Metabolic engineering of *Corynebacterium glutamicum* for the production of L-aspartate and its derivatives β-alanine and ectoine

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Abbreviations

Δ	Deletion
aa	Amino acids
ABC	ATP binding cassette
ADP	Adenosin diphosphate
Amp^R	Ampicillin resistance
ATP	Adenosin triphosphate
BCAA	Branched chain amino acids
BHI	Brain heart infusion
BHI-MOPS	Brain heart infusion, buffered with 100 mM MOPS pH=7
CDW	Cell dry weight
CGXII	Minimal medium
CoA	Coenzyme A
DTNB	5,5-dithiobis (2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
Kan^R	Kanamycin resistance
LB	Lysogeny broth complex media
mCGXII	Modified CGXII minimal medium
MOPS	3-(N-morpholino)propanesulfonic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PDHC	Pyruvate dehydrogenase complex
PEP	Phosphoenolpyruvate
P_i	Inorganic phosphate
PLP	Pyridoxal phosphate
PPP	Pentose phosphate pathway
PTS	Phosphotransferase system
$Spec^R$	Spectinomycin resistance
TAE	Tris-acetate EDTA buffer
TAPS	N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid
TCA	Tricarboxylic acid
TNB	2-nitro-5-thiobenzoeic acid
Tris	tris(hydroxymethyl)aminomethane
TSS	Transcriptional start point
U	Units (μmol/min*mg^{-1})

Summary

In living cells, aspartate is a central metabolite and the precursor for amino acids, cell wall components and the vitamin pantothenate. On the industrial scale, aspartate finds wide application in the artificial sweetener aspartame, but the chemical properties of aspartate and its derivatives allow further applications in the polymer, solvent, and the pharmaceutical market. Since decades, aspartate is synthesized by the enzymatic conversion of petrochemically synthesized fumarate, and is therefore directly dependent on limited fossil raw materials. *Corynebacterium glutamicum* is a well known as producer of amino acids and organic acids. It was engineered in this work for the overproduction of aspartate and its derivatives β-alanine and ectoine from renewable carbon sources.

To enable the overproduction of aspartate, a growth decoupled process was developed, which was modified focusing on enhancing the precursor supply, improving the transamination of aspartate precursors, the reduction of by-products, and increasing the productivity. *C. glutamicum* is not able to proliferate under anaerobic conditions and excretes mainly succinate, lactate, and acetate as fermentation products. The deletion of the succinate dehydrogenase and the lactate dehydrogenase genes led to the strain Δ*sdhCAB* Δ*ldhA*, which produces aspartate with a yield of 0.55 mol/mol glucose. The addition of sodium bicarbonate to the medium enhanced the precursor supply and increased the aspartate titer to 0.83 mol/mol glucose. Furthermore, the overproduction was enhanced by the expression of the aspartate aminotransferase AspB, which transaminates oxaloacetate to aspartate. The alanine-valine transaminase gene was deleted to reduce the by-product alanine, which improved the aspartate yield to 1.02 mol/mol glucose. The velocity of the process was increased 1.5-fold by the overexpression of the glycolytic glyceraldehyde dehydrogenase GapA. With the substrate maltose, the highest aspartate yield of 0.80 g/g and productivity of 0.176 mM/h*gCDW were achieved.

The non-proteinogenic amino acid β-alanine is derived from aspartate by decarboxylation by the aspartate-α-decarboxylase PanD. The decarboxylases of *Bacillus subtilis*, *Escherichia coli*, and the endogenous enzyme were tested in the aspartate producing strain *C. glutamicum* Δ*sdhCAB* Δ*ldhA* and enabled the overproduction of β-alanine with a final titer of 0.24 g/g glucose.

In contrast to the anaerobic production of aspartate and β-alanine, the biosynthesis of ectoine was achieved aerobically by the heterologous expression of the *ectABC* gene cluster of *Chromohalobacter salexigens*. Efficient overproduction of ectoine was already achieved with the wild type, and was further enhanced by introducing the ectoine biosynthetic genes into the lysine producing *C. glutamicum* DM1729. This strain produces ectoine with a yield of 0.146 g/g glucose, and was further modified to synthesize ectoine from the non-food carbon sources xylose, arabinose, and glycerol.

