCATTGATGTTCCA) and *ocd3*-RV (CCTggtaccTTAGGCAACCCGTCGGATAC, the stop codon was modified from TGA to TAA) or *ocd2*-RV (CCTggtaccTCAGGCAACCCGTCGGATAC). The amplified fragments were treated with *Kpn*I and *Pst*I and ligated with similarly treated pVWEx1. Plasmid pVWEx1-ocd_{Cg} was constructed similarly, however primers *ocd1*-FW (CTTctgcagAAGGAGATATAGATATGACCGCAACCTACACCACTG) and *ocd1*-RV (CCTggtaccTCAAGCCAGTGCGGGTG) were used for the amplification of *ocd* from *C. glutamicum* [NCBI-GeneID: 3343467]. The construction of pEKEx3-$argB_{A49VM54V}$ has been described elsewhere [20]. Plasmids pVWEx1, pVWEx1-ocd_{Cg}, pVWEx1-ocd_{Pp2}, pVWEx1-ocd_{Pp1}, pEKEx3, and pEKEx3-$argB_{A49VM54V}$ were transformed into ORN1 resulting in strains JJ001, JJ002, JJ003, JJ004, JJ005, and JJ006, respectively. Brain heart infusion broth (BHI, Roth Chemie GmbH, Karlsruhe, Germany) was used for inoculation of precultures, while CGXII minimal medium 40 g/L glucose or CGXIIm (CGXII but without ammonium sulfate) 35 g/L glucose was used for growth and proline production.

3.6.2 Culture conditions

C. glutamicum was inoculated to an OD of 1 in 50 mL minimal medium, 0.75 mM arginine, 1 mM Isopropyl-β-D-thiogalactopyranosid (IPTG), 25 µg/mL kanamycin, and when required 50 µg/mL spectinomycin on a rotary shaker (120 rpm) in baffled shake flasks at 30°C. Cultivations were always performed in triplicates. Growth was monitored measuring the OD at 600 nm using a spectrophotometer (V-1200, VWR, Radnor, PA, USA). 5 g/L urea and N-equimolar concentrations of ammonium sulfate and ammonium chloride were used for screening of nitrogen sources. For the screening of nitrogen sources and the Plackett-Burman design, cells were grown in 48-well flower plates using the Biolector microfermentation system (m2p-labs GmbH, Aachen, Germany). 1 mL medium was used per well with a shaking frequency of 1100 rpm. Biomass

formation was measured as backscattered light intensity sent at 620 nm with a signal gain factor of 20.

3.6.3 Utilization of proline as carbon and nitrogen source

C. glutamicum was inoculated to an OD of 1 in variants of CGXII medium. For the utilization of proline as carbon source CGXII with 20 g/L glucose or with a C-equimolar concentration of proline was used. For the utilization of proline as nitrogen source CGXII with 5 g/L urea and 20 g/L ammonium sulfate, with a N-equimolar concentration of proline, or with no nitrogen source added, was used.

3.6.4 Screening of medium components using the Plackett-Burman design

A Plackett-Burman design [39] for 19 factors (including seven dummies) with 20 runs was employed to screen for factors that significantly affect proline production through the ornithine biosynthetic pathway. The dummies serve as a measure for the error in estimating the main effects. The medium components were screened at a low (-) and a high (+) level, where the concentrations can be found in Table 6 and the design in Table 4. The concentrations of the two levels were selected based on a literature search and preliminary results. The effect of each factor on proline production was determined by the equation:

$$E(X_i) = 2\,(\Sigma Y_{+i} - \Sigma Y_{-i}) / \mathrm{N}$$

where $E(X_i)$ is the factor main effect, Y_{+i} and Y_{-i} are the proline concentrations in which the factors being tested are at their high and low levels respectively, N is the number of runs. The *t*-values of the factor main effects were plotted in a Pareto chart, and evaluated based on a *t*-value and a Bonferroni limit [40]. The t-test assesses the risk of declaring an effect significant, when it actually was caused by chance. The Bonferroni correction is a conservative adjustment that takes the number of estimated effects into account by dividing it into the desired probability for the risk value. Effects above the Bonferroni limit are likely not stacastic [40].

The experiment was designed, and obtained data analysed, using the software Design-Expert 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA). The experiment was carried out in duplicates, where the mean was considered the response (Table 6).

Table 6 Concentration levels used in the Plackett-Burman experiment

Factor	Name	Level	
		-	+
A (g/L)	Glucose	20	40
B (g/L)	Urea	2.5	10
C (g/L)	KH_2PO_4	0.5	2.0
D (g/L)	K_2HPO_4	0.5	2.0
E (g/L)	$CaCl_2$	$5.0 \cdot 10^{-3}$	$2.0 \cdot 10^{-2}$
F (g/L)	$MgSO_4$	$1.3 \cdot 10^{-1}$	$5.0 \cdot 10^{-1}$
G (g/L)	MOPS	31	52
H (mL/L)	Trace metals	$7.5 \cdot 10^{-1}$	2.0
I (g/L)	Biotin	$1.0 \cdot 10^{-4}$	$4.0 \cdot 10^{-4}$
J (g/L)	Prochatechuic acid	$1.5 \cdot 10^{-2}$	$6.0 \cdot 10^{-1}$
K (g/L)	IPTG	$1.2 \cdot 10^{-1}$	$3.6 \cdot 10^{-1}$
L (g/L)	Arginine	$1.3 \cdot 10^{-1}$	$1.7 \cdot 10^{-1}$
D1-D7	Dummy	0	0

3.6.5 Ornithine cyclodeaminase assay

BHI broth supplemented 25 μg/mL kanamycin and 1 mM IPTG was inoculated to an OD of 1 and grown for 4 h at 30 °C. Cells were harvested and washed in 20 mM KH_2PO_4 (pH 8.2). Then, cells were lysed by means of sonication (Ultraschalldesintegrator Sonoplus GM 200, Sonotrode M72, Bandelin electronic GmbH & Co KG, Berlin, Germany) for 6 min (cycle 0.5, amplitude 55) and centrifuged for 60 min at 4°C and 14600 rpm.

Crude extracts were purified using PD10 desalting columns (GE Healthcare, Chalfont St Giles, United Kingdom) with 20 mM KH_2PO_4 (pH 8.2). The reaction solution consisted of 20 mM KH_2PO_4 (pH 8.2) and 0.5 mM NAD^+. Tubes with reaction solution and between 0.08 and 0.4 mg protein pr. 250 μL reaction were equilibrated to 30°C for 3 min in a water bath. The reaction was initiated upon the addition of 25 mM L-ornithine. The reaction was stopped upon addition of 50% formic acid. The samples were then neutralized with 10 N KOH and precipitate was pelleted by centrifugation. Reactions were performed in triplicates with two enzyme concentrations. The conversion of ornithine to proline was measured by HPLC. Unpurified extracts were analysed by SDS-PAGE, and protein quantification was performed by the procedure of Bradford with bovine serum albumin as the standard [41].

3.6.6 *N*-Acetylglutamate kinase assay

Crude extracts were prepared as stated for the ornithine cyclodeaminase assay. The NAGK activity assay was performed as described by Haas and Leisinger [42]. The assay was performed in triplicates and carried out at 30°C at pH 7.2. One enzyme unit is the amount of enzyme that catalyses the formation of 1 μmol of product in 1 min.

3.6.7 Amino acid and glucose determination

Extracellular amino acids and carbohydrates were quantified by means of high-pressure liquid chromatography (1200 series, Agilent Technologies Deutschland GmbH, Böblingen, Germany). Samples were withdrawn from cultures, centrifuged (13,000 × *g*, 10 min), and the supernatant was used for analysis. For the detection of amino acids, samples were derivatised with 9-fluorenylmethyl chloroformate (FMOC) or *ortho*-phthaldialdehyde, separated on a system consisting of a pre-column (LiChrospher 100 RP18 EC-5μ (40 x 4 mm), CS-Chromatographie Service GmbH, Langerwehe, Germany) and a main column (LiChrospher 100 RP18 EC-5μ (125 x 4 mm), CS-Chromatographie), and detected with a fluorescence detector (FLD G1321A, 1200 series, Agilent Technologies). L-Asparagine was used as internal standard. For the detection of carbohydrates the separation of the analyte was achieved with a column for organic acids (300 x 8 mm, 10 μm particle size, 25 Å pore diameter, CS-Chromatographie) and a refractive index detector (RID G1362A, 1200 series, Agilent Technologies) was used. Derivatisation and quantification was carried out according to published methods [22] with the following modifications of the quantification of FMOC derivatised samples: The mobile phases used were A: 50 mM sodium acetate (pH 4.2) and B: acetonitrile. The gradient used was: 0 min 38% B, 5 min 38% B, 12 min 57% B, 14 min 76% B, 15 min 76% B, and 18 min 38% B.

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Chapter 4. L-citrulline production by metabolically engineered *Corynebacterium glutamicum* from glucose and alternative carbon sources.

Eberhardt, D., **Jensen, J.V.K.**, & Wendisch, V.F. (2014). L-citrulline production by metabolically engineered *Corynebacterium glutamicum* from glucose and alternative carbon sources. *AMB Express*, *4*, 85.

4.1 Abstract

L-citrulline plays an important role in human health and nutrition and is an intermediate of the L-arginine biosynthetic pathway. L-citrulline is a by-product of L-arginine production by *Corynebacterium glutamicum*. In this study, *C. glutamicum* was engineered for overproduction of L-citrulline as major product without L-arginine being produced as by-product. To this end, L-arginine biosynthesis was derepressed by deletion of the arginine repressor gene *argR* and conversion of L-citrulline towards L-arginine was avoided by deletion of the argininosuccinate synthetase gene *argG*. Moreover, to facilitate L-citrulline production the gene encoding a feedback resistant N-acetyl L-glutamate kinase $argB^{fbr}$ as well as the gene encoding L-ornithine carbamoylphosphate transferase *argF* were overexpressed. The resulting strain accumulated 44.1 ± 0.5 mM L-citrulline from glucose minimal medium with a yield of 0.38 ± 0.01 g · g^{-1} and a volumetric productivity of 0.32 ± 0.01 g · l^{-1} · h^{-1}. In addition, production of L-citrulline from the alternative carbon sources starch, xylose, and glucosamine could be demonstrated.

4.2 Introduction

L-citrulline is a natural non-proteinogenic amino acid whose name is derived from watermelon *Citrullus lanatus* (Wada 1930). In mammalians it serves as a precursor for L-arginine. In contrast to the proteinogenic L-arginine, which is not transferred to the blood stream, when ingested, L-citrulline can be converted to L-arginine, which is then released by the kidney into the blood stream. It is applied in several medical approaches e.g. as a pharmaconutrient (Rimando and Perkins-Veazie 2005; Curis et al. 2005).
Currently, biocatalytic and fermentative methods to produce L-citrulline using *Pseudomonas putida* (Kakimoto et al. 1971; Yamamoto et al. 1974) or *Bacillus subtilis* strains exist (Okumura et al. 1966). Additionally, extraction processes from watermelon have been established (Fish 2012). L-citrulline is an intermediate of L-arginine biosynthesis and accumulates as a by-product of engineered L-arginine producing *Corynebacterium glutamicum* strains (Ikeda et al. 2009; Schneider et al. 2011).

C. glutamicum is a workhorse for amino acid production and is employed for the annual production of several million tons of L-glutamate and L-lysine (Wendisch 2014). *C. glutamicum* has been engineered to produce a wide range of bioproducts, such as diamines, carotenoids, terpenes, proteins (Schneider and Wendisch 2010; Schneider et al. 2012; Heider et al. 2014a, b; Frohwitter et al. 2014; Kikuchi et al. 2009; Teramoto et al. 2011; An et al. 2013) and the L-glutamate family amino acids L-arginine, L-ornithine, and L-proline (Schneider et al. 2011; Ikeda et al. 2009; Georgi et al. 2005; Blombach et al. 2009; Jensen and Wendisch 2013). However, the production of L-citrulline as the only or major product has not been published yet.

Due to its natural ability to produce L-glutamate under several eliciting conditions, *C. glutamicum* is a suitable producer of L-glutamate-derived products (Sato et al. 2008; Radmacher et al. 2005; Kim et al. 2009, 2010; Delaunay et al. 1999; Wendisch et al. 2014). L-ornithine is a non-proteinogenic glutamate-family amino acid and an intermediate of L-arginine biosynthesis (Figure 1). An ornithine producer was obtained by deletion of *argR*, the gene encoding the genetic repressor of the arginine biosynthetic operon, and *argF* to prevent further processing of ornithine (Schneider et al. 2011). The production of L-proline from L-ornithine is possible by the heterologous overexpression of *ocd* from *Pseudomonas putida*, encoding ornithine cyclodeaminase (Jensen and Wendisch 2013). The diamine putrescine can be produced by overexpression of the *Escherichia coli* gene *speC*, which encodes ornithine decarboxylase (Schneider et al. 2012; Schneider and Wendisch 2010). As the arginine biosynthetic pathway is naturally regulated by feedback inhibition of N-acetylglutamate kinase (encoded by *argB*) by arginine, the use of feedback resistant enzyme variants in combination with deletion of *argR* has been described to overproduce L-arginine (Sakanyan et al. 1996; Ikeda et al. 2009; Schneider et al. 2011).

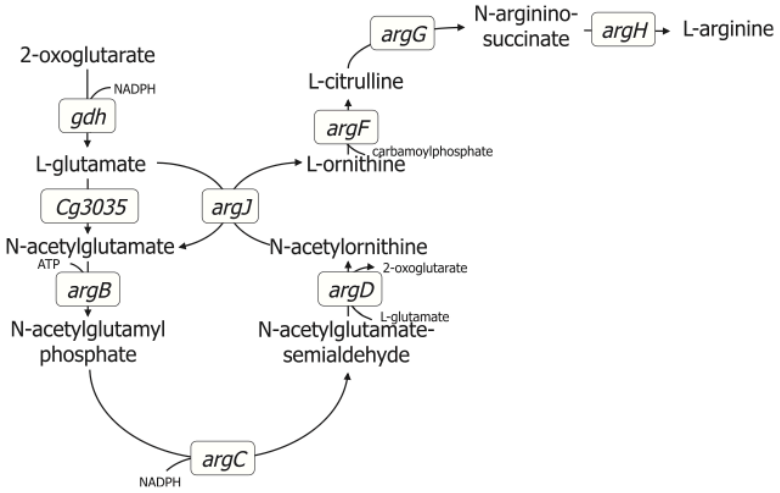

Figure 1 L-arginine pathway in *C. glutamicum* (modified from (Wendisch et al. 2014)). *gdh*: L-glutamate dehydrogenase, *cg3035*: anaplerotic N-acetylL-glutamate synthase, *argJ*: L-ornithine N-acetyltransferase, *argB*: N-acetylL-glutamate kinase; *argC*: N-acetyl-gamma-glutamyl-phosphate reductase; *argD*: acetylL-ornithine aminotransferase; *argE*: acetylL-ornithine deacetylase; *argF*: L-ornithine carbamoyltransferase; *argG*: argininosuccinate synthetase; *argH*: argininosuccinate lyase. Oxoglutarate is an intermediate of the central carbon metabolism.

C. glutamicum can utilize a variety of carbon sources. In contrast to many other microorganisms used in biotechnology, simultaneous utilization of carbon sources e.g. present in mixtures such as lignocellulosic hydrolysates is a hallmark of *C. glutamicum* (Blombach and Seibold 2010; Meiswinkel et al. 2013a, b). The natural substrate spectrum of *C. glutamicum* includes mono-saccharides, disaccharides, and organic acids as well as alcohols (Blombach and Seibold 2010; Arndt and Eikmanns 2008; Peters-Wendisch et al. 1998; Jolkver et al. 2009; Sasaki et al. 2011). To allow access to alternative carbon sources, *C. glutamicum* has also been engineered for utilization of glycerol, pentoses, and amino sugars as well as polysaccharides (Schneider et al. 2011; Rittmann et al. 2008; Seibold et al. 2006; Uhde et al. 2013; Gopinath et al. 2011; Matano et al. 2014).

One aim to reduce production cost is the use of complex sugar substrates for the production of biotechnological products. As an example of using a polymeric raw material without decomposition to its monomeric compounds e.g. by enzyme treatment, soluble starch could be used as a carbon source for the production of L-lysine and organic acids by engineered *C. glutamicum* (Seibold et al. 2006; Tateno et al. 2007; Tsuge et al. 2013). However, due to the growing world population and a correlating higher demand for food, biotechnological processes based on non-food derived carbon sources are sought. Xylose is a pentose sugar compound present in the hemicellulosic fraction of agricultural wastes as for example rice straw. Glucosamine, on the other hand, is a constituent of chitin, the second most abundant biopolymer in nature, which is accessible e.g. from shrimp shell waste accumulating in the food industry. *C. glutamicum* has been engineered to efficiently utilize both xylose and glucosamine as alternative carbon sources for growth and amino acid production (Gopinath et al. 2011; Meiswinkel et al. 2013a; Uhde et al. 2013; Matano et al. 2014).

In this study, the rational engineering of L-citrulline production by *C. glutamicum* is reported and the concept was extended to production of L-citrulline from the alternative carbon sources glucosamine, xylose, and starch.

4.3 Materials and methods

4.3.1 Microorganisms and growth conditions

Microorganisms and plasmids used in this study are listed in Table 1. *E. coli* DH5α was used for gene cloning. *C. glutamicum* and *E. coli* strains were routinely grown in lysogeny broth (LB) (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride) in 500-mL baffled flasks on a rotary shaker (120 rpm) at 30°C or 37°C. For growth experiments, CGXII minimal medium (Eggeling and Reyes 2005) was used for *C. glutamicum*. Growth was followed by measuring the optical density at 600 nm using a V-1200 Spectrophotometer (VWR, Radnor, PA, USA). An OD_{600} of 1 corresponds approximately to an estimated cell dry weight of 0.25 g/L.
When necessary, the growth medium was supplemented kanamycin (25 μg mL^{-1}), spectinomycin (100 μg mL^{-1}), tetracycline (10 μg mL^{-1}), isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM) and L-arginine (750 μM). The growth behavior and L-citrulline production of recombinant *C. glutamicum* strains were analyzed in 500 ml baffled flasks. Briefly, a 50 mL BHI (37 g L^{-1}) seed culture was inoculated from an agar plate and grown overnight. The cells were harvested by centrifugation (4,000 × g, 10 min) and washed twice with CGXII minimal medium lacking the carbon source. Subsequently, 50 mL CGXII medium, containing a given concentration of carbon source and necessary supplements, was inoculated to an optical density of 1.0. Detailed information on the carbon source concentrations employed is given in the Results chapter.

Table 1 Strains and plasmids used in this study

E. coli		
DH5α	F⁻ *thi*-1 *endA*1 *hsdr*17(r⁻, m⁻) *supE*44 Δ*lacU*169 (Φ80*lacZ*ΔM15) *recA*1 *gyrA*96 *relA*1	(Hanahan 1983)
C. glutamicum		
MB001	ATCC 13032 with in-frame deletion of prophages CGP1 (cg1507-cg1524), CGP2 (cg1746-cg1752), and CGP3 (cg1890-cg2071)	(Baumgart et al. 2013)
CIT0	MB001 with Δ*argF*, Δ*argG*, Δ*argR*	This study
CIT1	CIT0 carrying the pVWEx-*argFB*^{fbr} vector	This study
Plasmids		
pEKEx3	Spec^R, P_{tac}, lacI^q	(Stansen et al. 2005)
pVWEx1	Kan^R, P_{tac}, lacI^q	(Peters-Wendisch et al. 2001)
pEC-XT99A	Tet^R, P_{trc}, lacI^q	(Kirchner and Tauch 2003)
pK19Δ*argFR*	Kan^R, pk19mobsacB with the deletion construct of genes *argFR*	(Schneider et al. 2011)
pK19Δ*argG*	Kan^R, pk19mobsacB with the deletion construct of genes *argG*	This study
pEKEx3- *argB*^{fbr}	Spec^R, pEKEx3 carrying *argB* from *C. glutamicum* ATCC 13032 with amino acid exchanges A49VM54V	(Schneider et al. 2011)
pVWEx1-*argF*	Kan^R, pVWEx1 carrying *argF* from *C. glutamicum* ATCC 13032	This study
pVWEx1-*argFG*	Kan^R, pVWEx1 carrying *argF* and *argG* from *C. glutamicum* ATCC 13032	This study
pVWEx1-*argFB*^{fbr}	Kan^R, pVWEx1 carrying *argF* from *C. glutamicum* ATCC 13032 and *argB*^{fbr} from pEKEx3- *argB*^{fbr}	This study
pEKEx3-*nagB*	Spec^R, pEKEx3 carrying *nagB* from *C. glutamicum* ATCC 13032	(Uhde et al. 2013)
pEKEx3-*xylAB*	Spec^R, pEKEx3 carrying *xylA* from *Xanthomonas campestris* XCC1758 and *xylB* from *C. glutamicum* ATCC 13032	(Meiswinkel et al. 2013a)
pAMY	Tet^R, pEC-XT99A carrying *amy* from *Streptomyces griseus* IMRU 3570	(Seibold et al. 2006)

4.3.2 Molecular genetic techniques

Standard methods such as restriction digestions, and ligation were carried out as described elsewhere (Sambrook and Russell 2012). Digested DNA was purified by using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). *E. coli* cells were transformed by heat shock (Sambrook and Russell 2012) and *C. glutamicum* cells were transformed by electroporation (Eggeling and Reyes 2005). Isolation of genomic DNA was performed as previously described (Jensen and Wendisch 2013). Chromosomal changes in *C. glutamicum* were performed as described elsewhere (Eggeling and Reyes 2005).

4.3.3 Construction of strains and plasmids

The deletion of Δ*argFR* in MB001 was performed by using pK19mob*sacB*Δ*argFR*. Afterwards *argG* was deleted by using pK19mob*sacB*Δ*argG* to obtain CIT0. pK19mob*sacB*Δ*argG* contains the up- and downstream regions of *argG* in the ΔargFR strain. The plasmid was constructed by amplifying the upstream region with *argG*_up_f (CTTgaattcAGAAGCTGCGCCGCATG) and *argG*_up_r (agagacgacctaagccagtctAACGATGCGGTTAGTCATGAGG) and the downstream region with *argG*_down_f (agactggcttaggtcgtctctGCTAACAAGCGCGATCGC) and *argG*_down_r (CCTctgcagAACGACCAGCGCGCAGA). The two fragments were combined by crossover PCR using *argG*_up_f and *argG*_down_r and finally cloned into pK19mob*sacB* with PstI and EcoRI. pVWEx1-*argF* was constructed by amplifying *argF* with primers *argF*_f (CTTgtcgacAAGGAGATATAGATATGACTTCACAACCACAGGTTCG) and *argF*_r (CCTggatccTTACCTCGGCTGGTTGGC). The PCR product was treated with SalI and BamHI and ligated with similarly treated pVWEx1. pVWEx1-*argFG* was constructed by amplifying *argG* with primers *argG*_f (GGGgtcgacGAAAGGAGGCCCTTCAGATGACTAACCGCATCGTTCTTG) and *argG*_r (GGGgtcgacTTAGTTGTTGCCAGCTTCGCGA). The PCR product was treated with SalI and ligated with similarly treated pVWEx1-*argF*.
The plasmid vector pEKEx3-$argB^{fbr}$ (argBA49VM54V (Schneider et al. 2011)) was digested with BamHI and KpnI and the DNA fragment with a size of 0.9 kb harboring the $argB^{fbr}$ gene was cloned into the BamHI/KpnI digested vector pVWEx1-*argF*.

4.3.4 Determination of amino acid and carbohydrate concentrations

For the quantification of extracellular amino acids and carbohydrates, a high-performance liquid chromatography system was used (1200 series, Agilent Technologies Deutschland GmbH, Böblingen, Germany). Samples were withdrawn from the cultures, centrifuged (13,000 × g, 10 min), and the supernatant used for analysis.
Glucose and xylose were analyzed on a normal phase column (organic acid resin 300 × 8 mm, 10 µm particle size, 25 Å pore diameter; Chromatographie Service GmbH, Langerwehe, Germany) using 5 mM sulfuric acid as the mobile phase at a flow rate of 1 mL min−1 and were detected with a refractive index detector (RID G1362A, 1200 series, Agilent Technologies). Amino acids were

automatically modified by pre-column derivatisation with ortho-phthalaldehyde and separated as described previously (Georgi et al. 2005). L-ornithine was quantified using a pre-column (LiChrospher 100 RP18 EC-5 µ (40 × 4 mm), CS-Chromatographie Service GmbH, Langerwehe, Germany) and a reversed phase column (LiChrospher 100 RP18 EC-5 µ (125×4 mm), CS Chromatographie) as a main column and detected with a fluorescence detector at excitation at 230 nm and 450 nm emission (FLD G1321A, 1200 series, Agilent Technologies). For the determination of L-citrulline, a reverse-phase (RP) LiChrospher 100 RP8 EC-5 µ precolumn (40 × 4.6 mm) and a RP8 EC-5 µ (125 × 4.6 mm) main column (CS Chromatographie, Langerwehe, Germany) were used. 100 µM L-asparagine was used as an internal standard. The mobile phases used were in case of RP8 A: 0.25% Na-acetate pH 6, B: methanol. The gradient used was: 0 min 30% B, 1 min 30% B, 6min, 70% B, 11min 90% B, 14min 70% B, 16 min 30% B. In case of RP18, the mobile phases used were A: 0.1 M Na-acetate pH 7.2, B: methanol. The gradient used was: 0 min 20% B, 0.5 min 38% B, 2.5 min 46% B, 3.7 min 65% B, 5.5 min 70% B, 6 min 75% B, 6.2 min 85% B, 6.7 min 20% B.

4.4 Results

4.4.1 Engineering a prophage-free *C. glutamicum* strain for L-citrulline production

C. glutamicum has recently been cured of prophage sequences to yield MB001 (Baumgart et al. 2013). This strain was used as the parental strain because it can be transformed easily and plasmid-based gene overexpression is more efficient (Baumgart et al. 2013). As *C. glutamicum* ATCC 13032, this strain does not accumulate L-citrulline, an intermediate of L-arginine biosynthesis (Figure 1). The deletion of three genes of the L-arginine operon (L-ornithine carbamoyltransferase (EC 2.1.3.3) *argF*, argininosuccinate synthetase (EC 6.3.4.5) *argG*, and L-arginine biosynthesis operon repressor gene *argR*) in *C. glutamicum* MB001 yielded the L-arginine auxotrophic strain CIT0. When supplemented with 0.75 mM L-arginine, *C. glutamicum* CIT0 accumulated 25.2 ± 2.6 mM L-ornithine from 2% glucose. The deletion of *argF* and *argG* could be complemented by plasmid-borne expression of these genes since the complemented strain CIT0(pVWEx1-*argFG*) grew without L-arginine supplement while the empty vector carrying control CIT0(pVWEx1) did not (data not shown). Comparable growth rates and biomass concentrations were observed.

To enable L-citrulline accumulation, two plasmids were constructed and used to transform *C. glutamicum* CIT0. While pVWEx1-*argF* only carries *argF* encoding L-ornithine carbamoyltransferase, pVWEx1-*argFB*^{fbr} in addition carries *argB*^{fbr} encoding feedback-resistant *N*-acetyl L-glutamate kinase (NAGK, EC 2.7.2.8). When grown in minimal medium with 2% glucose

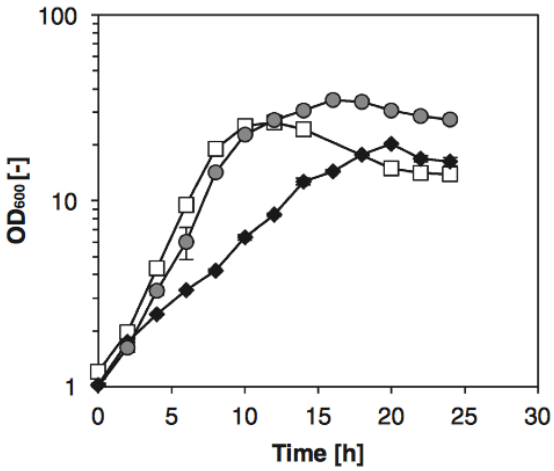

Figure 2 Biomass formation by various *C. glutamicum* strains. The cultivation was performed in CGXII minimal medium containing 20 g L-1 glucose, 1 mM IPTG, 750 µM L-arginine and 25 µg L-1 kanamycin. OD_{600} was determined of CIT0(pVWEx1) (open squares), CIT0(pVWEx1-*argF*) (gray circles) and CIT0 (pVWEx1-*argFB*^{fbr}) (black diamonds). Values and error bars represent the mean and the standard error of triplicates.

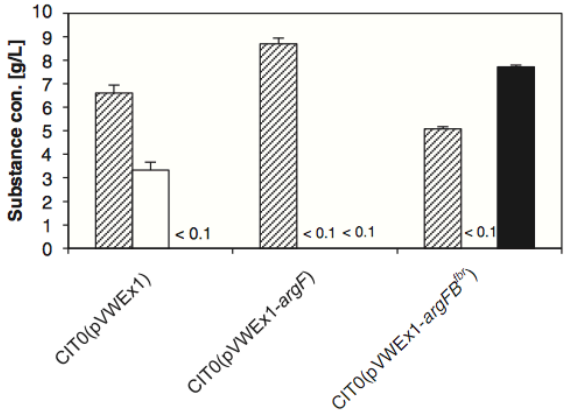

Figure 3 Biomass formation and production of ornithine and citrulline on glucose by various *C. glutamicum* strains: cell dry weight (hatched bars), L-ornithine concentration (open bars) and L-citrulline concentration (filled bars). The cultivation was performed in CGXII minimal medium containing 20 g/L glucose, 1 mM IPTG, 750 µM L-arginine and 25 µg/L kanamycin. The amino acid concentrations in the supernatant were determined after the consumption of glucose. Values and error bars represent the mean and the standard error of triplicates.

and 0.75 mM L-arginine *C. glutamicum* CIT0(pVWEx1-*argF*) grew to a higher OD than CIT0(pVWEx1) (Figure 2) and did not accumulate notable concentrations of L-citrulline. As opposed to CIT0(pVWEx1), CIT0(pVWEx1-*argF*) did not produce L-ornithine (Figure 3). By contrast, the combined overexpression of *argF* and *argB*^{fbr} entailed L-citrulline production and the respective strain was named CIT1. *C. glutamicum* CIT1 accumulated 44.1 ± 0.5 mM L-citrulline in minimal medium with 2% glucose (Figure 4).

When comparing the growth of C. glutamicum CIT0 (pVWEx1) to that of CIT0(pVWEx1-*argF*), similar growth rates (0.37 ± 0.01 h^{-1} and 0.35 ± 0.04 h^{-1}, respectively) were obtained, whereas L-citrulline formation by CIT0 (pVWEx1-*argFB*^{fbr}) was accompanied by a reduced growth rate (0.15 ± 0.01 h^{-1}) (Figure 2). Moreover, the final OD600 of CIT0(pVWEx1-*argFB*^{fbr}) was 20 ± 1 as compared to an OD600 of 26 ± 1 of CIT0(pVWEx1). By contrast, *C. glutamicum* CIT0(pVWEx1-*argF*) grew to a higher biomass concentration with a final OD600 of 35 ± 1. As shown in Figure 3, the lower growth rates of CIT0(pVWEx1) and CIT0(pVWEx1-*argFB*^{fbr}) correlated inversely with the formation of the respective amino acids L-ornithine and L-citrulline, whereas *C. glutamicum* CIT0(pVWEx1-*argF*) reaches a higher final biomass and neither produces L-ornithine nor L-citrulline.

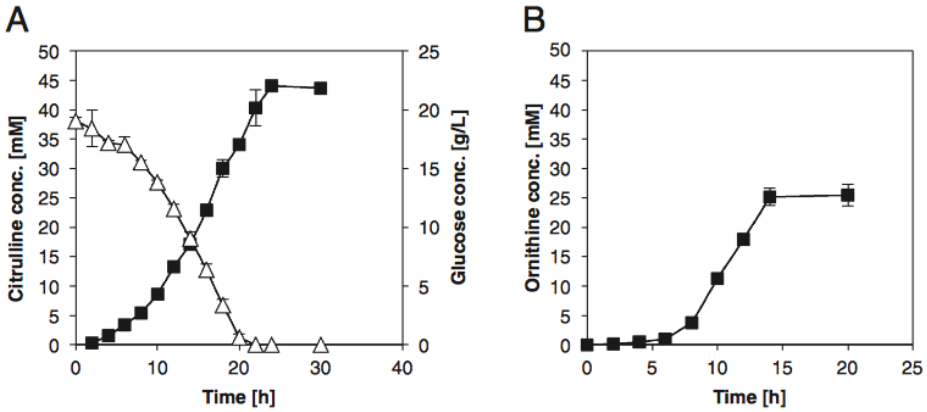

Figure 4 Amino acid production by various *C. glutamicum* strains. L-ornithine production by *C. glutamicum* CIT0(pVWEx1) (filled squares) **(A)** and L-citrulline accumulation (filled squares) and glucose consumption (open triangles) by strain CIT0(pVWEx1-*argFB*^{fbr}) **(B).** The experiments were performed in CGXII minimal medium with 20 g/L glucose, 1 mM IPTG, 25 µg/L kanamycin and supplemented with 750 µM L-arginine. Values and error bars represent the mean and the standard error of triplicates.

4.4.2 Production of L-citrulline from alternative carbon sources

Due to the high demand of biotechnological processes of using complex sugar substrates derived from raw materials and industrial wastes, the L-citrulline producer strain CIT1 was enabled to utilize the alternative carbon sources starch (as an example of a high molecular weight carbohydrate), xylose, and glucosamine (as an example of carbohydrates derived from forestry and food industrial wastes).

To enable *C. glutamicum* CIT1 to consume starch, the gene *amyA* from *Streptomyces griseus* was overexpressed. The combined overexpression of *xylA* from *Xanthomonas campestris* and

endogenous *xylB* allowed the utilization of xylose by *C. glutamicum* CIT1. The endogenous *nagB* was overexpressed ectopically to facilitate the consumption of glucosamine. The resulting strains were tested for growth and L-citrulline production.

When cultured in CGXII medium supplemented 0.75 mM L-arginine all strains engineered for alternative carbon source consumption grew with their respective substrate (Table 2). The empty vector carrying strain CIT1(pEKEx3) neither grew in xylose or glucosamine minimal medium nor consumed these substrates. By contrast, the recombinant strain CIT1(pEKEx3-*xylAB*) grew in xylose minimal medium with a growth rate of 0.03 ± 0.01 h^{-1} and reached a final OD600 of 6 ± 1 (Table 2). In glucosamine minimal medium, *C. glutamicum* CIT1(pEKEx3-*nagB*) grew to a final OD600 of 3±1 with a growth rate of 0.02 ± 0.01 h^{-1}. In minimal medium containing 1% starch and 0.25% glucose as carbon sources, the empty vector harboring strain CIT1(pEC-XT99A) formed roughly one third of the biomass as compared to *C. glutamicum* CIT1(pAmy) (Table 2). Growth of CIT1(pEC-XT99A) was slower (growth rate of 0.10 ± 0.01 h^{-1}) than that of CIT1(pAmy) (growth rate of 0.21 ± 0.01 h^{-1}). While strain CIT1(pEC- XT99A) only utilized glucose, but not starch, CIT1(pAmy) was able to consume both, glucose and starch.

Table 2 Growth on different carbon sources

C. glutamicum strain	Carbon source concentration	Maximum OD_{600}	Growth rate (h^{-1})
CIT1(pEKEx3-*xylAB*)	Xylose: 15 g/L	6 ± 1	0.03 ± 0.01
CIT1(pEKEx3-*nagB*)	Glucosamine: 10 g/L	3 ± 1	0.02 ± 0.01
CIT1(pAMY)	Soluble starch: 10 g/L Glucose: 2.5 g/L	9 ± 1	0.21 ± 0.01
CIT1(pEC-XT99A)	Soluble starch: 10 g/L Glucose: 2.5 g/L	3 ± 1	0.10 ± 0.01

Fermentations were performed in CGXII minimal medium containing the respective carbon source and were supplemented by 750 µM L-arginine. 1 mM IPTG and 25 µg/ml kanamycin and spectinomycin were added. Values and error bars represent the mean and the standard error of triplicates.

The strains engineered for utilization of xylose and glucosamine, respectively, also produced L-citrulline from these carbon sources (Figure 5). *C. glutamicum* CIT1(pEKEx3-*nagB*) accumulated 2.6 ± 0.3 mM L-citrulline which corresponds to a yield of 0.045 ± 0.002 g/g since glucosamine was utilized completely. Similarly, after complete utilization of xylose by *C. glutamicum* CIT1(pEKEx3-*xylAB*) 6.4± 0.1 mM L-citrulline accumulated corresponding to a yield of 0.075 ± 0.001 g / g xylose.

As the determination of the starch concentration by HPLC was not possible, residual starch content was assayed by the use of Lugols solution. However, as it is known that overexpression of *amyA* in *C. glutamicum* results in high molecular mass degradation products of starch, which remain in the medium and are not detectable by Lugols solution (Seibold et al. 2006), the L-citrulline concentration was measured until no change in OD600, starch content and L-citrulline concentration was observed. The starch utilizing strain CIT1(pAmy) was able to produce 11.9 ± 0.5 mM L-citrulline which corresponds to a yield of 0.167 g/g.

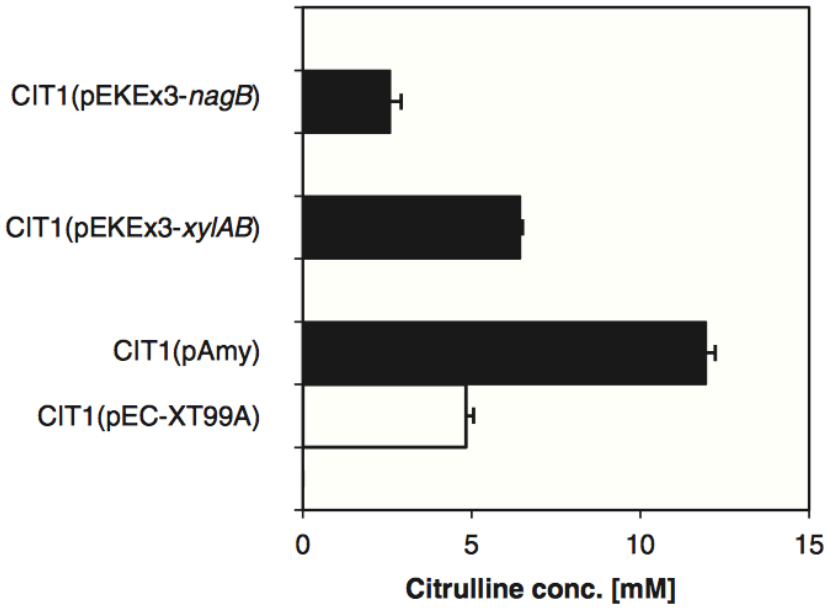

Figure 5 L-citrulline concentration in the engineered strains after the consumption of the respective carbon source. CIT1(pEC-XT99A), CIT1(pAmy) with 10 g/L soluble starch, 2,5 g/L glucose after 31 h. CIT1(pEKEx2-*xylAB*) with 15 g/L xylose after xylose consumption. CIT1(pEKEx3-*nagB*) with 10 g/L glucosamine after glucosamine consumption. Values and error bars represent the mean and the standard error of triplicates.

4.5 Discussion

C. glutamicum was engineered to accumulate L-citrulline as major product, both from glucose as well as from the alternative carbon sources starch, glucosamine and xylose.
Feedback insensitive *N*-acetyl L-glutamate kinase (encoded by *argB*^{fbr}; (A49VM54V)) was required for production of L-citrulline since CIT0(pVWEx1-*argF*) did not produce L-citrulline, while CIT0(pVWEx1-*argFB*^{fbr}) produced L-citrulline. It is unlikely that addition of L-arginine to CIT0(pVWEx1-*argF*) inhibited generation of L-ornithine, a precursor of L-citrulline, because strain CIT0(pVWEx1) produced L-ornithine when supplemented with L-arginine. However, it is possible that intracellular L-citrulline affects arginine biosynthesis. As overexpression of *argB*^{fbr} entailed L-citrulline formation, we assume that L-citrulline inhibits the NAGK of C. glutamicum, but this has not yet been described. As expected due to its structural similarity to L-arginine, L-citrulline inhibits NAGK of other microorganisms (Farago and Denes 1967; Haas and Leisinger 1975). In *Chlamydomonas reinhardtii*, NAGK is inhibited by several L-arginine structure analogs, including L-citrulline, however, inhibition was less pronounced than L-arginine inhibition (Farago and Denes 1967). NAGK from *Pseudomonas aeruginosa* lost two thirds of its activity in the presence of 2.5 mM L-citrulline, which was claimed to be too weak under physiological conditions (Haas and Leisinger 1975). However, it is conceivable that inhibition of NAGK by L-citrulline may play a role in recombinant *C. glutamicum* strains engineered for L-citrulline production, thus, possibly explaining the finding that L-citrulline production required overexpression of *argB*^{fbr} encoding NAGK feedback resistant to L-arginine. Commensurate with this notion, simultaneous production of L-arginine and L-citrulline resulted from *argB*^{fbr} overexpression in a Δ*argR* background (Ikeda et al. 2009). In this *argB*^{fbr} overexpressing strain, the ratio of L-citrulline to L-arginine was higher than by classically obtained strains, which solely contain native *argB* (Ikeda et al. 2009). Currently, it remains to be studied if L-citrulline inhibits NAGK from *C. glutamicum* and if (some) variants feedback resistant to L-arginine are also desensitized to L-citrulline.
Notably, about two fold more L-citrulline (about 7.7 g/L) was produced by strain CIT1 than L-ornithine was produced (about 3.3 g/L) by the isogenic strain CIT0 (pVWEx1). Both, overexpression of *argF* and *argB*^{fbr} may have contributed to this effect. It is more likely that *argB*^{fbr} is responsible as L-arginine supplementation may have limited flux in the arginine biosynthesis pathway of strain CIT0(pVWEx1) especially in the beginning of the cultivation. In *C. glutamicum* CIT1, only feedback resistant NAGK is present and additionally a gene dosage effect due ectopic overexpression of *argB*^{fbr} might have contributed to increase L-citrulline production.

Glucose, glucosamine, xylose, and starch were shown to be suitable substrates for the production of L-citrulline. Strain construction was based on previously established engineering strategies (Seibold et al. 2006; Uhde et al. 2013; Meiswinkel et al. 2013a; Gopinath et al. 2011). The achieved L-citrulline concentrations on these substrates were lower than with glucose as carbon source. However, L-citrulline production from xylose (6.44 ± 0.12 mM) by CIT1(pEKEx3-*xylAB*) was lower, but in a similar range as production of L-ornithine (19.6 ± 1.9 mM) and putrescine (15.1 ± 1.2 mM), respectively, from the same xylose concentration by the respective recombinant *C. glutamicum* strains (Meiswinkel et al. 2013a). Similarly, product yields with glucosamine as carbon source were lower for L-citrulline (0.067 g/g) than for putrescine (0.112 g/g) (Uhde et al. 2013).

Unexpectedly and hitherto not understood, the growth rate (0.02 ± 0.01 h^{-1}) and, thus, productivity by CIT1(pEKEx3-*nagB*) were very low. By contrast, a putrescine producing strain carrying pEKEx3-*nagB* showed only a slightly decreased growth rate (Uhde et al. 2013).
C. glutamicum strains carrying pAmy co-utilized starch with glucose (Seibold et al. 2006). Substrate co-utilization is observed with *C. glutamicum* WT as well as recombinant strains for almost all mixtures of carbon sources (Blombach and Seibold 2010). A L-lysine producing strain carrying pAmy showed increased biomass formation by addition of 10 g/L starch to 10 g/L glucose, whereas L-lysine production increased only upon addition of higher starch concentrations (Seibold et al. 2006).

In this study, the additional presence of starch increased the growth rate of CIT1 (from 0.15 to 0.21 h^{-1}) as well as L-citrulline production. Production of L-citrulline by CIT1(pAmy) from a starch glucose mixture was higher (11.95 ± 0.48 mM) than that by the empty vector carrying control strain (4.83 ± 0.4 mM) demonstrating that starch contributed to production of L-citrulline. It has to be noted that starch cannot be utilized completely by *C. glutamicum* strains overexpressing the α-amylase gene *amyA* because high-molecular-weight carbohydrates are generated from starch and remain unutilized in the medium (Seibold et al. 2006).
Taken together, production of L-citrulline as major product from glucose, starch, glucosamine, and xylose by recombinant *C. glutamicum* strains was achieved.

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Chapter 5. Modular pathway engineering of *Corynebacterium glutamicum* for production of the glutamate-derived compounds ornithine, proline, putrescine, citrulline, and arginine

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5.1 Abstract

The glutamate-derived bioproducts ornithine, citrulline, proline, putrescine, and arginine have applications in food and feed, the cosmetic, pharmaceutical, and chemical industries. Engineering an ornithine overproducing *Corynebacterium glutamicum* strain to accumulate a high ornithine yield could be useful not only for the production of ornithine, but also for production of the amino acids citrulline, proline, and arginine, and the diamine putrescine. We here demonstrate how the feedback alleviation of *N*-acetylglutamate kinase, tuning of the promoter of the glutamate dehydrogenase gene *gdh*, lowering expression of the phosphoglucoisomerase gene *pgi*, along with the introduction of a second copy of the arginine biosynthesic operon *argCJB*^{A49V,M54V}*D* into the genome resulted in a *C. glutamicum* strain with a yield of 0.52 g ornithine / g glucose, an increase of 71% as compared to the parental *ΔargFRG* strain.

Strains capable of efficiently producing citrulline, proline, arginine or putrescine were derived from ornithine producing strains by plasmid-based overexpression of appropriate pathway modules with one to three genes.

C. glutamicum is not only an excellent producer of glutamate but also of glutamate-derived products. Engineering targets beneficial for ornithine production were identified and the advantage of rationally constructing a platform strain for the production of bioproducts of the glutamate family was demonstrated.

5.2 Background

Corynebacterium glutamicum has for several decades been employed as workhorse for industrial production of amino acids and is especially known for its ability to secrete glutamate (Ikeda, 2003; Zahoor et al., 2012). The production of several other industrially relevant products of the glutamate family, namely ornithine, proline, citrulline, putrescine, and arginine, by *C. glutamicum* has been reported (Eberhardt et al., 2014; Hwang and Cho, 2014; Hwang et al., 2008; Ikeda et al., 2009; Jensen and Wendisch, 2013; Jiang et al., 2013a; Jiang et al., 2013b; Sakanyan et al., 1996; Schneider et al., 2012; Schneider et al., 2011; Schneider and Wendisch, 2010). These five compounds are of industrial interest due to their uses in for instance feed and food, cosmetics, pharmaceuticals, and in the case of putrescine as a monomer in the production of nylon-4,6 or nylon-4,10 (Jensen and Wendisch, 2013; Kaore et al., 2013; Scott et al., 2007; Shi et al., 2002; Zajac et al., 2010).

In *C. glutamicum,* glutamate is synthesized from the tricarboxylic acid cycle intermediate 2-oxoglutarate and five enzymatic steps are required for further conversion of glutamate to ornithine (Figure 1). The ornithine biosynthetic pathway in *C. glutamicum* is cyclic due to ornithine acetyltransferase (OAT), which catalyzes conversion of *N*-acetylornithine and glutamate to ornithine and *N*-acetylglutamate (NAG). NAG kinase (NAGK) then phosphorylates NAG in the second step of the pathway. Recently, *cg3035* was found to encode a new type of NAG synthase catalyzing acetyl-CoA-dependent acetylation of glutamate to NAG (Petri et al., 2013). It is assumed that most NAG is produced by OAT, while the role of the *cg3035* encoded NAG synthase is anaplerotic (Petri et al., 2013). Besides OAT and NAGK, *argC*-encoded *N*-acetylglutamate 5-semialdehyde dehydrogenase and *argD*-encoded *N*-acetylornithine aminotransferase are necessary for conversion of glutamate to ornithine (Figure 1). Once ornithine has been formed, citrulline can be produced by *argF*-encoded ornithine carbamoyltransferase (Eberhardt et al., 2014), proline by heterologous ornithine cyclodeaminase Ocd from *Pseudomonas putida* (Jensen and Wendisch, 2013), putrescine by heterologous ornithine decarboxylase SpeC from *Escherichia coli* (Schneider and Wendisch, 2010), and arginine by endogenous ornithine carbamoyltransferase and *argGH*-encoded argininosuccinate synthetase and argininosuccinate lyase (Ikeda et al., 2009; Park et al., 2014; Schneider and Wendisch, 2010) (Figure 1).