Zusammenfassung

Die Aminosäure Aspartat nimmt im Stoffwechsel von Organismen eine zentrale Rolle ein und dient unter anderem als Vorläufermolekül für Aminosäuren, Zellwandbausteine und dem Vitamin Pantothensäure. Im industriellen Maßstab wird Aspartat hauptsächlich zur Synthese des Süßstoffs Aspartam genutzt, allerdings erlauben die strukturellen Eigenschaften der Aminosäure und ihrer Derivate auch Anwendungen in der Polymersynthese und im kosmetischen Bereich. Heutzutage wird Aspartat durch die enzymatische Umsetzung von Fumarat erzeugt, das wiederum durch chemische Synthese aus fossilen Rohstoffen erzeugt wird. In dieser Arbeit wurde die Produktion von Aspartat und dessen Derivate β-Alanin und Ectoin auf Basis von nachwachsenden Rohstoffen mit dem Amniosäureproduzenten *Corynebacterium glutamicum* untersucht.

Um die Produktion von Aspartat zu ermöglichen, wurde ein vom Wachstum entkoppelter Prozess entwickelt, der mit Hinblick auf die Bereitstellung von Vorläufermolekülen, deren verbesserte Aminierung, der Reduktion von Nebenprodukten und der Erhöhung der Produktivität modifiziert wurde. Unter anaeroben Bedingungen findet in *C. glutamicum* keine Zellteilung statt und der Organismus wechselt zu einem fermentativen Metabolismus, der durch die Exkretion von Succinat, Laktat und Acetat gekennzeichnet ist. Die Deletion der Gene der Succinatdehydrogenase (*sdhCAB*) und die Laktatdehydrogenase (*ldhA*) führte zum Stamm Δ*sdhCAB* Δ*ldhA*, der Aspartat mit einem Ertrag von 0.55 mol/mol Glukose exkretiert. Durch die Zugabe von Natriumhydrogencarbonat konnte die Anaplerose verbessert, und der Ertrag auf 0.83 mol/mol gesteigert werden. Des Weiteren wirkte sich die Expression des Gens der Aspartat Aminotransferase AspB positiv auf die Ausbeute aus. Um die Bildung des Nebenprodukts Alanin zu verringern, wurde das Alanin-Valin Aminotransferase Gen deletiert, was den Ertrag auf 1.02 mol/mol Glukose erhöhte. Die Produktivität der Aspartatfermentation konnte durch die Expression der glykolytischen Glycerin-3-Phosphat-dehydrogenase um das 1,5-fache gesteigert werden. Zudem führte die Verwendung des Disaccharids Maltose zur höchsten Ausbeute von 0.80 g/g und höchsten Produktivität von 0.176 mM/h*gCDW.

Die nicht-proteinogene Aminosäure β-Alanin wird durch Decarboxylierung von Aspartat durch die Aspartat-α-Decarboxylase PanD gewonnen. Die Decarboxylasen aus *Bacillus subtilis*, *Escherichia coli* und das endogene Enzym wurden in den Aspartat-produzierenden Stamm Δ*sdhCAB* Δ*ldhA* eingebracht, und ermöglichten die Produktion von β-Alanin mit einem Ertrag von 0.24 g/g Glukose.

Durch die heterologe Expression der *ectABC* Gene aus *Chromohalobacter salexigens* konnte bereits im Wildtypstamm eine signifikante Menge des kompatiblen Soluts unter aeroben Bedingungen erzeugt werden. Dieser Ertrag konnte im Lysin-prozierenden Stamm *C. glutamicum* DM1729 auf 0,146 g/g gesteigert werden, der im weiteren Verlauf für die Verstoffwechslung der *non-food* Kohlenstoffquellen Xylose, Arabinose und Glycerin modifiziert wurde.

1 Introduction

1.1 Characteristics of *Corynebacterium glutamicum* and its application in biotechnology

Corynebacterium glutamicum was isolated in 1957 from soil samples during a screening for a natural glutamate excreting organism. The Gram-positive, immobile, non-sporulating bacterium is named after the typical club-shaped form and undergoes snapping division. It is facultative anaerobic, has a high G + C content and is generally regarded as safe (GRAS).