The regulation of the pathway is complex and involves allosteric regulation and transcriptional regulation. The transcriptional repressor ArgR represses transcription of the arginine operon *argCJBDFR* and is involved in regulation of *gltB* and *gdh* (Lee et al., 2010; Yim et al., 2011). Furthermore, the acyl-responsive transcriptional regulator FarR exerts control at the transcriptional level, although the mechanism is not fully comprehended (Lee et al., 2011). Lastly, NAGK is subject to allosteric inhibition as it is feedback inhibited by arginine. In addition, OAT is feedback inhibited by ornithine and citrulline (Hao et al., 2015; Sakanyan et al., 1996; Udaka, 1966). Alleviation of feedback inhibition is an important part of increasing product yield, evident by the number of publications dealing with this subject for the arginine pathway of *C. glutamicum* (Huang et al., 2015; Ikeda et al., 2009; Kim et al., 2015; Park et al., 2014; Schendzielorz et al., 2013; Schneider et al., 2011; Xu et al., 2012).

Feedback resistant NAGK from *E. coli* (EcNAGK) is a homodimer, whereas the so far investigated arginine sensitive NAGKs appear to be hexamers consisting of a trimer of dimers, where the dimers resemble EcNAGK (Ramon-Maiques et al., 2006). Arginine sensitive NAGKs like CgNAGK have an N-terminal extension. The extension was found to form a helix that interlaces with the helix of the adjacent dimer in *Pseudomonas aeruginosa* NAGK (PaNAGK) and *Thermotoga maritima* NAGK (TmNAGK). It was proposed that the N-helix plays an important modulatory role. Based on the crystal structure of TmNAGK, arginine was found to bind NAGK in a crevice near the interdimeric junction that flanks the N-helix and interacts with the C-terminal portion of it. The binding of arginine to the NAGK subunits results in an increased separation between the ATP and NAG sites, which appears to cause the inhibition. The inhibition by arginine is cooperative and NAGK has a high affinity for arginine (Fernandez-Murga and Rubio, 2008; Llacer et al., 2007; Ramon-Maiques et al., 2006).

Here we describe the construction and improvement of ornithine producing *C. glutamicum* strains by metabolic engineering. Furthermore, we show that ornithine producing strains can serve as platform for the overproduction of proline, putrescine, citrulline, and arginine. The study emphasizes the importance of improving ornithine yield, as it can be beneficial for at least four industrially relevant products.

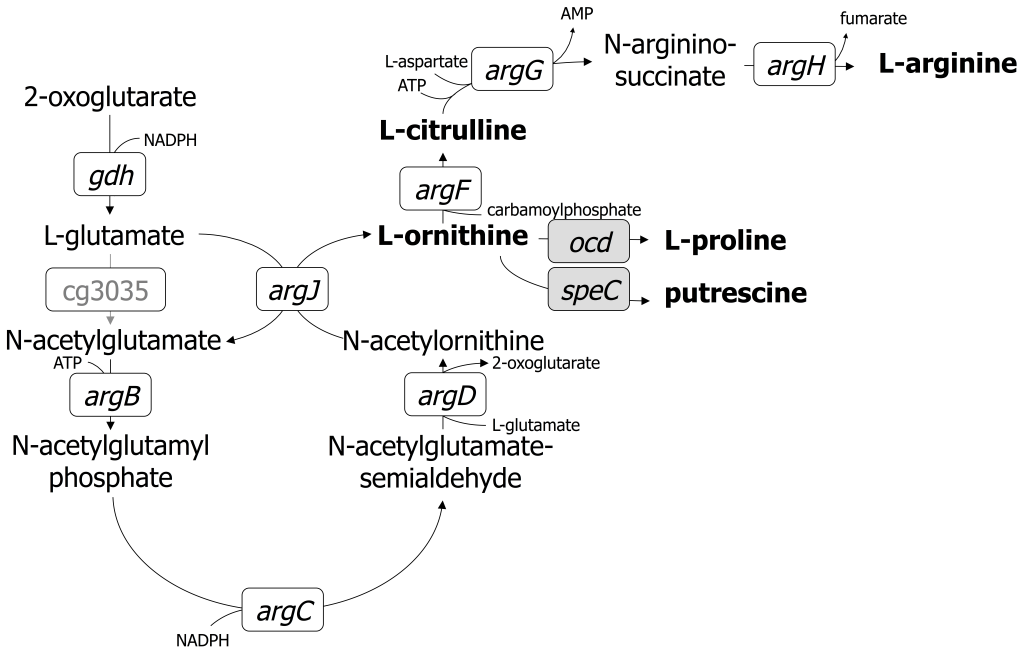

Figure 1: Pathways for the production of ornithine, proline, citrulline, putrescine, and arginine by *C. glutamicum* adapted from (Wendisch et al., 2014). Starting with ornithine the amino acids citrulline and proline as well as the diamine putrescine are synthesized in a single enzymatic step catalyzed by ornithine carbamoyltransferase (encoded by *argF*), ornithine cyclodeaminase (encoded by *ocd* from *Pseudomonas putida*) and ornithine decarboxylase (encoded by *speC* from *Escherichia coli)*. From citrulline arginine is formed by the reactions catalyzed by argininosuccinate synthase and argininosuccinate lyase encoded by *argG* and *argH*, respectively. The gene written in grey denotes its anaplerotic function, genes written in grey boxes originate from a different microorganism.

5.3 Materials and methods

5.3.1 Strains, plasmids, and primers

C. glutamicum MB001 and its derivatives have been used in this study (Table 1). *E. coli* DH5α or Stellar competent cells were used for the cloning procedures. All primers used were purchased from Metabion (Planegg/Steinkirchen, Germany) and can be found in the Appendix (supplementary material – chapter 5) along with the plasmids used.

Table 1: Strains used in this study

Strains	Relevant characteristics	Reference or source
Escherichia coli		
DH5α	*F-*, *endA1*, *supE44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *phoA*, *Φ80d lacZ*Δ *M15*, Δ *(lacZYA – argF) U169*, *hsdR17 (rK-, mK+) λ-*	(Hanahan, 1983)
Stellar	*F-*, *endA1*, *supE44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *phoA*, *Φ80d lacZ*Δ *M15*, Δ *(lacZYA – argF) U169*, Δ*(mrr – hsdRMS – mcrBC)*, Δ*mcrA*, *λ-*	Clontech Laboratories, Mountain View, CA, USA
Corynebacterium glutamicum		
MB001	ATCC 13032 with in-frame deletion of prophages CGP1 (cg1507-cg1524), CGP2 (cg1746-cg1752), and CGP3 (cg1890-cg2071)	(Baumgart et al., 2013)
MB001Δ*argFR*		This study
ORN1-pEKEx3-*argB*	ATCC 13032 Δ*argFR*, $Spec^R$	This study
ORN1-pEKEx3-$argB^{E19R,H26E,H268N}$	ATCC 13032 Δ*argFR*, $Spec^R$	This study
ORN2	MB001Δ*argFRG*	This study
ORN2-P_{gdh1}	P_{gdh1}: -35: TGGTCA -10: TATAAT	This study
ORN2-P_{gdh2}	P_{gdh2}: -35: TGGTCA -10: TGCTATAATGG	This study
ORN2-P_{gdh3}	P_{gdh3}: -35: TTGACA -10: TATAAT	This study
ORN2-P_{gdh4}	P_{gdh4}: -35: TTGCCA -10: TATAAT	This study
ORN3	ORN2-*Pgdh4-*$argB^{A49V,M54V}$	This study
ORN4	ORN3-pgi^{GTG}	This study
ORN5	ORN3-P_{tuf}-$argCJB^{A49V,M54V}D$	This study
ORN6	ORN4-P_{tuf}-$argCJB^{A49V,M54V}D$	This study
ORN2B	ORN2Δ*argB*	This study
ORN2B-pEKEx3	$Spec^R$	This study
ORN2B-pEKEx3-*argB*	$Spec^R$	This study
ORN2B-pEKEx3-$argB^{\Delta 1\text{-}23}$	$Spec^R$	This study
ORN2B-pEKEx3-$argB^{E19R}$	$Spec^R$	This study
ORN2B-pEKEx3-$argB^{H26E}$	$Spec^R$	This study

ORN2B-pEKEx3-*argB*^{H268N}	Spec^{R}	This study
ORN2B-pEKEx3-*argB*^{G287D}	Spec^{R}	This study
ORN2B-pEKEx3-*argB*^{A49V,M54V}	Spec^{R}	This study
ORN2B-pEKEx3-*argB*^{E19R,H26E,A49V,M54V}	Spec^{R}	This study
ORN2B-pEKEx3-*argB*^{A49V,M54V,H268N}	Spec^{R}	This study
ORN2B-pEKEx3-*argB*^{A49V,M54V,G287D}	Spec^{R}	This study
ORN2B-pEKEx3-*argB*^{E19R,H26E,H268N}	Spec^{R}	This study
ORN2B-pEKEx3-*argB*^{E19R,H26E,A49V,M54V,H268N}	Spec^{R}	This study
ORN2B-pEKEx3-*argB*^{E19R,H26E,A49V,M54V,H268N,G287D}	Spec^{R}	This study
ORN2B-pEKEx3*argB*$_{E.coli}$	Spec^{R}	This study
ORN2J	ORN2Δ*argJ*	This study
ORN2J-pEKEx3	Spec^{R}	This study
ORN2J-pEKEx3-*argJ*$_{Cg}$	Spec^{R}	This study
ORN2J-pEKEx3-*argJ*$_{Cc}$	Spec^{R}	This study
ORN2-pEKEx3	Spec^{R}	This study
ORN2-pEKEx3-*argJ*$_{Cg}$	Spec^{R}	This study
ORN2-pEKEx3-*argJ*$_{Cc}$	Spec^{R}	This study
ORN2D	ORN2Δ*argD*	This study
ORN2DD2	ORN2DΔ*argD2*	This study
ORN2DD2Δ*gabT*		This study
ORN2DD2Δ*bioA*		This study
ORN2DD2Δ*gapT*Δ*bioA*		This study
ORN2DD2-pEKEx3	Spec^{R}	This study
ORN2DD2-pEKEx3-*argD*	Spec^{R}	This study
ORN2DD2-pEKEx3-*argD2*$_{LA}$	Spec^{R}	This study
ORN2DD2-pEKEx3-*argD2*$_{LT}$	Spec^{R}	This study
ORN2DD2-pEKEx3-*argD2*$_{MA}$	Spec^{R}	This study
ORN2DD2-pEKEx3-*argD2*$_{MT}$	Spec^{R}	This study
ORN2-pEPR1	Kan^{R}	This study
ORN2-pEPR1-P$_{tuf}$	Kan^{R}	This study
ORN2-pEPR1-P$_{sod}$	Kan^{R}	This study
ORN2-pEPR1-P$_{argC}$	Kan^{R}	This study
ORN2Δ*argCJBD*		This study
ORN2Δ*argCJBD*-*argCJB*^{A49V,M54V}*D*	Insertion of *P$_{tuf}$*-*argCJB*^{A49V,M54V}*D*-*rrnB*T1T2 at site where prophage CGP1 was deleted	This study
ORN2Δ*argCJBD*-*argB*^{A49V,M54V}*JCD*	Insertion of *P$_{tuf}$*-*argB*^{A49V,M54V}*JCD*-*rrnB*T1T2 at site where prophage CGP1	This study

	was deleted	
CIT1	ORN2-pVWEx1-*argFB*^{A49V,M54V}, kan^{R}	This study
CIT2	ORN2-P$_{gdh4}$-pVWEx1-*argFB*^{A49V,M54V}, kan^{R}	This study
CIT3	ORN4-pVWEx1-*argFB*^{A49V,M54V}, kan^{R}	This study
PRO1	ORN2-pVWEx1-*ocd*$_{Pp}$, kan^{R}	This study
PRO2	ORN2-P$_{gdh4}$-pVWEx1-*ocd*$_{Pp}$, kan^{R}	This study
PRO3	ORN4-pVWEx1-*ocd*$_{Pp}$, kan^{R}	This study
PUT2	ORN2-pVWEx1-*speC*$_{Ec}$, kan^{R}	This study
PUT3	ORN2-P$_{gdh4}$-pVWEx1-*speC*$_{Ec}$, kan^{R}	This study
PUT4	ORN4-pVWEx1-*speC*$_{Ec}$, kan^{R}	This study
ARG5	ORN2-pVWEx1-*argGFB*^{A49V,M54V}, kan^{R}	This study
ARG6	ORN2-P$_{gdh4}$-pVWEx1-*argGFB*^{A49V,M54V}, kan^{R}	This study
ARG7	ORN4-pVWEx1-*argGFB*^{A49V,M54V}, kan^{R}	This study

5.3.2 Molecular Biology Techniques

Competent *E. coli* cells, transformation thereof and general molecular techniques were performed according to standard procedures (Sambrook and Russell, 2001). Chromosomal DNA from *C. glutamicum* was isolated as previously described (Jensen and Wendisch, 2013). The preparation of competent *C. glutamicum* cells and their transformation were performed according to published methods (Eggeling and Reyes, 2005). Modifications introduced into the genome of *C. glutamicum* were performed with non-replicative vectors via two-step homologous recombination as described previously (Niebisch and Bott, 2001). Plasmids were verified by sequencing (Sequencing Core Facility, Bielefeld University). The modifications introduced into the genome were verified by amplification of the modified region by PCR and sequencing of the PCR product. PCR amplification was performed with KOD Hot Start DNA Polymerase according to the manufacturer (Toyobo, Osaka, Japan). PCR purification and gel extraction were performed with the PCR purification kit and Minelute kit from Qiagen (Hilden, Germany). Plasmids were isolated using the Qiagen miniprep kit. Restriction enzymes were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dephosphorylation of plasmid DNA and ligation was performed with the Rapid DNA Dephos and Ligation Kit (Roche Diagnostics, Basel, Switzerland). Insert DNA was phosphorylated with polynucleotide kinase from Thermo Fisher Scientific. Colony PCR was performed with GoTaq polymerase (Promega, Madison, WI, USA). Site-directed mutagenesis was performed as previously described (Liu and Naismith, 2008) with *PfuTurbo* DNA polymerase (Agilent, Waldbronn, Germany).

5.3.3 Media and cultivation conditions

E. coli strains were cultured at 37°C in Lysogeny Broth (LB) or on LB-agar. *C. glutamicum* strains were cultured at 30°C in brain heart infusion broth (BHI, Roth Chemie, Karlsruhe, Germany) or CgXII minimal medium with 4% (w/v) glucose. Where appropriate kanamycin 25 µg/mL, spectinomycin 100µg/mL, 0.75 mM arginine, and 1 mM isopropyl ß-D-thiogalactopyranosid (IPTG) were added. For growth experiments the Biolector microfermentation system (m2p-labs, Aachen, Germany) was used. For growth experiments *C. glutamicum* strains were inoculated in BHI and grown 8-12 h on a rotary shaker, the culture was washed with 0.9% NaCl and inoculated in CgXII medium to an optical density (OD) of 1. OD was measured at 600 nm using a spectrophotometer (V-1200, VWR, Radnor, PA, USA). For cultivation in 48-well flower plates wells were filled with 1 mL medium and a shaking frequency of 1100 rpm was applied. Biomass formation was measured as backscattered light intensity sent at 620 nm with a signal gain factor of 20. The GFP fluorescence intensity was measured in the biolector with an excitation filter of 405 nm and an emission filter of 508 nm with a signal gain factor of 20. Cultivations were performed in triplicates with a minimum of three independent experiments unless otherwise mentioned.

5.3.4 Enzyme activity assays

For the determination of GDH, NAGS, and OAT activities crude extracts were used. Overnight cultures of all analyzed strains were inoculated in 50 mL BHI medium containing IPTG and antibiotics if appropriate. The cells were washed with 0.9% NaCl and inoculated to OD 1 in 50 mL BHI with or without 100 µg/mL spectinomycin and grown for 4h in 500 mL baffled shake flasks. Plasmid-based expression of target genes was induced by the addition of IPTG to a final concentration of 1 mM. For the NAGK assay cells were inoculated to OD 1 in CgXII medium with 4% (w/v) glucose, supplemented with 100 µg/mL spectinomycin, 1 mM IPTG, and 0.25 mM arginine. Crude extracts were obtained by sonication (Ultraschalldesintegrator Sonoplus GM 200, Sonotrode M72, Bandelin electronic GmbH & Co KG, Berlin, Germany) for 6 min (cycle 0.5, amplitude 55) and centrifugation at 4°C and 14600 rpm for 60 min. The GDH assay was performed as described by Hänßler *et al.* with an UV-1800 CE-230V UV-Spectrophotometer (Shimadzu, Kyoto, Japan) (Hanssler et al., 2009). The NAGK activity assay was performed as described by Haas and Leisinger (Haas and Leisinger, 1975). The assay was performed in duplicates and carried out at 30°C at pH 7.2. The reaction solution for the OAT assay consisted of 100 mM Tris-HCl (pH 7) and 6 mM glutamate. The reactions were initiated upon addition of 6 mM *N*-acetylornithine and stopped by boiling for 10 min. The conversion of *N*-acetylornithine to ornithine was quantified by HPLC. Protein concentrations were quantified by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976). One enzyme unit is the amount of enzyme that catalyzes the formation of 1 µmol product in 1 min.

5.3.5 Amino acid, putrescine and glucose quantification

Amino acids, putrescine, and glucose were quantified by means of high-pressure liquid chromatography (1200 series, Agilent). Quantifications of proline (Jensen and Wendisch, 2013), putrescine, citrulline, arginine (Klatte and Wendisch, 2014), and glucose (Jensen and Wendisch, 2013) were performed as previously described. Samples containing amino acids were derivatized with *ortho*-phthaldialdehyde, separated on a system consisting of a pre-column (LiChrospher 100 RP18 EC-5µ, 40 x 4 mm, CS-Chromatographie, Langerwehe, Germany) and a main column (LiChrospher 100 RP18 EC-5µ, 125 x 4 mm, CS-Chromatographie), and detected with a fluorescence detector (FLD G1321A, 1200 series, Agilent). For the detection of glutamate and ornithine the flow rate was 1.2 mL/min and the mobile phases used were A: 0.1 M sodium acetate (pH 7.2), B: methanol. The gradient used was: 0 min 20 % B, 0.5 min 38 % B, 2.5 min 46 % B, 3.7 min 65 % B, 5.5 min 70 % B, 6 min 75 % B, 6.2 min 85 % B, 6.7 min 20 % B, 8.9 min 20%.

5.4 Results and discussion

5.4.1 Construction of the platform strain MB001Δ*argFRG*

Recently, the genome-reduced *C. glutamicum* strain MB001 was constructed that lacks the prophages CGP1, CGP2, and CGP3 (Baumgart et al., 2013). One of the advantages of using this strain is its lack of the restriction-modification system (*cg1996-cg1998*) located within the large prophage CGP3 that entails a significantly increased transformation efficiency (Baumgart et al., 2013). Previously, we have described the production of ornithine by *C. glutamicum* ATCC 13032 Δ*argFR* named ORN1 (Schneider et al., 2011). Based on the prophage-free strain MB001, we describe here the construction of a platform strain for producing the five compounds ornithine, citrulline, proline, putrescine, and arginine. The absence of the regulator ArgR is necessary to enable efficient transcription of the *argCJBDFR* operon for the overproduction of ornithine and ornithine-derived products (Schneider et al., 2011). Here, we have deleted *argR* and genes *argF* and *argG* from the genome of MB001 by homologous recombination, resulting in an arginine auxotrophic strain that accumulates ornithine extracellularly. The resulting strain MB001Δ*argFRG* was named ORN2. When *C. glutamicum* ORN2 was grown in CgXII minimal medium with 4% (w/v) glucose and supplemented with 0.75 mM arginine, it produced 0.307 ± 0.003 g ornithine/g glucose. ORN2 served as the basis for further metabolic engineering to increase the ornithine yield.

5.4.2 Metabolic pull to increase the flux towards glutamate biosynthesis

The intermediate of the TCA cycle 2-oxoglutarate constitutes a branch point with either succinyl-CoA being formed by the 2-oxoglutarate dehydrogenase complex or glutamate being formed by glutamate dehydrogenase (GDH). Previously, Asakura *et al.* showed that tuning the *gdh* promoter had a positive effect on glutamate production (Asakura et al., 2007). This strategy was applied here to pull the flux towards glutamate biosynthesis and thereby towards ornithine biosynthesis. As the *gdh* transcript is monocistronic (Börmann et al., 1992), the change of the promoter sequence was assumed not to have any transcriptional effects on other genes. Four different constructs were tested for an increased transcription of *gdh* where the -10 and/or the -35 sequences were changed according to Table 2. A GDH activity assay with crude extracts of strains with each of the four mutated promoters revealed that all modified promoter regions resulted in an increased GDH activity. The highest activity was observed for ORN2-P_{gdh4} with "TATAAT" as -10 consensus sequence and "TTGCCA" as -35 sequence, i.e. 4.5-fold more than the activity of ORN2 crude extracts. When the strains with the altered *gdh* promoters were tested for ornithine production, a distinct difference between ORN2 and the mutants could be observed. A twofold increase in GDH activity in strain ORN2-P_{gdh1} resulted in a 20% increase in the ornithine yield. Further elevation of the GDH activity of strain ORN2-P_{gdh4} only led to a small further increase in the ornithine yield compared to ORN2-P_{gdh1}, implying that the glutamate levels are not limiting the flux towards glutamate derived products in these strains. Although the *gdh* expression level of ORN2-P_{gdh1} is sufficient to significantly increase the ornithine yield, further metabolic engineering downstream the GDH catalyzed step might require an increase in the level of activity needed. As

ORN2-P_{gdh4} and ORN2-P_{gdh1} had similar growth rates the higher specific activity of ORN2-P_{gdh4} does not appear to pose a metabolic burden to the cells and we used ORN2-P_{gdh4} for further engineering.

Table 2 - Effect of different promoters on GDH activity and ornithine yield. Specific activities were determined from cells grown in BHI and crude extracts from three independent experiments. Production of ornithine was performed in CgXII medium with 4% (w/v) glucose and 0.75 mM arginine until depletion of glucose. All values represent the mean and standard deviations of at least two independent experiments performed with biological triplicates.

Strain	-35	-10	Specific activity (U/mg)	Yield (g orn / g glc)
ORN2	TGGTCA	CATAAT	1.4 ± 0.3	0.312 ± 0.016
ORN2-P_{gdh1}	TGGTCA	TATAAT	2.8 ± 0.8	0.374 ± 0.007
ORN2-P_{gdh2}	TGGTCA	TGCTATAATGG	3.7 ± 1	0.383 ± 0.014
ORN2-P_{gdh3}	TTGACA	TATAAT	5.5 ± 1.5	0.386 ± 0.016
ORN2-P_{gdh4}	TTGCCA	TATAAT	6.3 ± 1.4	0.395 ± 0.018

5.4.3 Alleviation of feedback inhibition of NAGK

Based on previous studies with PaNAGK (Fernandez-Murga and Rubio, 2008), TmNAGK (Ramon-Maiques et al., 2006), CgNAGK (Xu et al., 2012), and *Corynebacterium crenatum* NAGK (Xu et al., 2011) different mutations and combinations thereof were selected to construct feedback resistant NAGKs (Table 3). The gene *argB* from *C. glutamicum* was cloned into the replicative IPTG-inducible expression plasmid pEKEx3, and the selected mutations were introduced by site-directed mutagenesis. In addition, *argB* from *E.coli* was tested. The plasmids were transformed into ORN2B with the genetic background Δ*argFRGB* to avoid formation of NAGKs consisting of a mixture of endogenously and plasmid expressed subunits.

Wild-type NAGK had the highest activity followed by NAGKs with the amino acid exchanges H268N or G287D (Table 3). The differences in the specific activities from the mutant NAGKs to wild-type NAGK are not in accordance with the studies performed with CgNAGKs and CcNAGKs purified from *E. coli* where all strains exhibited similar specific activities. The half-maximal inhibition of the wild-type enzyme was determined to be 2 mM which is in accordance with a previous report performed with crude extract (Sakanyan et al., 1996), but higher than the 0.4 mM obtained with purified His-tagged enzyme (Schendzielorz et al., 2013). Growth experiments with the various overexpressed *argB* genes resulted in similar yields for the strains ORN2B-pEKEx3-$argB^{E19R}$, ORN2B-pEKEx3-$argB^{G287D}$, ORN2B-pEKEx3-$argB^{A49V,M54V}$ and ORN2B-pEKEx3-$argB^{A49V,M54V,G287D}$. All these strains produced comparably high ornithine yields although their specific NAGK activities varied significantly (Table 3), a phenomenon previously observed (Schendzielorz et al., 2013).

Table 3: Specific activities of NAGK of various strains. Crude extracts were prepared from cells grown in CgXII medium with 4% glucose, 1 mM IPTG, 0.25 mM arginine, and 100 µg/mL spectinomycin. For the production of ornithine strains were grown in CgXII with 4% glucose, 1 mM IPTG, 0.75 mM arginine, and 100 µg/mL spectinomycin. ND = not determined. All values represent the mean and standard deviations of at least two independent experiments.

Strain	Specific activity (U/mg)	Yield (g ornithine / g glucose)	IC50 (mM)
ORN2B-pEKEx3	0	0	ND
ORN2B-pEKEx3-*argB*	1.58 ± 0.10	0.257 ± 0.044	2.0 ± 0.02
ORN2B-pEKEx3-$argB^{\Delta 1\text{-}23}$	0	0	ND
ORN2B-pEKEx3-$argB^{E19R}$	0.77 ± 0.18	0.307 ± 0.041	3.6 ± 0.06
ORN2B-pEKEx3-$argB^{H26E}$	0.26 ± 0.07	0.242 ± 0.028	2.5 ± 0.01
ORN2B-pEKEx3-$argB^{H268N}$	1.09 ± 0.31	0.276 ± 0.024	13 ± 2.0
ORN2B-pEKEx3-$argB^{G287D}$	1.20 ± 0.33	0.292 ± 0.039	>50
ORN2B-pEKEx3-$argB^{A49V,M54V}$	0.51 ± 0.03	0.300 ± 0.047	5.1 ± 1.7
ORN2B-pEKEx3-$argB^{E19R,H26E,A49V,M54V}$	0	0	ND
ORN2B-pEKEx3-$argB^{A49V,M54V,H268N}$	0.39 ± 0.14	0.268 ± 0.047	>50
ORN2B-pEKEx3-$argB^{A49V,M54V,G287D}$	0.42 ± 0.09	0.278 ± 0.019	>50
ORN2B-pEKEx3-$argB^{E19R,H26E,H268N}$	0	0	ND
ORN2B-pEKEx3-$argB^{E19R,H26E,A49V,M54V,H268N}$	0	0.114 ± 0.013	ND
ORN2B-pEKEx3-$argB^{E19R,H26E,A49V,M54V,H268N,G287D}$	0.15 ± 0.05	0.200 ± 0.005	>50
ORN2B-pEKEx3$argB_{E.coli}$	0.64 ± 0.05	0.300 ± 0.021	>50

Neither NAGK activity nor ornithine accumulation could be detected for ORN2B-pEKEx3-$argB^{\Delta 1\text{-}23}$ carrying the truncated NAGK. It was published that overexpression of pEKEx3-$argB^{A26VM31V}$ (here named pEKEx3-$argB^{\Delta 1\text{-}23}$) in the arginine producer *C. glutamicum* Δ*argR* resulted in a similar arginine concentration as the strain carrying pEKEx3-$argB^{A49V,M54V}$ (Schneider et al., 2011). Moreover, truncated versions of NAGK from *C. glutamicum* were reported to have significantly reduced activity compared to wild-type NAGK (Xu et al., 2012). It therefore appears that NAGK consisting of only truncated subunits results in an inactive protein or a protein with a very low activity, while heteromeric complexes of wild-type ArgB subunits and truncated ArgB subunits result in functionally active and feedback alleviated NAGK.

Furthermore, activity could not be measured in crude extracts from ORN2B-pEKEx3-$argB^{E19R,H26E,A49V,M54V}$ and ORN2B-pEKEx3-$argB^{E19R,H26E,H268N}$. Most likely the activity of these enzymes were too low to be measured and too low to result in extracellular ornithine accumulation. Interestingly, a study showed that the expression of $argB^{E19R,H26E,H268N}$ in arginine producing *C. crenatum* resulted in an increase in arginine production and the purified enzyme expressed in *E. coli* had an activity at the same level as the wild type (Xu et al., 2011). The amino acid sequences of CgArgB and CcArgB are identical so an explanation for the contradictory observations could be the presence of wild-type and mutant NAGK subunits in *C. crenatum*. To support this theory we performed a growth experiment in CgXII medium with 4% glucose with ORN1-pEKEx3-*argB* and ORN1-pEKEx3-$argB^{E19R,H26E,H268N}$ and observed accumulation of 11.4 ± 0.2 g/L and 12.1 ± 0.5 g/L

ornithine, respectively. The amino acid exchange H26E did not seem to be beneficial for ornithine production and strains harboring this exchange also had the lowest specific activities.

5.4.4 *N*-acetylornithine aminotransferase encoded by *argD* is not essential for ornithine biosynthesis

N-acetylornithine aminotransferase, encoded by *argD*, catalyzes the transfer of an amino group from glutamate to *N*-acetylglutamate semialdehyde yielding 2-oxoglutarate and *N*-acetylornithine. The overexpression of *argD2* (*cg2680*) encoding a putative aminotransferase was previously shown to be beneficial for arginine production (Kim et al., 2011). The amino acid sequence presented in the patent differs from the sequence of *C. glutamicum* ATCC 13032 (RefSeq NC_006958.1) at the second amino acid position. Since the start codon of *argD2* is TTG, four versions of the gene were cloned into pEKEx3 to test if the differences would have an effect on ornithine accumulation. The first two amino acids of the different plasmids with *argD2* are LA, LT, MA, and MT.

To begin with, the activity of the endogenous aminotransferases ArgD and ArgD2 were abolished to determine if *argD2* could complement the deletions. The deletion of *argD* (ORN2D) did neither affect growth nor production of ornithine, wherefore *argD* does not appear to be essential for ornithine synthesis (Table 4). The disruption of *argD2* in ORN2D did also not have an impact on growth or ornithine production. A BLAST analysis revealed that ArgD has two further homologs, namely 4-aminobutyrate aminotransferase (GabT) and adenosylmethionine-8-amino-7-oxononanoate aminotransferase (BioA). Neither the deletion of *gabT* nor of *bioA* affected ornithine production and growth of the strains (Table 4). Hence, the presence of one or several non-specific transaminases appears sufficient to maintain the level of ornithine production in the deletion mutants to the level of the parent strain.

In some microorganisms such as *Thermus thermophilus* the lysine and arginine biosynthesis pathways are interconnected (Ledwidge and Blanchard, 1999; Miyazaki et al., 2001). In *E. coli, argD* encodes an enzyme with activities of both *N*-acetylornithine aminotransferase of the arginine pathway and *N*-succinyldiaminopimelate aminotransferase of the lysine pathway (Ledwidge and Blanchard, 1999). *C. glutamicum* can synthesize lysine through the diaminopimelate and succinylase branches.

Although two different genes encode *N*-acetylornithine aminotransferase and *N*-succinyldiaminopimelate aminotransferase in *C. glutamicum* it is possible that the enzymes are promiscuous and therefore can catalyze the transfer of the amino group in both pathways. While it was already shown that a *C. glutamicum* strain deficient in *dapC*, *ddh*, and *argD* shows no growth deficiency (Hartmann et al., 2003), an additional deletion of *dapC* in the Δ*argDD2* or Δ*argDD2*Δ*gabT*Δ*bioA* background might reveal if *N*-succinyldiaminopimelate aminotransferase is capable of transferring the amino group to *N*-acetylglutamate-semialdehyde and thereby can substitute the *N*-acetylornithine aminotransferase activity of ArgD.

The overexpression of *argD* and the *argD2* variants in ORN2DD2 increased ornithine production (Table 4). There was no notable difference in the yields between strains with the overexpressed transaminase and putative transaminases. Moreover, in the particular genetic background it did not seem to make a difference if the start codon of *argD2* was ATG or TTG, or if the second N-

terminal amino acid was alanine or threonine. Thus, the increased expression of a gene for transaminase had a positive effect on ornithine accumulation.

Table 4: Ornithine yields of strains derived from MB001. Strains were cultured in CgXII with 4% glucose and supplemented 0.75 mM arginine, 1 mM IPTG, and 100 µg/mL spectinomycin when appropriate. All values represent the mean and standard deviations of three independent experiments performed in triplicates.

Strain	g ornithine / g glucose
ORN2	0.314 ± 0.002
ORN2D	0.311 ± 0.007
ORN2DD2	0.314 ± 0.015
ORN2DD2Δ*gabT*	0.320 ± 0.013
ORN2DD2Δ*bioA*	0.315 ± 0.002
ORN2DD2Δ*gabT*Δ*bioA*	0.313 ± 0.007
ORN2DD2-pEKEx3	0.277 ± 0.008
ORN2DD2-pEKEx3-*argD*	0.306 ± 0.009
ORN2DD2-pEKEx3-$argD2_{LA}$	0.310 ± 0.003
ORN2DD2-pEKEx3-$argD2_{LT}$	0.302 ± 0.009
ORN2DD2-pEKEx3-$argD2_{MA}$	0.291 ± 0.010
ORN2DD2-pEKEx3-$argD2_{MT}$	0.298 ± 0.022

5.4.5 Ornithine acetyltransferase another bottleneck in ornithine overproduction

OAT encoded by *argJ* in *C. glutamicum* is monofunctional and catalyzes the transfer of the acetyl group from *N*-acetylornithine to glutamate resulting in the formation of ornithine and *N*-acetylglutamate (Sakanyan et al., 1996). OAT was shown to be product inhibited by ornithine with an apparent K_i value of 5 mM (Sakanyan et al., 1996). Recently, it was shown that OAT is also inhibited by citrulline, where a concentration of 30 mM inhibited OAT activity to 50% (Hao et al., 2015). Crude extract of *C. crenatum* was reported to have a higher OAT specific activity than crude extract of *C. glutamicum* (Dou et al., 2011). Although such differences were not observed with the purified enzymes produced in *E. coli* we here tested the expression of OAT from *C. glutamicum* and *C. crenatum*.

The deletion of *argJ* in ORN2 resulted in no detectable extracellular accumulation of ornithine and overexpression of *argJ* from *C. glutamicum* and *C. crenatum* in ORN2Δ*argJ* (ORN2J) could both complement the *argJ* deletion (Table 5). OAT from both organisms exhibited a similar activity, which reflects the production yields of both strains. The overexpression of the *argJ* variants in ORN2 resulted in a significant increase in the ornithine yield compared to the empty vector control (Table 5). This reflects that the reaction catalyzed by OAT poses another bottleneck of the ornithine pathway in strain ORN2, which is in agreement with previous published data (Dou et al., 2011; Hao et al., 2015). Hence overexpression and/or feedback alleviation of OAT is an important part of improving ornithine yield.

Table 5: Impact of *argJ* overexpression on ornithine yield and specific activities of OAT. For the production of ornithine strains were cultured in CgXII with 4% glucose and supplemented 0.75 mM arginine, 1 mM IPTG, and 100 µg/mL spectinomycin when appropriate. Specific activities were determined from cells grown in BHI with 1 mM IPTG and 100 µg/mL spectinomycin. All values represent the mean and standard deviations of three independent experiments performed in triplicates.

Strain	g ornithine / g glucose	U/mg
ORN2J-pEKEx3	0	n.d.
ORN2J-pEKEx3-*argJ*$_{Cg}$	0.219 ± 0.025	0.06 ± 0.01
ORN2J-pEKEx3-*argJ*$_{Cc}$	0.207 ± 0.019	0.07 ± 0.01
ORN2-pEKEx3	0.307 ± 0.003	-
ORN2-pEKEx3-*argJ*$_{Cg}$	0.365 ± 0.015	-
ORN2-pEKEx3-*argJ*$_{Cc}$	0.361 ± 0.014	-

5.4.6 Introduction of multiple genomic changes resulted in a high ornithine producing strain

We have showed that a combination of tuning the *gdh* promoter and alleviating NAGK of feedback inhibition increased ornithine production remarkably. Therefore, the respective modifications were combined and introduced into the genome of ORN2. The amino acid changes A49V and M54V in ArgB were introduced into the genome of ORN2-P$_{gdh4}$ (ORN3). This increased the ornithine yield by 62% compared to the parent strain ORN2 (Figure 2).

The production of ornithine is at the expense of three molecules of NADPH and the subsequent conversion of ornithine to arginine requires one additional NADPH molecule. Therefore recent efforts have been put into increasing the NADPH availability in the cell (Hwang and Cho, 2014; Jiang et al., 2013a; Jiang et al., 2013b; Kim et al., 2015). One of the strategies employed, which proved to be beneficial for lysine production (Lindner et al., 2013), is reduced translation of *pgi* achieved by exchanging the start codon ATG to the less preferred GTG (Park et al., 2014). The *pgi*-encoded glucose-6-phosphate isomerase catalyzes the conversion of glucose-6-phosphate to fructose-6-phosphate. By reducing translation of *pgi* the flux into the pentose phosphate pathway is increased, which increases the NADPH level of the cell when glucose is used as substrate. Changing the translational start codon of *pgi* from ATG to GTG in strain ORN4 resulted in a 6.5% increased yield compared to ORN3 (Figure 2).

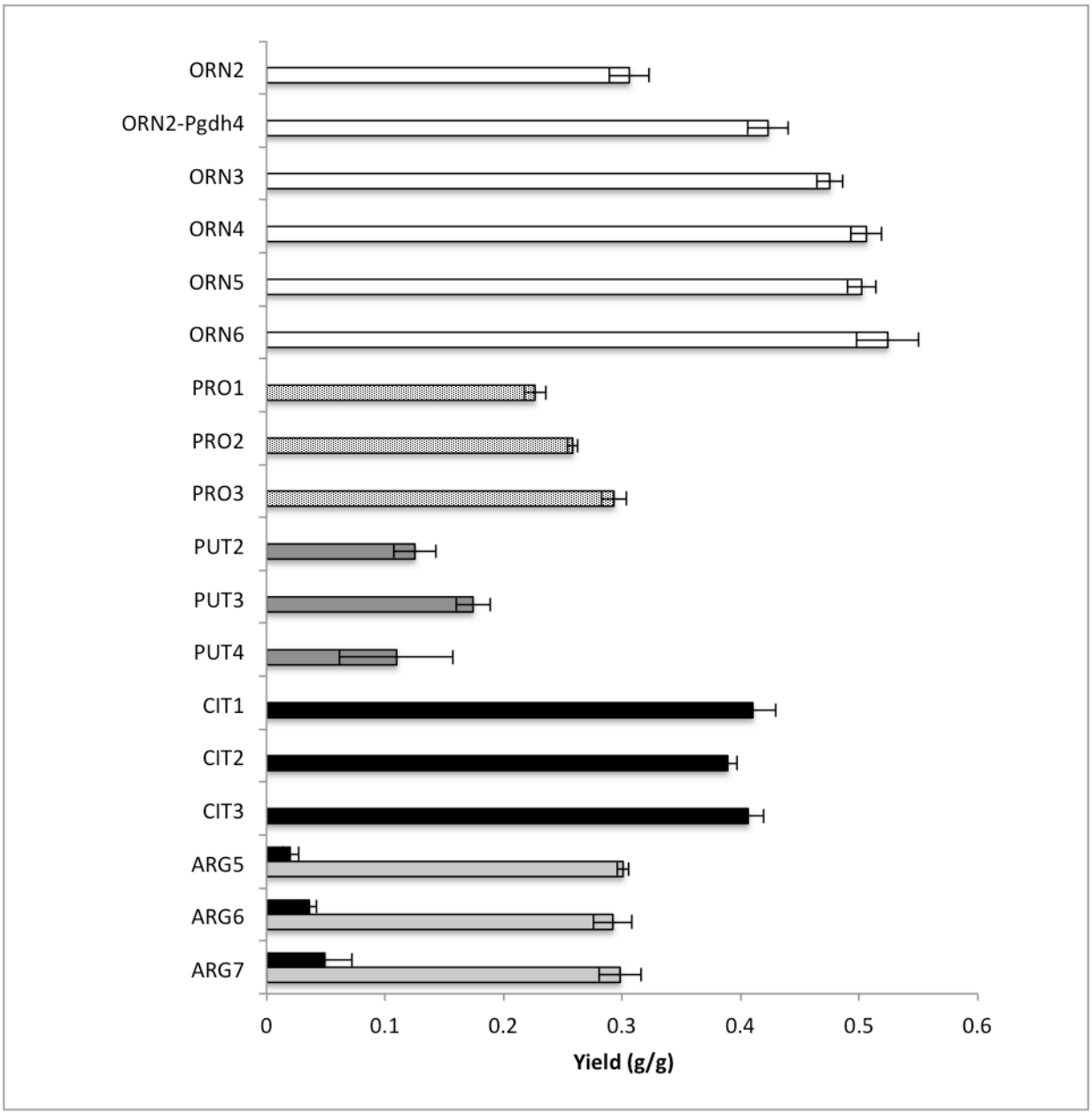

Figure 2: Yields of ornithine, proline, putrescine, citrulline, and arginine producing strains. The ornithine producing strains were cultured in CgXII medium with 4% glucose and supplemented 0.75 mM arginine. All other strains were cultured in CgXII medium with 2% glucose and supplemented 0.75 mM arginine (except for the arginine producing strain), 1 mM IPTG, and 25 µg/mL kanamycin. White bars, ornithine yield; dotted bars, proline yield; dark grey bars, putrescine yield; black bars, citrulline yield; light grey bars, arginine yield. All values represent the mean and standard deviations of at least two independent experiments performed in triplicates.

As overexpression of *argB*, *argD*, and *argJ* resulted in an increased ornithine yield (Tables 3, 4, and 5), a second copy of the *argCJB*^{A49V,M54V}*D* operon was introduced into the genome. To balance expression, the strength of the *argC* promoter was compared to the strengths of P_{tuf} and P_{sod}, as these are two strong constitutive promoters commonly used for overexpression in *C. glutamicum* (Becker et al., 2011; Park et al., 2014). The promoter strengths were assayed using the promoter

probe vector pEPR1 with GFP as reporter, and then monitoring fluorescence and growth simultaneously in the biolector microfermentation system. P_{tuf} drove the strongest expression (35.3 fluorescent units (FU)) followed by P_{argC} (19.1 FU) whereas P_{sod} driven expression resulted in the lowest fluorescence intensity (3.63 FU) compared to the empty vector control (1.18 FU)(errors below 10%; data not shown). Therefore, P_{tuf} was chosen to drive the expression of the second copy of the operon. We constructed two different operons both transcribed from P_{tuf} with the gene orders *argCJB*^{A49V,M54V}*D* and *argB*^{A49V,M54V}*JCD*. It has previously been shown that the order of the genes in an operon can affect the product yield (Hiroe et al., 2012), hence the synthetic operon *argB*^{A49V,M54V}*JCD* with a rearranged gene order was constructed.

These operons were introduced into the genome by homologous recombination at the former CGP1 locus, which previously has been used successfully for the introduction of the carotenoid biosynthetic pathway (Heider et al., 2014). Initially the operons were integrated into the genome of ORN2Δ*argCJBD*, resulting in strains ORN2Δ*argCJBD-argCJB*^{A49V,M54V}*D* and ORN2Δ*argCJBD-argCJB*^{A49V,M54V}*D*. The strains were grown in CgXII medium with 4% glucose supplemented with 0.75 mM arginine. No extracellular accumulation of ornithine by ORN2Δ*argCJBD* could be detected, but the introduction of both operons *argCJB*^{A49V,M54V}*D* and *argB*^{A49V,M54V}*JCD* could complement the deletion of *argCJBD*. ORN2Δ*argCJBD-argCJB*^{A49V,M54V}*D* produced 0.334 ± 0.011 g ornithine/g glucose (g/g) whereas ORN2Δ*argCJBD-argB*^{A49V,M54V}*JCD* produced 0.085 ± 0.008 g/g. ORN2Δ*argCJBD-argCJB*^{A49V,M54V}*D* accumulated slightly more ornithine than ORN2, which can be explained by the assumed increased expression of the operon by P_{tuf}. Rearranging the order of the genes of the operon resulted in a low yield; this effect could be due to the introduction of ribosomal binding sites in front of the genes, the altered gene order, or a combination of both, although the decrease of the yield compared to ORN2 is rather drastic.

Due to the lower yield obtained with the operon with the rearranged genes, *argCJB*^{A49V,M54V}*D* was introduced into the genome of ORN3, resulting in the strain ORN5 with two copies of the operon on the genome along with P_{gdh4} and feedback alleviated NAGK. The strains ORN4 and ORN5 had similar yields. By introducing the *pgi* start codon mutation to the genome of ORN5, the resulting strain ORN6, was capable of producing 71% more ornithine than ORN2. Experiments performed with ORN6 resulted in difficulties with retaining genetic stability, as recombination leaving only the promoter and the terminator at the CGP1 locus was a reoccurring event, and therefore a decrease in production was observed.

Compared to most published ornithine producing strains our strain does not carry a deletion of *proB* encoding the first enzyme of the proline pathway that also uses glutamate as precursor (Hwang and Cho, 2014; Jiang et al., 2013a; Jiang et al., 2013b; Kim et al., 2015). We chose not to introduce this deletion, as it would entail proline auxotrophy. Exchanging the translational start codon of *proB* to less preferred start codons was shown to increase putrescine production by *C. glutamicum* and circumvented proline supplementation, however, the best putrescine producing strain carried wild-type *proB* (Nguyen et al., 2015a). As exemplified by others, plasmid-based overexpression of *argCJBD* increases ornithine production, but to avoid the use of plasmids we integrated the operon into the chromosome (Hwang et al., 2008; Kim et al., 2015). Tuning the *gdh* promoter combined with the use of a gene variant of *argB* encoding feedback resistant NAGK was pivotal to achieve high ornithine production by *C. glutamicum*. The yield of *C. glutamicum* ORN6 of 0.524 ± 0.026 g/g described here is to the best of our knowledge the highest reported ornithine yield so far.

5.4.7 Production of Proline, Putrescine, Citrulline, and Arginine from Ornithine

An ornithine producing strain has the potential to be converted into a proline, putrescine, citrulline, or arginine producing strain by plasmid-based expression of appropriate pathway modules with one to three genes. Here we wanted to establish if the ornithine producing strains could be used as a platform for the production of the aforementioned compounds. First, plasmids for the appropriate pathway modules were constructed. The plasmids pVWEx1-*argFB*^{A49V,M54V}, pVWEx1-*ocd*$_{Pp}$, pVWEx1-*speC*$_{Ec}$, and pVWEx1-*argGFB*^{A49V,M54V} were transformed into ORN2, resulting in the citrulline producer CIT1, the proline producer PRO1, the putrescine producer PUT2, and the arginine producer ARG5, respectively. The same plasmids were also transformed into ORN2-P$_{gdh4}$ and ORN4, resulting in the next two generations of producers. These strains were all cultured in CgXII medium with 2% glucose, 1 mM IPTG, 25 µg/mL kanamycin, and 0.75 mM arginine (except for the arginine producing strains, that did not require arginine as supplement). Indeed, ornithine producing base strains transformed with the appropriate citrulline, proline, putrescine or arginine pathway modules accumulated the respective compounds in the supernatants as major products (Figure 2). Production of citrulline and arginine by CIT2, CIT3, ARG6, and ARG7, respectively did not improve compared to the yield by the CIT1 and ARG5 strains (Figure 2). This and the observation that strains CIT3 and ARG7 accumulated slightly more ornithine than CIT1 and ARG5, suggested that the reaction catalyzed by ornithine transcarbamylase and potentially other reactions are limiting citrulline and arginine production (Table 6).