Together with *Streptomycetales*, *Micrococcales* and *Actinomycetales*, the order *Corynebacteriales* belongs to the class *Actinobacteria* which contains more than 200 genera (Gao and Gupta, 2012; Zhi *et al.*, 2009). The order *Corynebacteriales* comprises six families which are characterized by the presence of mycolic acids in the cell wall. Only a few exceptions like *Corynebacterium amycolatum*, *C. atypicum* and *C. kroppenstedtii* lack this characteristic (Bernard and Goodfellow, 2012). Several organisms of the *Corynebacteriales* have been sequenced due to their importance as human pathogens like *Corynebacterium diptheriae* and *Mycobacterium tuberculosis* or their role in the biotechnological industry like *C. glutamicum* or *C. efficiens* (Cerdeno-Tarraga *et al.*, 2003; Cole *et al.*, 1998; Kalinowski *et al.*, 2003; Nishio *et al.*, 2003).

C. glutamicum is widely used in white biotechnology and synthesizes 2.2 million tons of glutamate and 1.7 million tons of lysine per year (Ajinomoto, 2010, 2011). Next to these, also pathways for the synthesis of branched chain, aromatic and other amino acids are developed (Becker and Wittmann, 2012; Blombach *et al.*, 2008; Hasegawa *et al.*, 2012; Ikeda *et al.*, 2009; Kelle *et al.*, 1996; Peters-Wendisch *et al.*, 2005; Schneider *et al.*, 2011). The portfolio of biotechnological produced compounds by *C. glutamicum* is steadily increasing and not limited to amino acids (Zahoor *et al.*, 2012). Pathways were also optimized for the biofuels ethanol and isobutanol (Blombach *et al.*, 2011; Inui *et al.*, 2004a; Sakai *et al.*, 2007; Smith *et al.*, 2010), and the diamines putrescine and cadaverine (Buschke *et al.*, 2011; Mimitsuka *et al.*, 2007; Schneider *et al.*, 2012; Schneider and Wendisch, 2010). The organism naturally excretes succinic and lactic acid under anoxic conditions. This scaffolding was used to engineer *C. glutamicum* for production of succinate, both under aerobic and anaerobic conditions (Litsanov *et al.*, 2012a; Litsanov *et al.*, 2012c; Okino *et al.*, 2008a). Under oxygen deprivation conditions both stereoisomers of lactic acid can be synthesized (Okino *et al.*, 2005; Okino *et al.*, 2008b). Recently, efficient production of the central precursor pyruvate was shown and also carotenoids can be overproduced (Heider *et al.*, 2012; Wieschalka *et al.*, 2012). Furthermore, the global demand for these products increases annually. The amino acid glutamate is used as a flavor

enhancer in Asian food, while lysine, threonine, and valine are fed to livestock to enhance growth. Increasing prices of oil favor the synthesis of biofuels from renewable and sustainable resources. In addition, the aforementioned diamines are used in polyamide synthesis, while lactic acid is polymerized and used as packing material. Succinic acid is mentioned as a top value added chemical from carbohydrates and serves as building block for commodity and specialty chemicals (Werpy *et al.*, 2004). This wide range of producible molecules substantiates the importance of the organism for industrial biotechnology.

The value of the organism is based on a variety of benefits. Cultivation of *C. glutamicum* is performed at moderate temperatures and high cell densities are obtained even in shake flasks. Furthermore, the biomass and metabolic activity remains stable, if the organism is arrested in growth due to nutrient limitation of the medium or a switch to anoxic conditions (Blombach *et al.*, 2007; Inui *et al.*, 2004b). In fed-batch cultivations, this feature enables the utilization of carbon source for product synthesis, and minimizes investments in retaining biomass. The organism is able to grow on a variety of carbon sources and shows with a few exceptions no catabolite repression (Arndt and Eikmanns, 2007). The mixture of glucose with gluconeogenetic carbon sources like pyruvate, lactate, and acetate, as well as the mixture with other six carbon sugars like fructose leads to a monophasic growth with simultaneous consumption of the carbon sources (Cocaign *et al.*, 1993; Dominguez *et al.*, 1997; Wendisch *et al.*, 2000).

C. glutamicum is accessible to genetic manipulations by a large tool box, with vectors for heterologous expression, promoter probe vectors, and suicide plasmids that enable gene deletions or single base exchanges as well as stable introduction of new genetic material (Nesvera and Patek, 2011). The sequencing of the 3.28 Mb genome was performed independently by two research groups, and paved the way for further improvements of the strain (Ikeda and Nakagawa, 2003; Kalinowski *et al.*, 2003). Transcriptomic and proteomic data is used to describe the physiology of the organism, and the knowledge about regulation and genetic organization allows fine tuning of specific pathways.