Table 6: Accumulation of glutamate and ornithine by the arginine and citrulline producing strains. All values represent the mean and standard deviations of two independent experiments performed in triplicates.

Strain	Glutamate (mM)	Ornithine (mM)
CIT1	1.55 ± 0.15	0.69 ± 0.28
CIT2	1.67 ± 0.15	0.92 ± 0.42
CIT3	1.87 ± 0.49	3.30 ± 1.45
ARG5	0.13 ± 0.01	0.23 ± 0.01
ARG6	0.15 ± 0.01	0.41 ± 0.05
ARG7	0.67 ± 0.06	2.31 ± 1.13

Moreover it cannot be excluded that the potential inhibition of OAT by ornithine and citrulline has an effect on the obtained yields. Both ORN2-P$_{gdh4}$ and ORN4 could be used as platform strains for the production of proline (Figure 2). While ORN2-P$_{gdh4}$ could be used for increased putrescine production (PUT3), introduction of pVWEx1-*speC*$_{Ec}$ into ORN4 resulted in a lower accumulation of putrescine (PUT4) (Figure 2). The putrescine producing strains all accumulated acetylputrescine in the range 7.11-10.44 mM. To construct a platform strain optimized for the production of all five compounds, further genetic modifications have to be performed. To avoid the formation of acetylputrescine it is necessary to delete *snaA* that recently was shown to be responsible for the

acetylation of putrescine (Nguyen et al., 2015b). Accumulation of citrulline as a byproduct by the arginine producing strain could be avoided by overexpression of *argGH* as previously shown (Park et al., 2014). Moreover strategies for increased citrulline production should be explored, this could include construction of feedback resistant OAT and specifically for citrulline production overexpression or deletion of transport proteins, and optimization of the carbamoylphosphate supply.

5.4 Conclusions

An ornithine producing strain was rationally engineered by feedback alleviation of *N*-acetylglutamate kinase, tuning of the *gdh* promoter, lowering expression of *pgi*, along with the introduction of a second copy of the ornithine operon *argCJB*^{A49V,M54V}*D* into the genome. These modifications resulted in an ornithine producing strain with a more than 70% higher yield than the parental strain.

Strains constructed for increased ornithine production could be used for improved proline and putrescine production. Conversion of the ornithine to citrulline or arginine producing strains was possible, however, the resulting strains were not superior to previously engineered citrulline and arginine producing strains. This may indicate bottlenecks in conversion of ornithine to citrulline and arginine. Thus, converting a platform strain capable of producing high yields of ornithine to strains overproducing proline, putrescine, citrulline, or arginine has to take into account optimization of the specific ornithine-converting reactions. Further metabolic engineering of *C. glutamicum* for production of citrulline and arginine could benefit from insight into glutamate, ornithine, and citrulline transport, along with exploring optimization of required precursors such as carbamoylphosphate and aspartate, and importantly elucidating the regulation of the arginine pathway. *C. glutamicum* has the potential to serve as an excellent platform strain for production of amino acids and putrescine. We have taken the first steps to construct superior platform strains with high ornithine yields and demonstrated how these strains easily can be converted into proline, putrescine, citrulline, and arginine overproducing strains. Notably, our approach was rational with employing classical mutagenesis or *in vivo* evolution. Thus, the ornithine platform strain and the citrulline, proline, putrescine or arginine producing strains derived from it by modular pathway engineering have the full potential for further improvements by classical mutagenesis or *in vivo* evolution**.**

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Chapter 6. Metabolic engineering of *Corynebacterium glutamicum* using the CRISPR/dCas9 system

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6.1 Abstract

Present-day approaches to the engineering of amino acid metabolic pathways in *C. glutamicum* can be tedious and cumbersome. In this work we developed the CRISPR/dCas9 technology for the quick and efficient screening of the effect that gene repression can have on the yields of amino acids.

By designing RNA-guided deactivated nucleases, we successfully repressed the expression of *pgi*, *pck* and *pyk*, resulting in increased yields of lysine and glutamate, comparable to those obtained by the deletion of these genes. We show that the sgRNA/dCas9 tool can be efficiently used for pathway modeling, without the need for gene deletions or mutations and selection thereof, in as little as four days.

6.2 Introduction

For the past 50 years, the industrialized world has relied on an extraordinary ability of the soil organism *Corynebacterium glutamicum* to synthesize and secrete incomparable amounts of amino acids [1]–[3]. These molecules are obtained by fermentation and are amongst the most relevant industrial bioproducts of the present time, to reach a market size of US $20.4 billion by 2020 [4]. They are mainly used as a component of animal feed and as flavor enhancers, while being also used in nutritional supplements and cosmetics as well [5]–[7].

Efforts aimed at increasing the amino acid production by *C. glutamicum* cell factories have historically been placed at creating random mutant strains [1]. These uncharacterized strains were obtained via chemical or UV-induced mutations. Despite their stronger producer phenotype they were often genetically unstable and had growth defects [1], [8]. With the advancement of molecular genetics and whole genome sequencing, many were finally characterized. Rather than working on largely uncharacterized mutant strains [1], specific genes could then be knocked out or introduced into the genetic makeup of an organism [9]. In 1991 the term *metabolic engineering* was coined to describe strain improvement mediated by genetic alterations [10].

Despite the need, the panoply of genetic engineering tools available for the manipulation of the chromosome of *C. glutamicum* remains scarce and tedious. In the last two decades many transposable elements were described for this organism, while enabling only random mutagenesis [8]. On the other hand, precise gene modifications rely on the integration of suicide vectors, with the low possibility of a double crossover event removing the plasmid backbone, based on the *sacB* counter selection. Cre/*loxP* has further enabled the deletion of chromosomal regions [8], [11].

Systems biology has made considerable contributions to understanding the intricacies of metabolic pathways [2], [12], [13]. By shedding light onto how pathways are interrelated, predictive strain design is within reach [14]–[18].

For the purpose of increasing production yields through pathway engineering, the ideal approach would be to quickly and efficiently test the phenotype resulting from single or multiple gene function loss. Nonetheless, no tool currently available is capable of quickly enabling such screening. Metabolic engineers still rely on deleting or mutating genes in order to observe or confirm a phenotype. This approach is often a time and resource sink due to technical difficulties in generating such mutants, and results often diverge from the intended.

There is the need for a quick, reliable and easy approach to knock out gene expression in *C. glutamicum*. The availability of such a tool would certainly equip metabolic engineers with the prospect of testing several and multi targets in a matter of days, instead of months. With such a straightforward method, one could potentially fine-tune the ability of strains to produce amino acids [19], [20].

With this goal in mind we adapted the deactivated version of the celebrated Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9) [21], [22], also known as CRISPR/dCas9 system for gene repression in *C. glutamicum* [23], [24].

Originally, the CRISPR/Cas9 system was described as being a bacterial immune-like system, enabling recognition and cleavage of foreign DNA at varying efficiency rates [21], [22], [25]. Traditionally, specialized Cas endonucleases (type I and III) or double stranded RNA ribonucleases triggered by trans-activating CRISPR targeting RNAs (tracrRNA) (type II systems) convert the pre-crRNA into mature CRISPR targeting RNA (crRNA). This product then assembles into a single (type II) or multi-Cas complex (type I and III), with the capacity to recognize and cleave crRNA-complementary DNA sequences [26]–[29]. The need for the additional processing step was supplanted by the realization that, in type II systems, a single RNA synthetic sequence (sgRNA) could suffice for Cas9-mediated cleavage. For efficient recognition of the target site, it was found that the base-pairing regions and a protospacer adjacent motif (PAM) containing a GG dinucleotide, adjacent to the crRNA-binding region in the target DNA sequence, sufficed. This simplified greatly the process of building customized sgRNAs [27].

As opposed to the cleaving CRISPR/Cas9 system, the deactivated Cas9 enzyme (dCas9) in the CRISPR/dCas9 system lacks the nuclease activity characteristic of its active counterpart. It nonetheless retains the capacity to complex with the sgRNA and bind to the homologous locus. Yet, instead of cleaving the DNA, this complex sterically blocks the progression of the RNA polymerase. As a result, the gene is not transcribed but also not mutated. The wild type phenotype of the organism is restored once induction ceases [23], [30].

As the intricacies of the metabolic pathways are disclosed, novel potential targets surface. Understandably so, the number of these possible targets and combinations thereof make their disruption a monumental and unappealing task. Such an easily multi-target scalable system could very well fasten strain improvement as well as pathway elucidation.

6.3 Results

6.3.1 sgRNA/dCas9-based regulation of amino acid production by *C. glutamicum*

To test whether the sgRNA/dCas9 system can be used for pathway regulation in *C. glutamicum*, we built several sgRNAs to sterically block gene transcription. We specifically selected genes known to indirectly impact the production of the two amino acids with the largest market size. We opted for *pgi*, whose repression results in high lysine titers [31], and *pyk* [32] and *pck* [33], which once repressed lead to higher titers of glutamate.

The lysine producing *C. glutamicum* strain DM1729 was transformed with the empty vector pAL374 or pAL-*pgi* targeting either the template or non-template strand of *pgi*. When cultured in CGXII minimal medium these strains all produced lysine to the same level (Figure 1). Simultaneously the DM1729 strains additionally harboring plasmid pZ8-T_*dcas9* were cultured in CGXII. By targeting the non-template strand of *pgi* in the presence of dCas9, the lysine titer was increased by a factor of 2.1 (Figure 1). This indicates a transcriptional repression by the sgRNA/dCas9 system. The same effect was not observed when the sgRNA targeted the template strand of the gene.

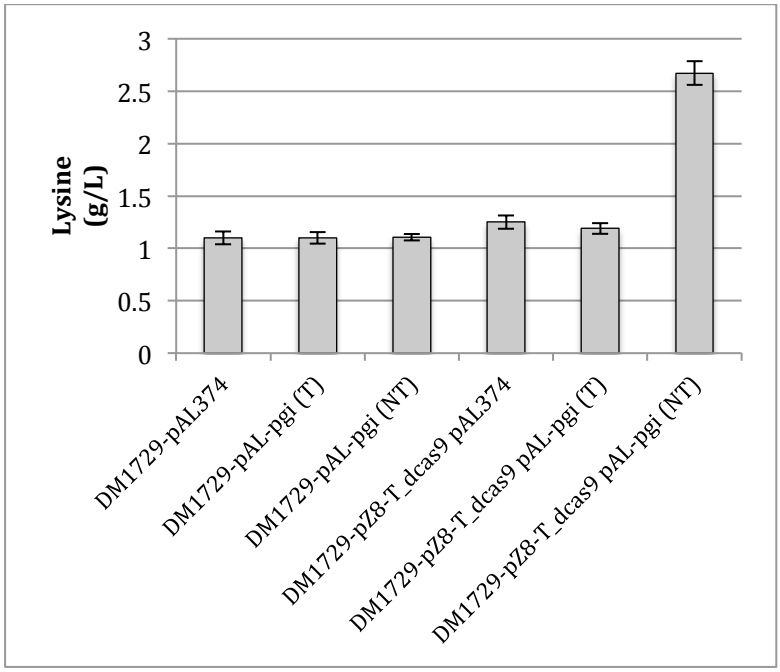

Figure 1. Lysine production by *Corynebacterium glutamicum* DM1729. Titers are the mean of three independent experiments performed in triplicates.

Then the sgRNA/dCas9 system was tested against genes whose repression is known to have a positive impact on the production of glutamate, another economically relevant amino acid. Here the template or non-template strands of genes *pck* or *pyk* were targeted.

C. glutamicum 13032 (*Cg*) was transformed with pAL374, pAL-*pck*, or pAL-*pyk* and with both pAL374, pAL-*pck*, or pAL-*pyk* and pZ8-T_*dcas9*.
Again no notable difference in glutamate accumulation was observed when strains only harboring plasmids with sgRNAs compared to the empty vector control were cultured (Figure 2). However it appears that both *Cg*-pAL-*pyk* (NT) and *Cg*-pZ8-T_*dcas9* pAL374 accumulated more glutamate than the other control strains. Since *Cg*-pAL-*pyk* (NT) and *Cg*-pZ8-T_*dcas9* pAL374 have similar glutamate titers and the standard deviations partially overlap with those of the other control strains these differences are likely due to the ethambutol-induced glutamate production. Compared to lysine production the standard deviations for glutamate production are much higher, which is not uncommon for ethambutol-induced glutamate production [34].
Contrary to the results obtained for lysine production by targeting *pgi*, targeting the template strands of *pck* and *pyk* resulted in an increase in the glutamate production (Figure 2).

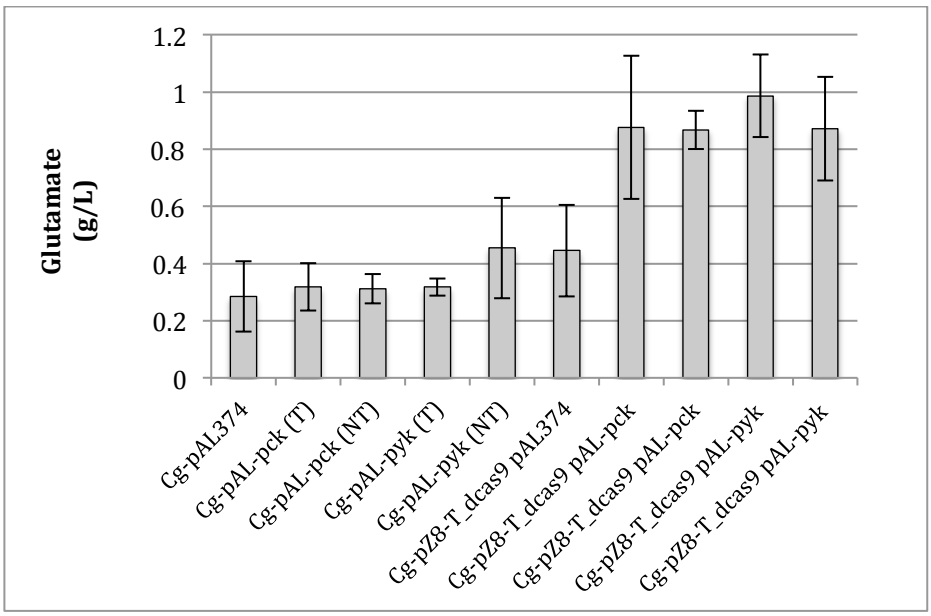

Figure 2. Glutamate production by *Corynebacterium glutamicum*. Titers are the mean of biological triplicates.

In fact, targeting either of the strands of *pck* or *pyk* resulted in elevated glutamate concentrations compared to *Cg*-pZ8-T_*dcas9* pAL374. There was a fold increase of 2.0, 1.9, 2.2, and 2.0 when targeting the template or non-template strands of *pck* or *pyk*, respectively.

6.3.2 Quantification of sgRNA/dCas9-based repression

We next evaluated the levels of transcription of each target gene. In order to obtain a picture of the levels of transcriptional repression, we sampled the *C. glutamicum* cultures in mid-exponential phase for relevant mRNA estimation.

No sgRNA/dCas9-mediated repression was observed when targeting the template strand of *pgi* in DM1729 during the exponential phase, targeting the non-template strand led to strong repression. The relative levels of mRNA were reduced by nearly 98%, resulting in a 33% increase in lysine production (Figure 3).

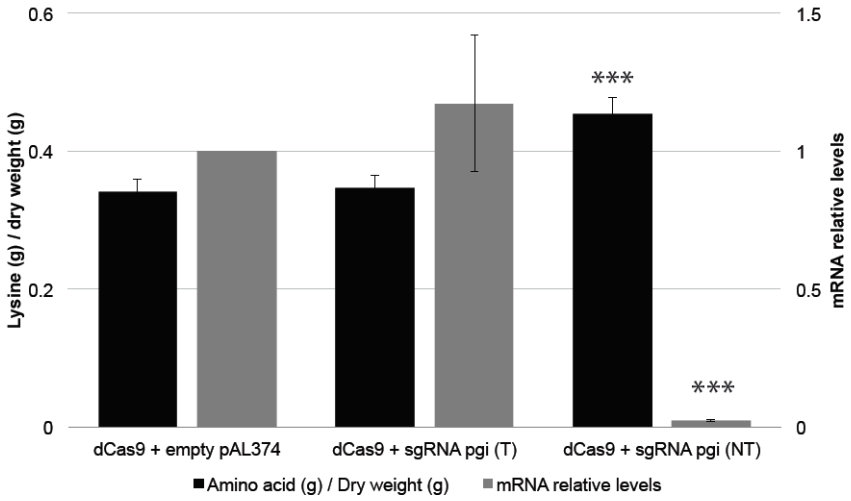

Figure 3. mRNA levels of sgRNA/dCas9 targeted *pgi* in DM1729. mRNA levels decrease, with an increase in the lysine levels. Results are the mean of three independent experiments performed in triplicates. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$, respectively.

When targeting the template strand of *pck* in *C. glutamicum*, a 70% reduction in its mRNA levels led to 28% more secreted glutamate. The transcriptional repression levels were however lower when dCas9 was led to the non-template strand of *pck*, resulting in 46% more glutamate with a 98% reduction in transcription (Figure 4).

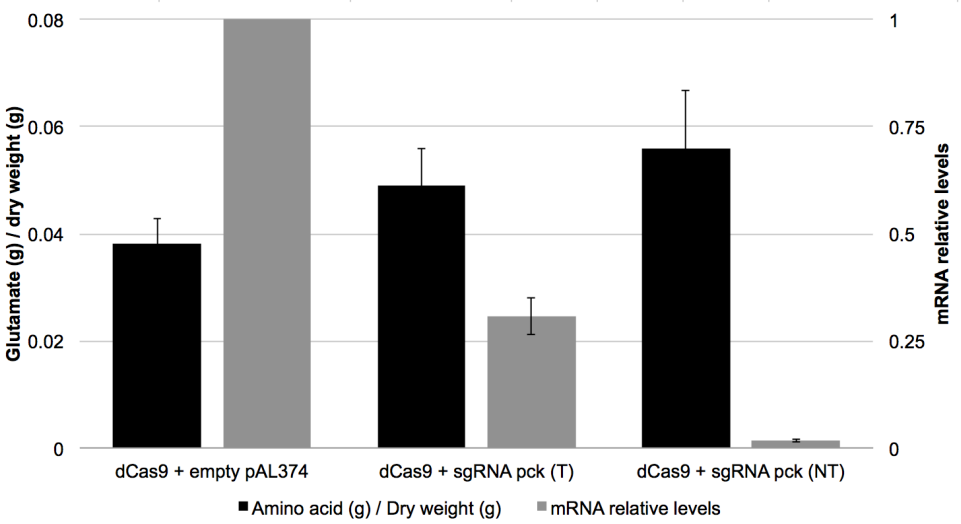

Figure 4. mRNA levels of sgRNA/dCas9 targeted *pck* in *C. glutamicum*. mRNA levels decrease, with an increase in the glutamate levels. Results are the mean of three independent experiments performed in triplicates.

When targeting the template strand of *pyk* in *C. glutamicum*, an 82% reduction in its mRNA levels led to 97% more secreted glutamate. The transcriptional repression levels were even lower when dCas9 was led to the non-template strand of *pyk*, resulting in 204% more glutamate with a 96% reduction in transcription (Figure 5).

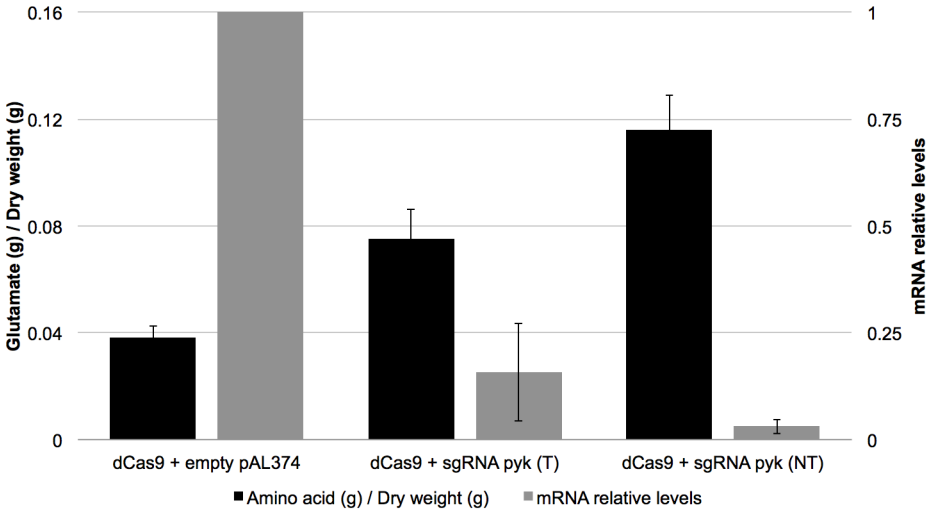

Figure 5. mRNA levels of sgRNA/dCas9 targeted *pyk* in *C. glutamicum*. mRNA levels decrease, with an increase in the glutamate levels. Results are the mean of three independent experiments performed in triplicates.

6.3.3 Impact of dCas9 on cell growth

We were concerned that the overexpression of dCas9 might result in cell toxicity, as previous attempts to express dCas9 driven by unrepressed Ptac in *C. glutamicum* resulted in cell death. Previous work in other bacterial hosts exhibited similar issues [35], [36].
To address this concern, we compared the growth rates of *C. glutamicum* carrying plasmid pZ8-T_*dcas9* and its dCas9-less version (pZ8-Ptac), with different concentrations of the inducer IPTG. No difference in the growth rates independent of the plasmid harbored and the concentration of IPTG used could be observed (Table 1). Therefore dCas9 appears not to affect the viability of *C. glutamicum* when expressed from pZ8-Ptac.

Table 1. Growth rates of *C. glutamicum* pZ8-Ptac and pZ8-T_*dcas9* cultured in CgXII medium with 2% (w/w) glucose, and 25µg/mL kanamycin. Results are the mean of three independent experiments performed in triplicates.

IPTG (mM)	WT pZ8-Ptac (h^{-1})	WT pZ8-T_*dcas9* (h^{-1})
0	0.35 ± 0.01	0.38 ± 0.02
0.5	0.36 ± 0.01	0.36 ± 0.02
1	0.36 ±0.02	0.37 ± 0.02
10	0.37 ± 0.2	0.36 ± 0.02

6.4 Discussion

The CRISPR/(d)Cas9 system has enabled researchers to edit DNA or regulate gene transcriptional levels from a range of organisms [37]–[39].
C. glutamicum plays a multi-billion dollar role on the worldly stage of production of lysine for animal feed and the savory additive glutamate. While the level of amino acids this organism has been engineered to produce is considerably high, we understand the need to increase it further. Yet, the panoply of tools available for engineering *C. glutamicum* remains scarce [11], [40]. It thus seemed germane to engineer the CRISPR/dCas9 system for use in this organism, given its simplicity of design and employment. In our system, the dCas9 and sgRNAs were expressed from independent replicative plasmids. It has previously been established that co-expression of both parts from a single plasmid can result in reduced host growth [35], having toxicity also been observed when overexpressing either of the parts [36]. Specifically, we expressed the sgRNA constitutively while controlling the expression of *dcas9* via the well-established $LaqI^q$ system.
We tested the usefulness of such tool for what is traditionally considered a cumbersome task: engineering metabolic pathways, towards increased titers of a given bio-product. In the case of *C. glutamicum*, it was clear that the product should be amino acids. Yet, our goal was not to further engineer pathways, but to test the performance and suitability of this tool for pathway engineering. To establish this, we decided to target genes *pgi*, *pck* and *pyk* using the sgRNA [27] and the nuclease-inactivated dCas9 enzyme [41]. Following this approach we were able to obtain the *C. glutamicum* strains for testing in 4 days from the initial cloning *in E. coli* to the final *C. glutamicum* colonies carrying dCas9 and the sgRNA.

It is well characterized how the repression of these genes indirectly results in an increased production of lysine when targeting *pgi* [31], and glutamate when targeting *pck* [33] or *pyk* [16]. We found the repression level caused by the steric blockage of *pck* and *pyc* to the progression of the RNA polymerase efficient whether dCas9 annealed onto the template or non-template strands of the coding region. Nonetheless, such observation seemed to be gene or target-sequence specific, as we were not able to extend such observation to all three genes.

Despite observing a significant increase of amino acid production mediated by any of the sgRNAs in the presence of dCas9, the binding of the complex to the non-template strand of the gene exhibited a stronger phenotype for *pgi*, *pck,* and *pyk* in the eksponential growth phase. Earlier publications accounted for an ineffective template-strand targeting [23], [27], which has been repeatedly demystified as reports of efficient template-based repression arise [24], [36].
Our results compare with the described ratio of increase in lysine production when deleting *pgi* (2.1 vs. 1.7) [31]. This ratio for *pck* sgRNA/dCas9 targeting both target and non-target strands was half of the published ratio when deleting *pck* (2.0 and 1.9 vs. 4.5) [16]. Lastly, the ratio for *pyk* sgRNA/dCas9 targeting went slightly beyond the published results for gene deletion (2.2 and 2.0 vs. 1.25) [33]. The final amounts of amino acids produced were however not as high as the previously published titers [16], [31], [33].

As expected, in the absence of dCas9 neither of the sgRNAs interfered with the production of the two amino acids, the indirect result of tempering with the levels of Pck, Pyk or Pgi available in the cell. As the presence of the sgRNA alone, both in lysine and glutamate production experiments, was not sufficient to perturb gene expression, the presence of dCas9 is essential for gene repression.
The presence of dCas9 and the sgRNAs significantly drove up the production of glutamate, when compared with the strains carrying the sgRNAs alone.

Following the amino acid production experiments, the mRNA quenching capabilities of the sgRNA/dCas9 system of *pgi*, *pck*, and *pyk* were quantified.
In the specific case of *pgi*, the levels of amino acid production in mid-exponential phase and *pgi* mRNA levels were inversely proportional when the sgRNA targeted the non-template strand. This is in line with the stationary phase observations made. On the other hand, the sgRNA towards the template strand had a much smaller impact on production. In fact, the results were found not to be statistically significant.
The sgRNA toward the template strands of *pck* and *pyc* and especially toward the non-template strands were efficient at repressing transcription, also reflected in the levels of the glutamate produced.
Overall the experiments show that the CRISPR/dCas9 system could efficiently be used in *C. glutamicum* for increasing amino acid production by lowering the transcription of the targeted genes.

It is important to mention that the steric transcriptional control is indeed merely steric. The likelihood of a complete shutdown is low, given that with each cell division the bacterium needs to again express dCas9, which subsequently needs to be guided by the sgRNA towards the specific target locus. When using this tool to study the impact of such gene repressions, it is important to take into account that despite the versatility of this tool, even a lowered expression level of certain metabolic genes could contribute with enough enzyme activity to maintain flux at the wild-type level. Owing to this, some residual wild type phenotype is would not be surprising, though it will likely not impede the observation of the repression-based phenotype, as shown in this manuscript.

In contrast to the traditional gene knockout approach, which if unlucky can take months, it takes as few as four days to obtain the strain of interest for testing when taking advantage of the transcriptional repression power of sgRNA/dCas9. The use of dCas9 instead of Cas9 further shortens the time-to-screen, as it omits the tedious need to select for the rarer sgRNA/Cas9-derived mutants [25]. The system has great potential for applications in metabolic engineering of *C. glutamicum*, as it can significantly speed up the process of identifying targets and combinations of targets for increased production of industrially relevant bioproducts.

6.5 Materials and Methods

6.5.1 Microorganisms, plasmids and growth conditions

All plasmids were transformed into and maintained in *E. coli* DH5α, grown in Lysogeny broth (for liquid cultures, Miller, LabExpress, USA) or agar (for plates, with 15 % agar, Apex), with the appropriate antibiotics (Table 2), and incubated at 37°C. *C. glutamicum* strains were also maintained on LB agar plates, supplemented with the appropriate antibiotic when necessary (Table 2), but incubated at 30°C. Liquid cultures were prepared using Brain-Heart Infusion (BHI, Sigma-Aldrich, Germany) medium.

Table 2. List of strains and plasmids used in this study.

Strain	Properties	Source
C. glutamicum ATCC 13032	Wild type, biotin auxotroph	ATCC
C. glutamicum DM1729	DM1729 is an aminoethylcysteine-resistant mutant of ATCC 13032; pyc(P458S) hom(V59A) lysC(T311I) - L - Lysine overproducer	Evonik Industries AG
E. coli DH5α	Cloning strain	[42]
Plasmids	**Properties**	**Source**
pDSW204	*E. coli* IPTG inducible expression vector, AmpR	[43]
pZ8-1	*E. coli* – *C. glutamicum* Ptac constitutive expression shuttle vector, KanR	[44]
pAL374	*E. coli* – Corynebacterineae expression shuttle vector, SpecR	[45]
pZ8-T_*dcas9*	pZ8-1 plasmid carrying *dcas9*, driven by the IPTG-inducible *tac* promoter, KanR	This study
pZ8-Ptac	IPTG inducible version of pZ8-1 plasmid, by cloning of *lacIq*, KanR	This study
pPP208	Donor of *dcas9*	[41]
pAL-*pgi* (T)	pAL374 plasmid carrying the *pgi* (T) sgRNA, targeting the template strand of *pgi*, SpecR	This study
pAL-*pgi* (NT)	pAL374 plasmid carrying the *pgi* (NT) sgRNA, targeting the nontemplate strand of *pgi*, SpecR	This study
pAL-*pck* (T)	pAL374 plasmid carrying the *pck* (T) sgRNA targeting, the template strand of *pck*, SpecR	This study
pAL-*pck* (NT)	pAL374 plasmid carrying the *pck* (NT) sgRNA, targeting the nontemplate strand of *pck*, SpecR	This study
pAL-*pyk* (T)	pAL374 plasmid carrying the *pyk* (T) sgRNA targeting the template strand of *pyk*, SpecR	This study

pAL-*pyk* (NT)	pAL374 plasmid carrying the *pyk* (NT) sgRNA targeting the nontemplate strand of *pyk*, SpecR	This study
pK19mob*sacB*	Integrative plasmid carrying the counter-selection gene *sacB*, KanR	[46]

The plasmid pZ8-Ptac, which is an inducible version of plasmid pZ8-1, was obtained by cloning *lacIq* from pDSW405 upstream the *tac* promoter. The Isothermal Assembly Method[47] was used for building the vector. The primers used are listed in Table 3.

Table 3. Primers used for the construction and verification of plasmids.

Template	Strand	Primer sequence (5' – 3')
lacIq for pZ8-Ptac	F	GCGTCAGGCAGCCATCGGAAGCTGTGGTATGGCCGCTATCGCTACGTGACTGG
	R	CGAATTATGCAGTGATTTACGACCTGCACAGTCACTGCCCGCTTTCCAGTCG
pZ8-1 backbone for pZ8-Ptac	F	CGACAGGTTTCCCGACTGGAAAGCGGGCAGTGACTGTGCAGGTCGTAAATCACTGC
	R	GCAGCCATGACCCAGTCACGTAGCGATAGCGGCCATACCACAGCTTCCGATGG
dcas9 for pZ8-Ptac	F	CACACAGGAAACAGAATTCATGGACTACAAAGACCATGACG
	R	GCAGGTCGACGGATCCCCGGTCAGCCCACCTTCCTCTTCTTC
pZ8-1 backbone for pZ8-Ptac	F	GCTAGGAGGTGACCCCAAGAAGAAGAGGAAGGTGGGCTGACCGGGGATCCGTCGACCTGC
	R	CATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCCATGAATTCTGTTTCCTGTGTGA
pAL374 backbone	F	CTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTATGGAATTGGATCCGTGGAAG
	R	CATTATACGAGCCGATGATTAATTGTCAACAGCTCATTTCAGAATATTTGCC
pgi - qPCR	F	TCATTGGTTTCGCTCGTCCA
	R	AGCGTTCTTACCGAAAGCCA
pck - qPCR	F	ATTGGCTACAACGCTGGTGA
	R	CCACTTCAGAACGCGAGAGT
pyk - qPCR	F	GATACCGCAAAGCGTGTGG
	R	GACAGGTGGACACAGGAAGG
16S rRNA - qPCR	F	TTACCTGGGCTTGACATGGAC
	R	GCTGGCAACATAAGACAAGGG

The gene coding for a nuclease-inactivated version of Cas9 - dCas9 - from *Streptococcus pyogenes* was cloned into the *C. glutamicum* replicative plasmid pZ8-Ptac, under the control of an IPTG inducible promoter.

The short, synthetic version of the sgRNA was designed to contain a 24 bp region of homology to the transcriptional template or non-template strands of the target DNA including the seed sequence, followed by the dCas9 handle and the *S. pyogenes* terminator. The expression of this set was driven by an unrepressed *tac* promoter, ordered as a gBlock from IDT and cloned into the replicative plasmid pAL374. The base-pairing regions, handle, and terminators used were the same as those previously described for the sgRNA [23]. The target sequences are listed in Table 4. All sgRNAs were cloned into pAL374 by the Isothermal Assembly Method, having the plasmid backbone been amplified using the primers listed in Table 3, which also removed the *trc* promoter.

Table 4. Genes and sequences targeted by dCas9/sgRNA.

Gene targeted (ID)	Strand targeted	Target sequence (5' – 3')	PAM (5' – 3')
pgi (AGT04848.1)	T	TGACCGATCATTACTCAAACTTCC	AGG
	NT	TTGCCTGGAAGTTTGAGTAATGAT	CGG
pck (AGT06569.1)	T	AGGGCGAGGCGCCGACCAAGAATA	AGG
	NT	TCCAGTTCAGCAGTTCCTTATTCT	TGG
pyk (AGT05824.1)	T	AGATTGTATGTACCCTAGGCCCAG	CGG
	NT	ATTCCATCTGCACTAGCCACCGCT	GGG

6.5.2 sgRNA/dCas9-based regulation of amino acid production by *C. glutamicum*

Genes *pgi* (AGT04848.1), *pck* (AGT06569.1) and *pyk* (AGT05824.1), coding for enzymes known to affect the production of L-lysine (*pgi*) and L-glutamate (*pck* and *pyk)* were selected as individual targets for repression by the sgRNA/dCas9 complex. The sgRNAs were designed to anneal onto the first 150 bp of the template and non-template strands of genes *pgi*, *pck* or *pyk*.

Prior to every amino acid production experiment, *C. glutamicum* cells from glycerol stocks were streaked onto LB agar plates with the appropriate antibiotics and incubated at 30°C overnight. These cells were subsequently used to inoculate BHI broth. Upon overnight growth, the uninduced precultures were harvested by centrifugation (4000 rpm, 5 min, 4°C) and washed with 0.9% NaCl. Ten mL CgXII minimal medium[48] with 2% (w/v) glucose, supplemented with 30 µg/L protocatechuic acid and 1 mM of the *dcas9* inducer IPTG were inoculated to an optical density (OD) of 1 at 610 nm. When appropriate, kanamycin and/or spectinomycin were added to a final concentration of 25 µg/mL and 100 µg/mL, respectively.

The cultures were grown in 125mL flasks at 30°C, with a shaking frequency of 200 rpm, until glucose depletion, measured using Quantofix® Glucose strips (Machery-Nagel, Germany).

The strain *C. glutamicum* ATCC 13032 was used for L-glutamate production. It required supplementing CgXII with 20 mg/L ethambutol, a trigger for L-glutamate secretion [49]. For the experiment where samples where taken after glucose depletion, the preculture was also induced by adding 1 mM IPTG.

Lysine production did not require any additional supplements, having been performed by *C. glutamicum* DM1729. This strain is an S-aminoethyl-L-cysteine-resistant mutant of ATCC 13032, previously developed for the specific purpose of L-lysine overproduction.

Growth of all cultures was monitored over time by measuring their OD throughout the experiment, using an Infinite 200 Pro microplate reader (Tecan, Switzerland). Three independent experiments were performed in triplicate.

The OD_{610nm} conversion to cell dry weight (CDW) followed the ratio previously described of $OD_{610nm}1 = 0.25$ g CDW [50].

6.5.3 Quantification of sgRNA/dCas9-based repression

In order to determine the level of dCas9/sgRNA-based transcriptional repression, quantitative reverse transcription PCR (qRT-PCR) was performed on total RNA samples. One mL aliquots of each culture were taken at mid-exponential phase for total RNA extraction.

All aliquots were centrifuged at 17.000 rpm for 15 seconds, the pellets were immediately flash frozen and stored at -80°C until further processing. The cells were lysed by resuspension of pellets in RA1 buffer and bead beating 2 x 15 sec. (Mini-Beadbeater-16, Biospec Products, USA) with intermittent cooling on ice. The steel beads used were RNase-free and 0.2 mm in diameter (KSE Scientific, USA).

The total RNA was extracted using the Illustra RNAspin Mini Kit according to the recommendations of the manufacturer (GE Life Sciences, UK). To remove residual co-extracted DNA, the purified RNA was treated with DNase (Turbo DNA-free kit, Life Technologies, USA).

qRT-PCR was performed using the KapaTM SYBR®Fast One-Step qRT-PCR kit (Kapa Biosystems, USA), the LightCycler® 96 System (Roche, USA), 50 ng of total RNA extracted, 16S rRNA as reference and the primers indicated in Table 2. The results were analyzed following the Livak method [51].

6.5.4 Amino acid quantification

Samples for amino acid quantification were taken in mid-exponential phase and directly after glucose depletion. The samples were centrifuged at 17.000 rpm for 15 seconds and automatically derivatized using the Fluoraldehyde™ *o*-Phthaldialdehyde Reagent Solution (Thermo Scientific, USA), prior to entering the chromatographic separation column. Compound separation was performed by high-pressure liquid chromatography (HPLC, 1200 series, Agilent) with a RP18 column (Eclipse XDB-C18, 4.6 x 150 mm, Agilent) and a fluorescence detector (FLD G1321A, 1200 series, Agilent).

The mobile phases were 0.1 M sodium acetate at pH 7.2 (A) and 100% methanol (B). The gradient used was as follows: 0 min 20% B, 0.5 min 38% B, 2.5 min 46% B, 3.7 min 65% B, 5.5 min 70% B, 6 min 75% B, 6.2 min 85% B, 9.7 min 20% B and 11.9 min 20%, at a flow rate of 1.2 mL/min.

Ornithine was used as internal standard, and standard curves for the determination of the amino acid concentrations in the supernatants was determined using solutions of glutamate and lysine with known concentrations.

6.5.5 Statistical treatment of data

Results were tested for significance using the parametric unpaired T-Student test. The level of significance of the differences observed between the control and test samples was expressed as one, two or three stars, for $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$, respectively.

6.6 References

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Chapter 7. Discussion

The members of the glutamate family; ornithine, proline, citrulline, putrescine, and arginine are highly relevant industrial products with wide applications in the food and feed, cosmetic, chemical, pharmaceutical, and plastics industries [1]–[5]. Production of proline, ornithine, and arginine by microorganisms has been explored for more than five decades [6]. Most of the strains used in these processes were constructed by means of random mutagenesis and selection [6]–[10]. Although these strains can accumulate high concentrations of amino acids, they often accumulate undesired mutations resulting for instance in auxotrophy, low productivity, low stress tolerance, or sensitivity to external perturbations [11]. Over the years DNA technologies have developed and cloning tools for *Corynebacterium glutamicum* have been established [12]. This has entailed a number of recent publications on ornithine-, putrescine-, and arginine-producing strains constructed using metabolic engineering [13]–[19].

In this work the first rationally engineered citrulline producing *C. glutamicum* strain was constructed, the first rationally engineered proline producing strain along with optimization of proline production was published, and different strategies to increase ornithine production by the platform strain were explored. Following this work we tried to translate the increased ornithine production into increased production of proline, citrulline, putrescine, and arginine. Lastly we showed how the CRISPR/dCas9 system could be employed for metabolic engineering in *C. glutamicum*

Some of the discoveries of this thesis will be discussed more thoroughly here in context to previous findings. However, first the thermodynamics of the biochemical reactions of the arginine pathway will be examined.

7.1 Considering the thermodynamics

The laws of thermodynamics apply to biochemical reactions and therefore the feasibility of a reaction pathway can be evaluated with thermodynamics [20]. This makes it possible to pinpoint potential difficulties of the pathway. The overall change in entropy determines the spontaneity of a process. Only reactions with a negative change in Gibbs free energy (Δ_rG) proceed spontaneously. Increasing substrate concentration and/or decreasing product concentration can force the direction in which a reaction occurs [20].

In the cell, reactant and product concentrations do not necessarily remain steady; moreover their concentrations are not likely to be those of the standard conditions (pH of 7, ionic strength of 0.1 mM, a temperature of 25°C, and concentrations of reactants and products of 1 M). Erroneous conclusions can therefore be made when evaluating the thermodynamic feasibility based on those values [20]. Concentrations resembling reality should always be used when a thermodynamic analysis is performed because reactant and product concentrations can have a large effect on Δ_rG' (the change in Gibbs free energy of a reaction taking pH and ionic strength into account). Moreover the laws of thermodynamics only apply to Δ_rG' and not $\Delta_rG'°$ (Δ_rG' in standard conditions) [21]. The concentration ranges of metabolites should be measured experimentally to calculate Δ_rG'. The reactions catalyzed by the enzymes of the arginine pathway, along with enzymes catalyzing the

formation of proline and putrescine can be found in Table 1. Since the intracellular concentrations of the metabolites of the arginine pathway are not readily available for *C. glutamicum* the values listed in Table 2 are the $\Delta_rG'^m$ ($\Delta_rG'°$ but with concentrations of reactants and products of 1 mM) and Δ_rG' of reactions calculated with different concentrations of reactants and products. The software eQuilibrator [21] was used to calculate the net feasibility of the reactions.

Table 1. Genes, reactions, and enzymes of glutamate derived molecules.

Gene	Enzyme	Step	Reaction
gdh	Glutamate dehydrogenase (GDH)	1	2-oxoglutarate + NADPH + NH_3 -> glutamate + $NADP^+$ + H_2O
cg3035	*N*-acetylglutamate synthase (NAGS)	2	Acetyl-CoA + glutamate <-> CoA + *N*-acetylglutamate
argB	*N*-acetylglutamate kinase (NAGK)	3	ATP + *N*-acetylglutamate <-> ADP + *N*-acetylglutamatephosphate
argC	*N*-acetylglutamylphosphate reductase (ArgC)	4	NADPH + *N*-acetylglutamatephosphate <-> $NADP^+$ + orthophosphate + glutamate semialdehyde
argD	*N*-acetylornithine aminotransferase (ArgD)	5	Glutamate + glutamate semialdehyde <-> 2-oxoglutarate + *N*-acetylornithine
argJ	Ornithine acetyltransferase (OAT)	6	Glutamate + *N*-acetylornithine <-> ornithine + *N*-acetylglutamate
argF	Ornithine transcarbamylase (OT)	7	Ornithine + carbamoylphosphate <-> orthophosphate + citrulline
argG	Argininosuccinat synthetase (ASS)	8	ATP + aspartate + citrulline <-> diphosphate + AMP + argininosuccinate
argH	Argininosuccinate lyase (AL)	9	Argininosuccinate <-> arginine + fumarate
carAB	Carbamoylphosphate synthase (CPS)	-	2 ATP + glutamine + CO_2 + H_2O <-> 2 ADP + orthophosphate + glutamate + carbamoylphosphate
ocd	Ornithine cyclodeaminase (Ocd)	-	Ornithine <-> NH_3 + proline
speC	Ornithine decarboxylase (ODC)	-	Ornithine <-> CO_2 + putrescine

Although we cannot make a thermodynamic analysis based on the values in Table 2 they still provide an understanding of the reactions. The $\Delta_rG'^m$ of the reactions catalyzed by NAGK, ArgD, OAT, and ASS are positive, which means that in theory they should proceed in the opposite direction of the reactions shown in Table 2.

If all concentrations of the reactants are set to 10 mM and concentrations of the product to 0.1 mM only the Δ_rG' of the NAGK catalyzed reaction remains positive. The reaction will be at equilibrium with high reactant concentrations of 41 mM and concentrations of 0.1 mM of the products. If however the concentrations of the products are lowered to 1 µM only 1 mM of the reactants are needed for the forward reaction to proceed (Δ_rG' = -4.4 ± 6.0 kJ/mol). This indicates that low concentrations of *N*-acetylglutamatephosphate or very high concentration of *N*-acetylglutamate are important for the NAGK catalyzed reaction to take place. From a thermodynamic point of view the NAGK catalyzed reaction could be limiting in production of glutamate derivatives.

Table 2. Feasibility of the reactions of the glutamate derived molecules.

Reaction catalyzed by	$\Delta_rG'^{m}$ (kJ/mol)	$\Delta_rG'^{a}$ (kJ/mol)	$\Delta_rG'^{b}$ (kJ/mol)
GDH	-21.8 ± 0.7	-50.3 ± 0.7	6.7 ± 0.7
NAGS	-27.4 ± 6.5	-50.2 ± 6.5	-4.6 ± 6.5
NAGK	29.8 ± 6.0	7.0 ± 6.0	52.6 ± 6
ArgC	-25 ± 3.2	-53.5 ± 3.2	3.5 ± 3.2
ArgD	11 ± 6.5	-11.8 ± 6.5	33.8 ± 6.5
OAT	3.1 ± 5.8	-19.7 ± 5.8	25.9 ± 5.8
OT	-28.9 ± 5.8	-51.7 ± 5.8	-6.1 ± 5.8
ASS	1.7 ± 2.9	-32.5 ± 2.9	35.9 ± 2.9
AL	-4.7 ± 5.8	-21.8 ± 5.8	12.5 ± 5.8
CPS	-23.7 ±5.2	-75.0 ± 5.2	27.7 ± 5.2
Ocd	-39.5 ± 8.2	-56 ± 8.2	-22.4 ± 8.2
ODC	-60.4 ± 10.6	-77.5 ± 10.6	-43.2 ± 10.6

[m]Reactants set to 1 mM and products set to 1 mM
[a]Reactants set to 10 mM and products set to 0.1 mM
[b]Reactants set to 0.1 mM and products set to 10 mM

Reactions with large negative energy such as those catalyzed by NAGS and OT are often considered key control points in a pathway, as they are essentially irreversible based on their $\Delta_rG'°$. This means that in the case of the NAGS catalyzed reaction, even when low levels of glutamate or high levels of *N*-acetylglutamate are present the reaction will still take place.

The reaction catalyzed by ASS is for instance rather close to equilibrium, this makes it relatively sensitive to substrate and product concentrations as can be seen from Table 2. Hence, it can rapidly communicate changes in flux.

Notably the reactions catalyzed by Ocd and ODC have a large negative $\Delta_rG'^{m}$. Interestingly it was found that ornithine production could be enhanced by Ocd in proline supplemented cultures [22]. Or rather, it was assumed that the reaction was taking place based on the experiments performed, although the conversion of proline and ammonia to ornithine was not directly demonstrated. From the thermodynamic calculations performed with eQuilibrator and our findings on Ocd enzyme activity, this assumption can be challenged. In order for the reaction catalyzed by Ocd to proceed in the reverse direction more than 290 mM proline and 290 mM ammonia are required if the concentration of ornithine is 0.01 mM. As the intracellular glutamate concentration in a lysine producing strain was determined to be 190 mM, which was by far the highest concentration of all the proteinogenic amino acids measured, it seems unlikely that the reaction in the opposite direction can take place [23]. The maximum concentration of proline that was added to the medium in the study to promote the conversion of proline and ammonia to ornithine was 30 mM; this is unlikely to be a high enough concentration for the reaction to proceed. Moreover when we performed an Ocd activity assay no activity was observed, which indicated that the enzyme did not have this putative function (Chapter 3). Taken together, these findings indicate that ornithine production was not and cannot be enhanced by Ocd in proline supplemented cultures of *C. glutamicum*.