1.2 The central metabolism of *C. glutamicum*

The central metabolism of *C. glutamicum* consists of glycolysis, the tricarboxylic acid cycle (TCA) and the pentose phosphate pathway (PPP) (Figure 1). This network generates energy and delivers precursors for several anabolic reactions. Energy in the form of the reduction equivalents NADH or menaquinone is used to establish a proton motif force at the cytoplasmatic membrane, while NADPH is used for assimilatory purposes, e. g. the reductive amination of 2-oxoglutarate to glutamate by the glutamate dehydrogenase Gdh (*gdh*, cg2280).

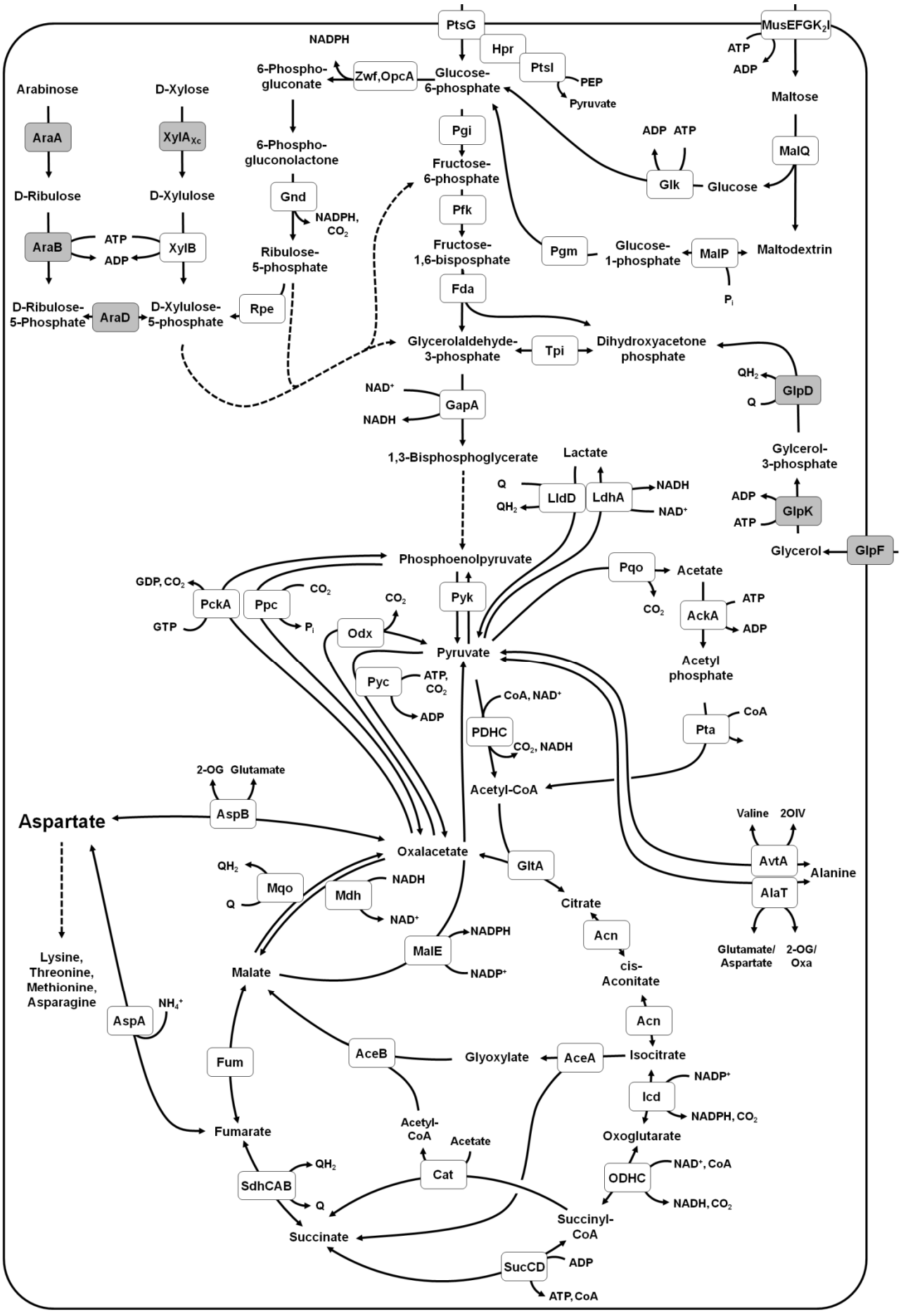
PtsG
Hpr
PtsI
PEP
Pyruvate
Glucose-6-phosphate
NADPH
6-Phospho-gluconate
Zwf,OpcA
6-Phospho-gluconolactone
Gnd
NADPH, CO2
Ribulose-5-phosphate
Rpe
D-Xylulose-5-phosphate
D-Ribulose-5-Phosphate
AraD
Arabinose
D-Xylose
AraA
XylA Xc
D-Ribulose
D-Xylulose
AraB
XylB
ATP
ADP
Pgi
Fructose-6-phosphate
Pfk
Fructose-1,6-bisphosphate
Fda
Glycerolaldehyde-3-phosphate
Tpi
Dihydroxyacetone phosphate
NAD+
NADH
GapA
1,3-Bisphosphoglycerate
Lactate
Q
QH2
LldD
LdhA
NADH
NAD+
Phosphoenolpyruvate
GDP, CO2
GTP
PckA
Ppc
CO2
Pi
Odx
Pyk
Pyruvate
Pyc
ATP, CO2
ADP
PDHC
CoA, NAD+
CO2, NADH
Acetyl-CoA
Pqo
Acetate
AckA
Acetyl phosphate
Pta
CoA
Oxalacetate
GltA
Citrate
Acn
cis-Aconitate
Isocitrate
Icd
NADP+
NADPH, CO2
Oxoglutarate
ODHC
NAD+, CoA
NADH, CO2
Succinyl-CoA
SucCD
ADP
ATP, CoA
Succinate
SdhCAB
Fumarate
Fum
Malate
MalE
NADPH
NADP+
Mqo
Mdh
AceB
Glyoxylate
AceA
Cat
Acetyl-CoA
Acetate
AspB
2-OG
Glutamate
Aspartate
AspA
NH4+
Lysine, Threonine, Methionine, Asparagine
AvtA
Valine
2OIV
Alanine
AlaT
Glutamate/ Aspartate
2-OG/ Oxa
MusEFGK2I
Maltose
MalQ
ADP ATP
Glk
Glucose
Pgm
Glucose-1-phosphate
MalP
Maltodextrin
Pi
GlpD
Gylcerol-3-phosphate
GlpK
Glycerol
GlpF