This section on thermodynamics was written to illustrate that metabolic engineering is not only about introducing mutations, insertions, or knockouts, and repressing or overexpressing genes; it is also about understanding the chemistry and physics of the cell. In the iterative cycle of metabolic engineering an important part of evaluating what steps are to be taken next is the characterization of the strain. Here thermodynamic analysis combined with methods such as metabolic control analysis and flux analysis can be valuable to determine flux distributions. This would be valuable for the planning of further metabolic engineering strategies, and would shed light on the where the actual bottlenecks are located.
Making such an analysis of the arginine pathway could give insight into where the bottlenecks are located especially before and after feedback alleviation of NAGK and OAT. In the literature there is still some uncertainty as to where the bottlenecks of the arginine pathway are and in which order they should be addressed when performing metabolic engineering. Currently the bottlenecks are considered to be the reactions catalyzed by NAGK and OAT, the supply of glutamate, carbamoylphosphate, and NADPH, and the expression level of *argGH*.

7.2 Production of glutamate derivatives by *Corynebacterium glutamicum*

Hwang *et al.* (2008) first published a rationally constructed ornithine producing *C. glutamicum* strain. This strain carried a deletion of *argF* whose gene product is responsible for the conversion of ornithine to citrulline, and hence important for ornithine accumulation. Moreover *argR* encoding the repressor ArgR that regulates the transcription of the genes of the arginine pathway was deleted to further boost the ornithine titer. These deletions are common for most of the efficient ornithine producers published in literature and were also used in this work to construct the initial strain used for all further modifications (Chapters 3, 4, and 5).

7.2.1 Feedback inhibition

In the process of metabolic engineering for the production of amino acids abolishing feedback inhibition is commonly one of the first targets to be addressed as it controls the flux through the pathway [24]–[26]. The finding that acetylglutamate, the substrate of NAGK, accumulates intracellularly in *C. glutamicum* wild-type and *C. glutamicum* Δ*argFR* [27] indicates that the step of the pathway catalyzed by NAGK is a bottleneck of the arginine and ornithine biosynthetic pathways. Studies performed on *C. glutamicum* [28], *Corynebacterium crenatum* [29], *Pseudomonas aeruginosa* [30], and *Escherichia coli* [31] have revealed mutations that release the enzymes NAGK, or in the case of *E. coli*, NAGS from inhibition by arginine, the end product of the arginine pathway. Based on published data we could show that the expression of a variety of feedback resistant NAGKs was beneficial for ornithine overproduction (Chapter 5). The NAGK variants had lower specific enzyme activities than wild-type NAGK, in one case even a 4 times lower activity. But once these *argB* variants were overexpressed they resulted in higher ornithine yields than when wild-type *argB* was overexpressed. This indicates that in order to increase the ornithine titer in the Δ*argFRGB* background it is more important to reduce the level of feedback

inhibition of NAGK than to maintain the specific enzyme activity to the level of wild-type NAGK. Overexpression of *argB*^{A49VM54V}, encoding feedback alleviated NAGK in proline producing *C. glutamicum* increased the proline titer but also decreased the growth rate and the total biomass formed (Chapter 3). Introduction of such mutations in NAGK have previously been connected to a lowered carbon consumption rate in arginine overproducing *C. glutamicum* and hence the feasibility of the mutations should be evaluated [14].

7.2.2 Increasing the precursor supply

The glutamate supply has also been assumed to be limiting for overproduction of ornithine, hence a push-strategy was employed to assess if this was the case [13]. The gene *pck* encoding phosphoenolpyruvate kinase catalyzing the first step of gluconeogenesis was deleted and *pyc* encoding pyruvate carboxylase catalyzing the carboxylation of pyruvate to form oxaloacetate was overexpressed. This resulted in accumulation of glutamate in the Δ*argFR*Δ*proB* background but did not translate into increased ornithine production [13]. This finding, in combination with the observation that glutamate accumulated extracellularly as byproduct in proline production (Chapter 3) meant we initially assumed that glutamate availability was not limiting in the ornithine pathway. However, a study on medium optimization for the production of ornithine indicated that the addition of the surfactant Tween 80, known to elicit glutamate overproduction, had a positive effect on ornithine accumulation [32]. In an attempt to determine if a more direct approach to increase the intracellular glutamate concentration was beneficial for ornithine production we tuned the promoter of *gdh* (Chapter 5). We showed that this resulted in increased enzyme activity and that this could not only be translated into increased production of ornithine but also to increased production of putrescine and proline. To this end it is not clear why the push-strategy did not result in a higher ornithine titer while tuning the *gdh* promoter did. Unknown regulatory mechanisms could possibly be involved.

7.2.3 Aminotransferases involved in ornithine/arginine biosynthesis

Aminotransferases are enzymes involved in, but not limited to, amino acid biosynthesis [33]. These enzymes are known to have overlapping substrate specificity, which has already been demonstrated for instance in *E. coli*. Here enzymes encoded by *tyrB*, *ilvE*, and *aspC* and involved in tyrosine, phenylalanine, and aspartic acid biosynthesis respectively can overlap each others function [34]. Another example is the overlapping functions of the enzymes encoded by *avtA* and *ilvE* in valine biosynthesis in *C. glutamicum* [33]. 20 putative aminotransferases were identified in *C. glutamicum* with a bioinformatic approach [35]. One of them, acetylornithine aminotransferase encoded by *argD* was not essential for ornithine production by *C. glutamicum* MB001Δ*argFRG* (Chapter 5). Hence we expected another enzyme to be able to overtake its function, however additional deletions of the aminotransferases encoded by *argD2, gapT*, and *bioA* did also not result in ornithine auxotrophy. This finding was surprising as the deletion of *argD* in *C. glutamicum* RES167 resulted in arginine auxotrophy [35]. Moreover it was later shown that *C.*

glutamicum Δ*argDR* accumulates acetylglutamate semialdehyde, the substrate of ArgD, which neither the wild-type nor *C. glutamicum* Δ*argFR* did [27].
C. glutamicum has no annotated genes involved in arginine catabolism and no published findings on an arginine degradation system, hence it should not be possible for arginine to be converted into a compound that can be converted into ornithine. Although it is not surprising that ArgD is not essential for ornithine production, as aminotransferases are known to have overlapping substrate specificity, it is interesting that this finding is contradiction to results published in literature. A recent study showed that the overexpression of *gabT* resulted in an improved ornithine titer, more interestingly the overexpression of the gene almost tripled specific aminotransferase activity [36]. Additionally we showed that overexpression of *argD2* improved the ornithine yield. Although speculative an upregulation of expression of a gene encoding an aminotransferase may be responsible for the lack of ornithine auxotrophy observed in the MB001Δ*argFRGD* strain. Further experiments have to be performed to determine if genetic differences between the strains can explain the results.

7.2.4 Medium optimization

The medium used for industrial fermentations should allow maximum concentration of product or biomass per unit mass of substrate, minimum production of byproducts, and a consistent product quality to ensure a cost-efficient process [37]. Defined media are often used for experiments where chemical, physical, and physiological parameters have to be standardized or growth has to be assessed, as all components of the medium are chemically defined [37]. Fermentation media for industrial processes often contain cheap complex compounds like hydrolysates and extracts of waste products with an undefined chemical composition. The cheap substrates such as cane molasses, corn steep liquor, yeast hydrolysate etc. are used to keep production costs low [38].
Often medium optimization is required for optimal conversion of substrate to product; here design of experiments (DOE) can be used to reduce the resources and time spent compared to using a single factor at a time method. Such a systematic approach allows one to determine the optimum medium composition and other variables that might influence the fermentation process, such as inoculum size [37].
For cultivation of *C. glutamicum* in defined medium, CGXII is commonly used. The medium is rich in N-source, with 20 g/L ammonium sulfate and 5 g/L urea. When *C. glutamicum* Δ*argFR* with plasmid-based overexpression of *ocd* to facilitate proline production was cultured in CGXII both glutamate and ornithine accumulated (Chapter 3). The reaction catalyzed by Ocd releases both proline and ammonia, hence the ammonia required for the GDH catalyzed reaction converting 2-oxoglutarate to glutamate is recycled once released with the formation of proline. Thereby the overall ammonium requirement of the cell should be reduced and hence reduce the amount of N-source needed in the growth medium. We therefore performed a DOE to determine if medium optimization could reduce accumulation of intermediates in the culture medium and thereby increase the proline yield. Once the medium was modified to contain 5 g/L urea as sole nitrogen source and 35 g/L glucose instead of 40 g/L, proline production was increased by 25% and byproduct formation was reduced (Chapter 3).

To achieve the best possible production of each of the five compounds dealt with in this thesis it may be necessary to optimize the medium for each of these compounds seperately. It is only in proline production that the ammonia consumed by the GDH catalyzed reaction can be recycled, therefore the optimal production of ornithine, citrulline, and arginine are likely to have a higher demand for a nitrogen source. This is supported by previous reports on medium optimization for the production of ornithine and arginine where the final media contained 35 and 60 g/L ammonium sulfate, respectively [32], [39].
Along with strain construction and optimizing the fermentation process, the medium composition plays a role in establishing an overall optimized production process of a compound and therefore contributes to establishing a cost efficient production process.

7.2.5 Citrulline and arginine production

For the first time, we demonstrated how *C. glutamicum* could be engineered to accumulate citrulline extracellularly. Surprisingly we found that deleting both *argG* coding for argininosuccinate synthase responsible for the conversion of citrulline into argininosuccinate, and plasmid-based overexpression of *argF*, neither resulted in citrulline production nor accumulation of ornithine. Overexpression of an *argB* variant encoding feedback resistant NAGK (*argB*^{fbr}) was necessary for citrulline accumulation in *C. glutamicum* MB001Δ*argFRG* (Chapter 4).
Around the time we published these results another paper dealing with citrulline production by *C. glutamicum* was published. The authors observed accumulation of 0.15 g/L citrulline extracellularly and a deletion of *argR* and *argG* further increased the titer to 5.43 g/L [40]. This was unexpected as we had not been able to detect extracellular accumulation of citrulline in wild-type *C. glutamicum*, when using a fluorescence detector to detect amino acid derivatives; a more sensitive instrument than the UV-detector used in their study.
Theoretically the genetic background of our strain only differed from their strain in the deletion of the three prophages and *argF*. Although unlikely, it cannot be excluded that the differences observed were due to the fact that we had deleted *argF* and overexpressed it on a plasmid. Other differences could be determined by whole-genome sequencing along with transcriptomics and could potentially reveal regulation that causes our strain not to accumulate citrulline extracellularly.
One theory that could explain why neither ornithine nor citrulline accumulated extracellularly in cultures of *C. glutamicum* MB001Δ*argFRG* overexpressing *argF*, is that citrulline inhibits NAGK. If the intracellular citrulline concentration is not sufficient to activate a putative citrulline exporter but conversely high enough to inhibit NAGK, then neither ornithine nor citrulline can accumulate in the medium. Although this is rather speculative it would be in line with an earlier study that showed that the deletion of *argR* in *C. glutamicum* was not enough to produce arginine extracellularly, the overexpression of *argB*^{fbr} was also required [19].
We were not able to increase arginine or citrulline production by increasing the glutamate level of the cell even though it increased ornithine production notably (Chapter 5). This indicates that in those strains the reactions following ornithine formation are limiting. Park *et al.* solved this by removing at least one of the bottlenecks downstream the ornithine pathway. This was achieved by exchanging the *argG* promoter with the strong *tuf* promoter, and the *carA* promoter with the strong *sod* promoter. After the introduction of these modifications citrulline no longer accumulated as

byproduct in arginine production [14]. If the modifications performed above to construct an arginine producing strain were combined with the tuned *gdh* promoter that increases the supply of glutamate, this increased supply could possibly be translated into increased citrulline or arginine production.

7.2.6 Summary

Bottlenecks of the ornithine/arginine pathway include, but are not necessarily limited to; the glutamate supply and the reactions catalyzed by NAGK and OAT, the carbamoylphosphate supply, and the expression level of *argGH*. For the GDH and NAGK catalyzed reactions it appears that at a certain threshold level an increase of the enzymatic activity does not equal an increase in the production of ornithine. This indicates that these reactions are no longer limiting for ornithine production when the expression of *gdh* is increased and NAGK is alleviated of feedback inhibition by arginine. The bottlenecks located in the arginine specific part of the pathway prevent an increased level of ornithine production to be translated into an increased production of citrulline or arginine. Citrulline production required the presence of feedback alleviated NAGK, possibly suggesting that the enzyme is not only regulated by arginine, but also by citrulline. ArgD was not essential for ornithine production indicating that another transaminase can substitute its function. Lastly it was shown that medium optimization could be an important part of improving the conversion of substrate into product. By the example of proline production its was shown that the reduction of the nitrogen content in the medium had a positive effect. It was also argued that optimal medium composition for ornithine, putrescine, citrulline, and arginine likely differs from proline production medium as these compounds have a different demand for nitrogen.
In the next subsection it will be discussed which next steps can be taken to further increase the production of the glutamate derivatives, and the potential of the platform strain.

7.2.7 Improving the platform strain

Ornithine aminotransferase (OAT) is feedback inhibited by ornithine and citrulline. OAT may be the major bottleneck of the ornithine pathway once NAGK is feedback alleviated and sufficient concentrations of glutamate are supplied. When *argJ* encoding OAT was overexpressed in ornithine producing strains, the specific enzyme activity remained largely unchanged in one report and more than doubled in another [13][36]. Based on our studies plasmid-based overexpression of *argJ* had a major impact on the ornithine yield and could reflect an increased enzyme activity (Chapter 5). We were able to construct an ornithine producing strain with a high yield of 0.52 g ornithine / g glucose without the use of a feedback resistant OAT. Based on the findings described above further experiments are required to determine whether overexpression of *argJ* is sufficient for increased production of the glutamate derivatives or if alleviation of feedback inhibition of OAT can boost the production even further. Thus far no protein engineering efforts have been made to remove the potential bottleneck feedback inhibition might be. Though the amino acid change G330D of OAT has been reported, the effect of the exchange on feedback inhibition has not been investigated [36].

To determine if overexpression of *argJ* encoding feedback alleviated OAT is more beneficial for overproduction of the glutamate derivatives than overexpression of wild-type *argJ*, the following experiments could be performed; Feedback resistant mutants could be obtained by the classical approach where *C. glutamicum* is treated with a mutagen or UV-irradiation and mutants are selected based on their resistance to toxic ornithine analogues such as α-methylornithine or difluoromethylornithine. The mutants can then be tested for growth and ornithine production using for instance the biolector microfermentation system as it allows simultaneous cultivation and online monitoring of growth for up to 48 strains. Although more challenging and resource intensive rational protein engineering by construction of a homology model based on published crystal structures can be performed. By rationally selecting amino acid(s) to exchange, a number of different mutations can be tested to construct a feedback resistant enzyme. Although this is a targeted approach, still a large number of clones would have to be screened since every target amino acid can be exchanged with up to 19 other amino acids.

Increased levels of ornithine should entail increased production of the other glutamate derivatives; this was not the case for the production of putrescine, citrulline, and arginine. Therefore further changes have to be made to facilitate this. For increased putrescine production the deletion of *snaA* to avoid the conversion of putrescine to acetylputrescine along with replacing the promoter of the gene coding for the putrescine exporter with a stronger one should be performed. It should be determined if citrulline inhibits NAGK and NAGK resistant to feedback inhibition by arginine. To remove the bottlenecks downstream the ornithine pathway the carbamoylphosphate supply could be increased by overexpression of *carAB* and the flow from the ornithine pathway into the arginine pathway could be improved by overexpressing the *argF(GH)* genes.
It could also be beneficial to engineer the transport systems for increasing the production of the glutamate derivatives. LysE is a carrier protein shown to export both lysine and arginine at similar rates across the membrane of *C. glutamicum*, but it does not export ornithine [41]. When the N-terminal of the protein was extended with the amino acids methionine, valine, and isoleucine, the export of lysine and arginine was improved. In the Δ*argFRGH* background it also led to increased ornithine production [42]. The experiments performed in the study indicated that the modified protein could facilitate the export of ornithine. It was claimed that this was the result of a change in the secondary structure. Hence it could be beneficial to introduce the modified *lysE* gene in the platform strain and test its effect on the production of ornithine, citrulline, and arginine. However if ornithine can be exported this modification could be detrimental to citrulline and arginine production.
Lastly the product range of the platform strain has the potential to be extended even further by establishing the overproduction of spermidine, a polyamine synthesized from putrescine.

7.3 Metabolic engineering

A major challenge in metabolic engineering is selecting the right targets at the right step in the process. Although several targets for metabolic engineering can usually easily be identified by looking at the pathway along with information in the literature, the modification of the chosen target will not necessarily have the desired effect. For one there might be a bottleneck

upstream the target that has to be solved first, in that case improving the target could show a small effect or non at all. On the other hand improving one target and then moving on in the iterative metabolic engineering cycle does not mean that the solved bottleneck might not become a bottleneck later on. When a target is optimized, it will necessarily be optimized for the particular genetic background of that strain. Once the strain has undergone several more rounds of alterations, the previously engineered targets may no longer be optimal for the new genetic background of the strain. Metabolic engineering can be seen as an endless tinkering of metabolism to obtain the best producing strains. Performing genetic alterations on the genome such as deletions can at times be quite cumbersome if the same or multiple targets have to be tested in different genetic backgrounds. Computational simulations may be able to facilitate a better engineering of the strain if it is based information such as kinetic data and 13C flux analysis [43].
Technology-wise much has happened since Bailey coined the term 'metabolic engineering' in 1991. New exciting technologies and fields such as synthetic biology have come along the way. This has the potential to take metabolic engineering to the next level where cell factories can be constructed in a more predictive, fast, and efficient way.
An example of a new genetic engineering tool is the Clusters of Regularly Interspaced Short Palindromic Repeats (CRISPR)/dCas9 system. This technology opens new avenues for metabolic engineering.

7.3.1 Increasing the pace of pathway engineering with the CRISPR/(d)Cas9 system

The CRISPR/dCas9 system allows for a quick determination of whether modifications are beneficial alone or in combination with other targets and can speed up the strain construction process. We for the first time showed that the CRISPR/dCas9 system is functional in *C. glutamicum* (Chapter 6). We were able to repress selected genes beneficial for glutamate and lysine production by targeting non-template and in some cases template strands. In the case of lysine production we repressed the expression of *pgi*, redirecting the flux into the pentose phosphate pathway, thereby generating more NADPH know to be beneficial for lysine production [44]. We showed that the perturbation of *pgi* resulted in nearly complete repression, where we observed a fold increase in lysine production comparable to that of a *pgi* deletion mutant. When we targeted genes *pyk* and *pck* whose repression is beneficial for glutamate production we also observed a nearly complete repression when targeting non-template strands. When template strands of *pyk* and *pck* were targeted an efficient but not complete repression was obtained and glutamate production was significantly increased. This study exemplifies how the CRISPR/dCas9 system can be used in *C. glutamicum* to evaluate the feasibility of different targets for metabolic engineering.
The CRISPR/(d)Cas9 system has great potential to take genetic engineering to a new level, allowing metabolic engineers to significantly reduce the time needed for strain construction. Especially the potential the system has for multiplex targeting, which allows one to evaluate the combination of different targets simultaneously is valuable. The multiplex targeting can easily be achieved by cloning several small guide RNAs (sgRNA) into one plasmid [45].

There is still much room for optimization of the system for *C. glutamicum*. For one the system we constructed was based on the IPTG inducible *tac* promoter, this system is known to be leaky [46]. We also used a propionate inducible promoter in an attempt to reduce basal expression of *dcas9*, but this system was also too leaky (unpublished results). For some applications it might be necessary to have a completely tight system; to achieve this other inducible systems such as the tetracycline inducible system could be used. Another option is to put the sgRNA under the control of an inducible promoter to reduce sgRNA/dCas9 repression under uninduced conditions.

The system can also be developed to activate transcription of genes and could thereby be used to increase transcription of weakly expressed genes. This was achieved by fusing dCas9 with the omega subunit of RNA polymerase in *E. coli*, that had a deletion of *rpoZ* encoding the omega subunit [47]. With the help of the sgRNA the dCas9-ω fusion protein is guided to the promoter region, where the ω subunit recruits the RNA polymerase by interacting with the β' subunit [47]. It would be even more useful to develop the CRISPR/Cas9 system for genome editing in *C. glutamicum*. This was already accomplished in *E. coli* and *Streptococcus pneumonia* where the introduction of marker-free mutations was demonstrated [48]. This was achieved by introducing a system targeting a locus in the genome along with a template to recombine into the targeted locus. We already constructed a plasmid capable of replicating in *C. glutamicum* with *cas9* under the control of Ptac. Other than Cas9 and sgRNA, an editing template is required. Two challenges of the system are the potential low efficiency of editing the target of choice, as it is dependent on a rare homologous recombination event to take place and to select against non-edited cells. A more general limitation of the system is the need of a protospacer-adjacent motive (PAM) an NGG sequence required for the sgRNA to recognize the target sequence [45]. The sgRNA is designed based on where a PAM can be found, if there is no PAM in the promoter sequence or in the 5' region of the gene, then the maximum achievable repression might not be as high as desired. Nonetheless research within the use of the CRISPR/(d)Cas9 system is progressing rapidly, advancing the technique and so its current limitations may soon belong to the past.

The CRISPR/dCas9 system would be a powerful tool for further engineering of the ornithine producing platform strain. For instance the simultaneous perturbation of expression of the genes encoding the enzymes catalyzing the first step of proline and glutamine biosynthesis that both compete with the arginine pathway for glutamate could be tested for increased titers of ornithine, proline, and putrescine. This could be even further extended to construct a plasmid with sgRNAs targeting the genes *proB, aceA, pck, odhA* and *pgi*. Where the perturbation of *aceA*, *pck*, and *odhA* should direct the flux into the arginine pathway.

7.4 Final remarks

These times are truly exciting for metabolic engineers. Not only can we use well established tools such as metabolic control analysis, flux balance analysis, 13C metabolic flux analysis, RNA sequencing (transcriptomics), metabolomics, proteomics, but newer exciting tools have also been developed. The CRISPR/Cas9 system has made precise genetic modifications possible in a range of organisms, including mammalian cells. This system has now been established for the use in *C. glutamicum* and has the potential to significantly reduce the strain construction process. The concept of constructing a platform strain for the production of a range of bioproducts is not new, but like the CRISPR/dCas9 system, it can shorten the strain construction process as the different products have a common precursor.

A challenge in the development of a platform strain for the production of the glutamate derivatives is the very different co-factor, substrate, and energy requirements for synthesis of ornithine, proline, and putrescine versus synthesis of arginine and citrulline. This makes the engineering of these compounds more challenging and requires even further tinkering of the metabolism to achieve high yields of the individual compounds.

7.5 References

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Appendix

Supplementary material – chapter 5

Table S1. Plasmids used and constructed in this study.

Plasmids	Reference
pK19mobs*acB*	[1]
pK19mobs*acBΔargFR*	[2]
pK19mobs*acBΔargG*	This study
pK19mobs*acBΔargCJBD*	This study
pK18mobs*acBΔargB*	[3]
pK18mobs*acBΔargJ*	[3]
pK19mobs*acBΔargD*	This study
pK19mobs*acBΔargD2*	This study
pK19mobs*acBΔgabT*	This study
pK19mobs*acBΔbioA*	This study
pK19mobs*acB*-P_{gdh}	This study
pK19mobs*acB*-P_{gdh1}	This study
pK19mobs*acB*-P_{gdh2}	This study
pK19mobs*acB*-P_{gdh3}	This study
pK19mobs*acB*-P_{gdh4}	This study
pK19mobs*acB*-pgi^{GTG}	Provided by Prof. Dr. Bernhard Eikmanns
pK19mobs*acB*-$argB^{A49V,M54V}$	This study
pK19mobs*acB*-CGP1.1-P_{tuf}-$argCJB^{A49V,M54V}D$-*rrnB*T1T2-CGP1.2	This study
pK19mobs*acB*-CGP1.1-P_{tuf}-$argB^{A49V,M54V}JCD$-*rrnB*T1T2-CGP1.2	This study
pEPR1	[4]
pEPR1-P_{tuf}	This study
pEPR1-P_{sod}	This study
pEPR1-P_{argC}	This study
pEKEx3	[5]
pEKEx3-*argB*	This study
pEKEx3-$argB^{\Delta 1\text{-}23}$	[2]
pEKEX3-$argB^{E19R}$	This study
pEKEX3-$argB^{H26E}$	This study
pEKEX3-$argB^{H268N}$	This study
pEKEX3-$argB^{G287D}$	This study
pEKEX3-$argB^{A49V,M54V}$	[2]
pEKEX3-$argB^{E19R,H26E,A49V,M54V}$	This study
pEKEX3-$argB^{A49V,M54V,H268N}$	This study
pEKEX3-$argB^{A49V,M54V,G287D}$	This study
pEKEX3-$argB^{E19R,H26E,H268N}$	This study
pEKEX3-$argB^{E19R,H26E,A49V,M54V,H268N}$	This study
pEKEX3-$argB^{E19R,H26E,A49V,M54V,H268N,G287D}$	This study
pEKEX3$argB_{E.coli}$	[2]
pEKEx3-*argD*	This study
pEKEx3-$argD2_{LA}$	This study
pEKEx3-$argD2_{LT}$	This study
pEKEx3-$argD2_{MA}$	This study
pEKEx3-$argD2_{MT}$	This study
pEKEx3-$argJ_{Cg}$	This study
pEKEx3-$argJ_{Cc}$	This study
pVWEx1	[6]
pVWEx1-$argFB^{A49V,M54V}$	This study

pVWEx1-*argGFB*^{A49V,M54V}	This study
pVWEx1-ocd_{Pp}	[7]
pVWEx1-$speC_{Ec}$	[8]

Table S2. Primers used for the construction of plasmids. Bases in italics denote restriction recognition sites, bases in lower case denote overlapping regions, bases in bold denote mutations.

#	Primer	Primer sequence 5'->3'	Purpose
1	*argG*-up-fw	CTT*gaattc*AGAAGCTGCGCCGCATG	Deletion of *argG*
2	*argG*-up-rv	agagacgacctaagccagtctAACGATGCGGTTAGTCATGAGG	Deletion of *argG*
3	*argG*-dw-fw	agactggcttaggtcgtctctGCTAACAAGCGCGATCGC	Deletion of *argG*
4	*argG*-dw-rv	CCT*ctgcag*AACGACCAGCGCGCAGA	Deletion of *argG*
5	*argCJBD*-up-fw	CTT*gaattc*ACTTCCAGCTCCTCCAGCAG	Deletion of *argCJBD*
6	*argCJBD* -up-rv	agagacgacctaagccagtctGCAAATTATTCATGCATAAATTTTTG	Deletion of *argCJBD*
7	*argD*-up-fw	CTT*gaattc*CATTAACGCCGATACCGCA	Deletion of *argD*
8	*argD*-up-rv	agactggcttaggtcgtctctAGTTTCCAGCGTGCTCATTTAC	Deletion of *argD*
9	*argD*-dw-fw	agactggcttaggtcgtctctGACGCAGTCAAGGCTATTGCC	Deletion of *argCJBD* and *argD*
10	*argD*-dw-rv	CCT*ggatcc*AGTCTGGATGCTTGAAAAGGTGG	Deletion of *argCJBD* and *argD*
11	*argD2*-up-fw	CTT*gaattc*TGGATGCACAACCCAGATGT	Deletion of *argD2*
12	*argD2*-up-rv	agagacgacctaagccagtctGTAACCCTTCAATGCCAAACCA	Deletion of *argD2*
13	*argD2*-dw-fw	agactggcttaggtcgtctctGCTGTCGAGCTGACCTTC	Deletion of *argD2*
14	*argD2*-dw-rv	CCT*ctgcag*CATGCTGGCCAAATGGTTGT	Deletion of *argD2*
15	*gabT*-up-fw	CTT*gaattc*CGATCGCGATGACCCCTG	Deletion of *gabT*
16	*gabT*-up-rv	agagacgacctaagccagtctGTATGAGAGATCTTCCACGGTTCC	Deletion of *gabT*
17	*gabT*-dw-fw	agactggcttaggtcgtctctCTAGAGCGCGAAACCGC	Deletion of *gabT*
18	*gabT*-dw-rv	CCT*ggatcc*CCATGGGGTGATCGCCAG	Deletion of *gabT*
19	*bioA*-up-fw	CTT*gaattc*TGGTTACTGAGATCGCACCG	Deletion of *bioA*
20	*bioA*-up-rv	agagacgacctaagccagtctCAAGCTGGGGTTTTCCATTTTC	Deletion of *bioA*
21	*bioA*-dw-fw	agactggcttaggtcgtctctTGCACTGCGCTTCATGCTG	Deletion of *bioA*
22	*bioA*-dw-rv	CCT*ggatcc*GCATTCAAAGCGGAGGCAAC	Deletion of *bioA*
23	P_{gdh}-fw	CTT*gaattc*GTCGGTGGGGGAGTTGGA	Amplification of *gdh* promoter

			region
24	P_{gdh}-rv	CCT*ggatcc*CAAGGCACACGGAAGATGAG	Amplification of *gdh* promoter region
25	P_{gdh1}-fw	ACACTGC**T**ATAATTGAACGTGAGCATTTACCA	SDM of *gdh* promoter region
26	P_{gdh1}-rv	CAATTATAGCAGTGTCGCACAGATATGACCA	SDM of *gdh* promoter region
27	P_{gdh2}-fw	CGAC**TG**TGC**T**ATAAT**G**GAACGTGAGCATTTACCA	SDM of *gdh* promoter region
28	P_{gdh2}-rv	CCATTATAGCACAGTCGCACAGATATGACCACAA	SDM of *gdh* promoter region
29	P_{gdh3}-rv	CAATTATAGCAGTGTCGCACAGATATG**T**C**A**AC	SDM of *gdh* promoter region
30	P_{gdh4}-rv	CAATTATAGCAGTGTCGCACAGATATG**G**C**A**AC	SDM of *gdh* promoter region
31	*argB**-fw	ATGAATGACTTGATCAAAGATTTAGGC	Amplification of $argB^{A49V,M54V}$
32	*argB**-rv	TTACAGTTCCCCATCCTTGT	Amplification of $argB^{A49V,M54V}$
33	*argCJBD*-fw	CTT*catatg*GAAAGGAGGCCCTTCAGATGACAATCAAGGTTGCAATCG	Construction of $argCJB^{A49V,M54V}D$ operon
34	*argCJBD*-rv	CCT*catatg*TTATGCGATTGTCTCGGCAATAG	Construction of $argCJB^{A49V,M54V}D$ operon
35	CGP1.1-fw	ACGCCAAGCTTGCATGCCCAGTGAAGGATCGGTGCG	Construction of $argB^{A49V,M54V}JCD$ operon
36	CGP1.1-rv	GTAACGGCCACCTATCTGCTGGCCGGTG	Construction of $argB^{A49V,M54V}JCD$ operon
37	P_{tuf}-fw	AGCAGATAGGTGGCCGTTACCCTGCGAATG	Construction of $argB^{A49V,M54V}JCD$ operon
38	P_{tuf}-rv	c*acgcgt*agagacgacctaagccagtct*catatg*cTGTATGTCCTCCTGGACTTC	Construction of $argB^{A49V,M54V}JCD$ operon
39	*argB*-fw	CCAGGAGGACATACAGCAGAAAGGAGGCCCTTCAGATGAATGACTTGATCAAAGATTTAGGC	Construction of $argB^{A49V,M54V}JCD$ operon
40	*argB*-rv	GCCTTTTTCTGCCATCTGAAGGGCCTCCTTTCTTACAGTTCCCCATCCTTGT	Construction

			of *argB*^{A49V,M54V}*JCD* operon
41	*argJ*-fw	GATGGGGAACTGTAAGAAAGGAGGCCCTTCAGATGGCAGAAAAAGGCATTACC	Construction of *argB*^{A49V,M54V}*JCD* operon
42	*argJ*-rv	AACCTTGATTGTCATCTGAAGGGCCTCCTTTCTTAAGAGCTGTACGCGGA	Construction of *argB*^{A49V,M54V}*JCD* operon
43	*argC*-fw	GCGTACAGCTCTTAAGAAAGGAGGCCCTTCAGATGACAATCAAGGTTGCAATCG	Construction of *argB*^{A49V,M54V}*JCD* operon
44	*argC*-rv	TTCCAGCGTGCTCATCTGAAGGGCCTCCTTTCTTAAGGTGCGACGCCGAC	Construction of *argB*^{A49V,M54V}*JCD* operon
45	*argD*-fw	GGCGTCGCACCTTAAGAAAGGAGGCCCTTCAGATGAGCACGCTGGAAACTTGG	Construction of *argB*^{A49V,M54V}*JCD* operon
46	*argD*-rv	TCTCATCCGCCAAAAcACGCGTTATGCGATTGTCTCGGCAATAG	Construction of *argB*^{A49V,M54V}*JCD* operon
47	*rrnB*T1T2-fw	g*catatg*agactggcttaggtcgtctct*acgcgt*gTTTTGGCGGATGAGAGAAG	Construction of *argB*^{A49V,M54V}*JCD* operon
48	*rrnB*T1T2-rv	CTAGGTACAGCAAAAGAGTTTGTAGAAACGCA	Construction of *argB*^{A49V,M54V}*JCD* operon
49	CGP1.2-fw	AACTCTTTTGCTGTACCTAGCGCAAGTAGTAAGA	Construction of *argB*^{A49V,M54V}*JCD* operon
50	CGP1.2-rv	AACGACGGCCAGTGAATTCTGCTCATCCTTCAACAAC	Construction of *argB*^{A49V,M54V}*JCD* operon
51	*argB*-fw	GAAAGGAGGCCCTTCAGATGAATGACTTGATCAAAGATTTAGGC	Amplification of *argB*
52	E19R-rv	GCT**CG**GGCGTTGCCATGGTTGCAGCACTTCCGCG	SDM of *argB*
53	H26E-fw	TGGCAACGCCTCAGCGAGGACATTTGCGCG	SDM of *argB*
54	H26E-rv	GCTGAGGCGTTGCCATGGTTGCAG**G**A**A**TTCCGCG	SDM of *argB*
55	H268N-RV	CAATGACAT**T**AGCAGCGCTTACTCCCCCACG	SDM of *argB*
56	H268N-FW	GCTGCT**A**ATGTCATTGACGGCCGCATCGC	SDM of *argB*
57	G287D-fw	CATGGGTG**AT**ATTGGCACGATGGTGCTG	SDM of *argB*
58	G287D-rv	CAATATCACCCATGGTCAAAAGCTCCAGC	SDM of *argB*
59	E19R-H26E-fw	GCT**CG**GGCGTTGCCATGGTTGCAG**G**A**A**TTCCGCG	SDM of *argB*

60	E19R-H26E-rv	TGGCAACGCC**CG**AGCGAGGACATTTGCGCG	SDM of *argB*
61	*argD*-fw	CTT*gtcgac*AAGGAGATATAGATATGAGCACGCTGGAAACTTGG	Amplification of *argD*
62	*argD*-rv	CCT*gaattc*TTATGCGATTGTCTCGGCAATAG	Amplification of *argD*
63	$argD2_{LA}$-fw	CTT*gtcgac*AAGGAGATATAGATTTGGCATTGAAGGGTTACACCA	Amplification of *argD2*
64	$argD2_{LT}$-fw	CTT*gtcgac*AAGGAGATATAGATTTGACCTTGAAGGGTTACACCA	Amplification of *argD2*
65	$argD2_{MA}$-fw	CTT*gtcgac*AAGGAGATATAGATATGGCATTGAAGGGTTACACCA	Amplification of *argD2*
66	$argD2_{MT}$-fw	CTT*gtcgac*AAGGAGATATAGATATGACCTTGAAGGGTTACACCA	Amplification of *argD2*
67	*argD2*-rv	CCT*cccggg*TTAGAACAACGCCCCAGCG	Amplification of *argD2*
68	$argJ_{Cg}$-fw	CG*ggatcc*GAAAGGAGGCCCTTCAGATGGCAGAAAAAGGCATTACCG	Amplification of *argJ*
69	$argJ_{Cg}$-rv	GG*gaattc*TTAAGTGCTGTACGCGGAGTTG	Amplification of *argJ*
70	$argJ_{Cc}$-rv	GG*gaattc*TTAAGAGCTGTACGCGGAGTTG	Amplification of *argJ*
71 a	$argJ_{Cc}$-mut-rev	TCAAGGCATACTGCTTTATCATTGAAGAAC	Introduction of internal mutation
71 b	$argJ_{Cc}$-mut-for	GTTCTTCAATGATAAAGCAGTATGCCTTGA	Introduction of internal mutation
72	*argF*-fw	CTT*gtcgac*AAGGAGATATAGATATGACTTCACAACCACAGGTTCG	Amplification of *argF*
73	*argF*-rv	CCT*ggatcc*TTACCTCGGCTGGTTGGC	Amplification of *argF*
74	*argG*-fw	GGG*gtcgac*GAAAGGAGGCCCTTCAGATGACTAACCGCATCGTTCTTG	Amplification of *argG*
75	*argG*-rv	GGG*gtcgac*TTAGTTGTTGCCAGCTTCGCGA	Amplification of *argG*
76	P_{tuf}-fw	TGGCCGTTACCCTGCGAATG	Amplification of P_{tuf}
77	P_{tuf}-rv	TGTATGTCCTCCTGGACTTC	Amplification of P_{tuf}
78	P_{sod}-fw	TAGCTGCCAATTATTCCGGG	Amplification of P_{sod}
79	P_{sod}-rv	GGGTAAAAAATCCTTTCGTAGGTT	Amplification of P_{sod}
80	P_{argC}-fw	AAATTCATGCTTTTACCCACTTG	Amplification of P_{argC}
81	P_{argC}-rv	AGTTACACCATACACGTTATGCATG	Amplification of P_{argC}

Construction of plasmids used in the study

pK19mob*sacB*Δ*argG* was constructed by amplifying up- and downstream regions of *argG* with primers 1 and 2, and 3 and 4, respectively. The two resulting fragments were combined by crossover PCR using primers 1 and 4. The final fragment was treated with PstI and EcoRI and ligated with similarly treated pK19mob*sacB*.

pK19mob*sacB*Δ*argCJBD* was constructed by amplifying up- and downstream regions of *argCJBD* with primers 5 and 6, and 9 and 10, respectively. The two resulting fragments were combined by crossover PCR using primers 5 and 10. The final fragment was treated with BamHI and EcoRI and ligated with similarly treated pK19mob*sacB*.

pK19mob*sacB*Δ*argD* was constructed by amplifying up- and downstream regions of *argCJBD* with primers 7 and 8, and 9 and 10, respectively. The two resulting fragments were combined by crossover PCR using primers 7 and 10. The final fragment was treated with BamHI and EcoRI and ligated with similarly treated pK19mob*sacB*.

pK19mob*sacB*Δ*argD2* was constructed by amplifying up- and downstream regions of *argD2* with primers 11 and 12, and 13 and 14, respectively. The two resulting fragments were combined by crossover PCR using primers 11 and 14. The final fragment was treated with PstI and EcoRI and ligated with similarly treated pK19mob*sacB*.

pK19mob*sacB*Δ*gabT* was constructed by amplifying up- and downstream regions of *gabT* with primers 15 and 16, and 17 and 18, respectively. The two resulting fragments were combined by crossover PCR using primers 15 and 18. The final fragment was treated with BamHI and EcoRI and ligated with similarly treated pK19mob*sacB*.

pK19mob*sacB*Δ*bioA* was constructed by amplifying up- and downstream regions of *bioA* with primers 19 and 20, and 21 and 22, respectively. The two resulting fragments were combined by crossover PCR using primers 19 and 22. The final fragment was treated with BamHI and EcoRI and ligated with similarly treated pK19mob*sacB*.

pK19mob*sacB*-P$_{gdh}$ was constructed by amplifying the upstream region of *gdh* with primers 23 and 24. The final fragment was treated with BamHI and EcoRI and ligated with similarly treated pK19mob*sacB*.

pK19mob*sacB*-P$_{gdh1}$ was constructed by site-directed mutagenesis (SDM) using primers 25 and 26 with pK19mob*sacB*-P_{gdh}.

pK19mob*sacB*-P$_{gdh2}$ was constructed by site-directed mutagenesis (SDM) using primers 27 and 28 with pK19mob*sacB*-P_{gdh}.

pK19mob*sacB*-P$_{gdh3}$ was constructed by site-directed mutagenesis (SDM) using primers 25 and 29 with pK19mob*sacB*-P_{gdh}.

pK19mob*sacB*-P$_{gdh4}$ was constructed by site-directed mutagenesis (SDM) using primers 25 and 30 with pK19mob*sacB*-P_{gdh}.

pK19mob*sacB*-*argB*^{A49V,M54V} was constructed by amplifying *argB*^{A49V,M54V} from pEKEx3-*argB*^{A49V,M54V} using primers 31 and 32. The final fragment was phosphorylated and ligated with SmaI digested pK19mob*sacB*.

pK19mob*sacB*-CGP1.1-P$_{tuf}$-*argCJB*^{A49V,M54V}*D-rrnB*T1T2-CGP1.2: the CGP1.1 upstream region was amplified from genomic *C. glutamicum* MB001 DNA with primers 35 and 36 , P_{tuf} was amplified from genomic *C. glutamicum* ATCC 13032 DNA with primers 37 and 38, *rrnB*T1T2 was amplified from pEKEx3 with primers 47 and 48, and the CGP1.2 upstream region was

amplified from genomic *C. glutamicum* MB001 DNA with primers 49 and 50. pK19mob*sacB* was treated with PstI and EcoRI and pK19mob*sacB*-CGP1.1-P_{tuf}-*rrnB*T1T2-CGP1.2 was assembled by the Gibson assembly method. *argCJB*^{A49V,M54V}*D* was amplified from genomic ORN3 DNA with primers 33 and 34. The resulting fragment was digested with NdeI and ligated with similarly treated pK19mob*sacB*-CGP1.1-P_{tuf}-*rrnB*T1T2-CGP1.2.

pK19mob*sacB*-CGP1.1-P_{tuf}-*argB*^{A49V,M54V}*JCD*-*rrnB*T1T2-CGP1.2: *argB*^{A49V,M54V} was amplified from pEKEx3-*argB*^{A49V,M54V} with primers 39 and 40, *argJ* was amplified from genomic *C. glutamicum* ATCC 13032 DNA with primers 41 and 42, *argC* was amplified from genomic *C. glutamicum* ATCC 13032 DNA with primers 43 and 44, and *argD* was amplified from genomic *C. glutamicum* ATCC 13032 DNA with primers 45 and 46. pK19mob*sacB*-CGP1.1-P_{tuf}-*rrnB*T1T2-CGP1.2 was treated with MluI and NdeI and pK19mob*sacB*-CGP1.1-P_{tuf}-*argB*^{A49V,M54V}*JCD*-*rrnB*T1T2-CGP1.2 was constructed by the Gibson assembly method.

pEPR1-P_{tuf} was constructed by amplifying P_{tuf} with primers 76 and 77, phosphorylating the resulting PCR product and ligating it with SmaI digested pEPR1.

pEPR1-P_{sod} was constructed by amplifying P_{sod} with primers 78 and 79, phosphorylating the resulting PCR product and ligating it with SmaI digested pEPR1.

pEPR1-P_{argC} was constructed by amplifying P_{argC} with primers 80 and 81, phosphorylating the resulting PCR product and ligating it with SmaI digested pEPR1.

pEKEx3-*argB* was constructed by amplifying *argB* with primers 51 and 32, phosphorylating the resulting PCR product and ligating it with SmaI digested pEKEx3.

pEKEX3-*argB*^{E19R} was constructed by site-directed mutagenesis (SDM) using primers 52 and 60 with pEKEx3-*argB*.

pEKEX3-*argB*^{H26E} was constructed by site-directed mutagenesis (SDM) using primers 53 and 54 with pEKEx3-*argB*.

pEKEX3-*argB*^{H268N} was constructed by site-directed mutagenesis (SDM) using primers 55 and 56 with pEKEx3-*argB*.

pEKEX3-*argB*^{G287D} was constructed by site-directed mutagenesis (SDM) using primers 57 and 58 with pEKEx3-*argB*.

pEKEX3-*argB*^{E19R,H26E,A49V,M54V} was constructed by site-directed mutagenesis (SDM) using primers 59 and 60 with pEKEx3-*argB*^{A49V,M54V}.

pEKEX3-*argB*^{A49V,M54V,H268N} was constructed by site-directed mutagenesis (SDM) using primers 55 and 56 with pEKEx3-*argB*^{A49V,M54V}.

pEKEX3-*argB*^{A49V,M54V,G287D} was constructed by site-directed mutagenesis (SDM) using primers 57 and 58 with pEKEx3-*argB*^{A49V,M54V}.

pEKEX3-*argB*^{E19R,H26E,H268N} was constructed by site-directed mutagenesis (SDM) using primers 55, 56, 59, and 60 with pEKEx3-*argB*.

pEKEX3-*argB*^{E19R,H26E,A49V,M54V,H268N} was constructed by site-directed mutagenesis (SDM) using primers 55, 56, 59, and 60 with pEKEx3-*argB*^{A49V,M54V}.

pEKEX3-*argB*^{E19R,H26E,A49V,M54V,H268N,G287D} was constructed by site-directed mutagenesis (SDM) using primers 57 and 58 with pEKEX3-*argB*^{E19R,H26E,A49V,M54V,H268N}.

pEKEx3-*argD* was constructed by amplifying *argD* with primers 61 and 62. The PCR product was treated with SalI and EcoRI and ligated with similarly treated pEKEx3.

pEKEx3-*argD2*$_{LA}$ was constructed by amplifying *argD2* with primers 63 and 67. The PCR product was treated with SalI and XmaI and ligated with similarly treated pEKEx3.

pEKEx3-*argD2*$_{LT}$ was constructed by amplifying *argD2* with primers 64 and 67. The PCR product was treated with SalI and XmaI and ligated with similarly treated pEKEx3.

pEKEx3-*argD2*$_{MA}$ was constructed by amplifying *argD2* with primers 65 and 67. The PCR product was treated with SalI and XmaI and ligated with similarly treated pEKEx3.

pEKEx3-*argD2*$_{MT}$ was constructed by amplifying *argD2* with primers 66 and 67. The PCR product was treated with SalI and XmaI and ligated with similarly treated pEKEx3.

pVWEx1-*argF* was constructed by amplifying *argF* with primers 72 and 73. The PCR product was treated with SalI and BamHI and ligated with similarly treated pVWEx1.

pVWEx1-*argFB*^{A49V,M54V} was constructed by purification of BamHI and KpnI digested argB^{A49V,M54V} from pEKEx3-argB^{A49V,M54V} and ligated with similarly treated pVWEx1-*argF*.

pVWEx1-*argFGB*^{A49V,M54V} was constructed by amplifying *argG* with primers 74 and 75. The PCR product was treated with SalI and ligated with similarly treated pVWEx1-*argFB*^{A49V,M54V}.

pEKEx3-*argJ*$_{cg}$ was constructed by amplifying *argJ* with primers 68 and 69. The PCR product was treated with BamHI and EcoRI and ligated with similarly treated pEKEx3.

pEKEx3-*argJ*$_{cc}$ was constructed by introducing N-terminal and internal mutations into *argJ* from *C. glutamicum* by using primers 68 and 71a as well as 71b and 70. The Fragments were linked by crossover PCR by using primers 68 and 70, treated with BamHI and EcoRI and ligated with similarly treated pEKEx3.

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