Figure 1: The central metabolism of *C. glutamicum*.
White boxes: native proteins of *C. glutamicum*; grey boxes: heterologous proteins to broaden substrate spectrum. Carbon source uptake and utilization: PtsG: PTS transporter glucose; Hpr, PtsI: unspecifc PTS components; MusEFGK2I: ABC-transporter for maltose uptake; MalQ: 4-α-glucanotransferase; MalP: maltodextrin phosphorylase; Pgm: α-phosphoglucomutase; Glk: glucokinase; AraA: L-arabinose isomerase; AraB: L-ribulokinase; AraD: L-ribulose-5-phosphate-4-epimerase; XylAXc: xylose isomerase; XylB: xylulose kinase; GlpF: glycerol facilitator; GlpK: glycerol kinase; GlpD: glycerol-3-phosphate dehydrogenase; Pentose phosphate pathway: Zwf, opcA: glucose-6-phosphate dehydrogenase; Gnd: 6-phosphogluconate dehydrogenase; Rpe: ribulose-5-phosphate epimerase; Glycolysis and subsequent reactions: Pgi: phosphoglucose isomerase; Pfk: phosphofructokinase; Fda: fructose-1,6-bisphosphate aldolase; GapA: glyceraldehyde-3-phosphate dehydrogenase; Pyk: pyruvate kinase; LdhA: lactate dehydrogenase (NAD-dependent); LldD: lactate dehydrogenase (quinone-dependent); Pqo: pyruvate-quinone oxidoreductase; AckA: acetate kinase; Pta: phosphotransacetylase; AlaT: alanine transaminase; AvtA: alanine valine transaminase; Anaplerosis: Ppc: PEP-carboxylase; PckA: PEP-carboxykinase; Pyc: pyruvate carboxylase; Odx: oxaloacetate decarboxylase; MalE: malic enzyme; TCA-cycle and subsequent reactions: PDHC: pyruvate dehydrogenase complex; GltA: citrate synthase; Acn: aconitase; Icd: isocitrate dehydrogenase; AceA: isocitrate lyase; AceB: malate synthase; ODHC: 2-oxoglutarate dehydrogenase complex; Cat: Succinyl-CoA:Acetate CoA transferase; SucCD: succinyl-CoA synthetase; SdhCAB: succinate dehydrogenase; AspA: aspartase; Fum: fumarase; Mqo: malate quinone oxidoreductase; Mdh: malate dehydrogenase; AspB: aspartate aminotransferase.

1.2.1 Carbon source uptake

C. glutamicum is able to utilize several carbon sources, ranging from C2-sources like ethanol to disaccharides like sucrose (Cocaign *et al.*, 1993; Dominguez *et al.*, 1997; Yokota and Lindley, 2005). Gluconeogenetic carbon sources are activated to actetyl-CoA and are metabolized in the TCA cycle. For example, D- and L-lactate are oxidized to pyruvate by the quinone dependent lactate dehydrogenases (DlD and LldD, respectively) and activated at the pyruvate dehydrogenase complex (PDHC). The general uptake system for carbohydrates is the phosphotransferase system, transferring the phosphate group of phosphoenol pyruvate (PEP) to the sugar, synthesizing sugar-6-phosphates (Figure 1). It consists of the general uptake proteins E1 and HPr, encoded by *ptsI* and *ptsH*, respectively, and substrate specific transporters for glucose (*ptsG*), fructose (*ptsF*) and sucrose (*ptsS*). PTS independent uptake of glucose was shown in PTS-deficient mutants, with the inositol transporters IolT1 and IolT2 (Lindner *et al.*, 2011). These permeases import glucose, fructose, and inositol and subsequent phosphorylation is performed by the glucokinases Glk or PpgK. The utilization of the disaccharide maltose in *C. glutamicum* was uncovered recently. It is initiated with the import of maltose by the ABC-transport system MusEFGK$_2$I (Henrich *et al.*, 2013). The 4-α-glucanotransferase MalQ catalyzes the addition of maltose to maltodextrin, thereby liberating one glucose molecule which is phosphorylated to glucose-6-phosphate by the glucokinase Glk. Maltodextrin is a chain of α-1,4-linked glucose molecules and serves as storage polymer. Its degradation involves the maltodextrin phosphorylase MalP and the α-phosphoglucomutase Pgm. MalP synthesizes glucose-1-phosphate that is converted by Pgm to glucose-6-phosphate, which can be metabolized in glycolysis (Seibold *et al.*, 2009).

Next to these food carbon sources, the substrate spectrum of *C. glutamicum* was enlarged to non-food carbon sources by the integration of heterologous pathways. Lignocellulosic plant material contains 25-30 % hemicelluloses, consisting mainly of arabinose and xylose. Recently, utilization of arabinose was enabled by expression of the *E. coli araBAD* operon, encoding the L-arabinose isomerase AraA, the L-ribulokinase AraB and the L-ribulose-5-phosphate-4-epimerase AraD (Kawaguchi *et al.*, 2008). Furthermore, the type strain *C. glutamicum* ATCC 31831 is able to utilize arabinose and additionally carries an arabinose transporter encoded by *araE* (Kawaguchi *et al.*, 2009). Xylose utilization was first shown in *C. glutamicum* by the expression of the *E. coli* genes for the xylose isomerase *xylA* and for the xylulose kinase *xylB* (Kawaguchi *et al.*, 2006). *C. glutamicum* possesses an endogenous kinase encoded by cg0147, while no functional isomerase was discovered so far. In a screening for xylose isomerases, accelerated xylose consumption was found with XylA of *Xanthomonas campestris* (Meiswinkel *et al.*, 2013). Both arabinose and xylose are converted to D-xylulose-5-phosphate and enter the pentose phosphate pathway.

Glycerol is a stoichiometric waste product in biodiesel (fatty-acid-methyl-ester, FAME) synthesis, and expression of the genes of *E. coli* encoding the glycerol facilitator (*glpF*), the glycerol kinase (*glpK*) and the glycerol-3-phosphate dehydrogenase (*glpD*) enables growth and amino acid production in *C. glutamicum* based on this carbon source (Rittmann *et al.*, 2008). Glycerol is phosphorilated to glycerol-3-phosphate and oxidized to dihydroxyacetone phosphate, which feeds glycolysis.

1.2.2 Glycolysis

The monosaccharides glucose and fructose are utilized in their phosphorilated forms via the glycolysis and supply the organism with precursors for amino acid synthesis or energy generation. Phosphorylation of the sugars is mediated during the uptake by the PTS and subsequent activation and substrate level phosphorylation is performed according to the Embden Meyerhoff Parnas pathway. Glycolytic genes are scattered across the genome, only one transcriptional unit was detected, containing the genes for the glyceraldehyde-3-phosphate dehydrogenase (*gapA*), the 3-phosphoglycerate kinase (*pgk*), the triosephosphate isomerase (*tpi*) and the phosphoenol pyruvate carboxylase (*ppc*), which catalyzes anaplerotic reactions (Eikmanns, 1992; Schwinde *et al.*, 1993). Several genes of the glycolysis are controlled by the master regulators SugR and RamA (Engels *et al.*, 2008; Toyoda *et al.*, 2009). The transcriptional regulation was studied in detail on GapA, a key enzymes of glycolysis which catalyzes the NAD-dependent phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. SugR was shown to bind to the promoter region of *gapA* and to repress expression in the absence of sugars, while RamA serves as activator of the gene, being able to bind to three different sites in the promoter region (Toyoda *et al.*, 2008, 2009). Moreover, the activity of many

glycolytic enzymes is regulated by feedback inhibition. GapA and its gluconeogenetic counterpart GapB are regulated inversely by the level of ATP and ADP and GapA is additionally inhibited by NADH, thereby controlling the glycolytic flux (Danshina *et al.*, 2001; Dominguez *et al.*, 1998; Omumasaba *et al.*, 2004). Feedback inhibition is also found for the key enzyme pyruvate kinase (*pyk)*. In this case, high ATP concentrations inhibit its activity, while AMP activates the enzyme (Ozaki and Shiio, 1969).

1.2.3 Pentose phosphate pathway

The main functions of the PPP are the supply of reduction equivalents and the synthesis of precursors for histidine, aromatic amino acids, and nucleotide synthesis. The PPP is connected to the glycolysis by fructose-6-phosphate and glyceraldehyde-3-phosphat. In the oxidative part of the PPP, two moles of NADPH per mol glucose are generated by the glucose-6-phosphate dehydrogenase encoded by *zwf* and *opcA* and the 6-phosphogluconate dehydrogenase encoded by *gnd*. This feature is exploited in the industrial synthesis of lysine, as four moles of the reduction equivalent are needed to synthesize one mol of the amino acid (Marx *et al.*, 1997; Schrumpf *et al.*, 1991; Sonntag *et al.*, 1993). Therefore flux is redirected from glycolysis to the PPP by disruption of the gene for the glycolytic phosphoglucose isomerase (*pgi*) or by the expression of the gluconeogenetic fructose-1,6-bisphosphatase (*fbp*) (Becker *et al.*, 2005; Georgi *et al.*, 2005; Marx *et al.*, 2003). In a more direct approach, the flux was redirected by the expression of *zwf* (Becker *et al.*, 2007). The Gnd irreversibly decarboxylates 6-phosphogluconate to ribulose-5-phosphate (Ru-5P) and marks the end of the oxidative part (Moritz *et al.*, 2000). The non-oxidative part is performed by four enzymes and leads to ribose-5-phosphate as precursor for nucleotide and histidine synthesis and to erythrose-4-phosphate as precursor for the synthesis of phenylalanine, tyrosine and tryptophan. Another important intermediate of the PPP is xylulose-5-phosphate, as it serves as the entry point of the lignocellulosic carbon sources arabinose and xylose (Kawaguchi *et al.*, 2008; Kawaguchi *et al.*, 2006).

1.2.4 Anaplerosis

The anaplerotic node links glycolysis and the TCA cycle by the molecules pyruvate, phosphoenol pyruvate (PEP), oxaloacetate and malate to each other. The synthesis of amino acids of the aspartate family is draining off oxaloacetate from the TCA, which is replenished by the carboxylation of the glycolytic molecules by the PEP carboxylase Ppc (*ppc*) and the pyruvate carboxylase Pyc (*pyc*) (Eikmanns *et al.*, 1989; Peters-Wendisch *et al.*, 1997) (Figure 1). To enable gluconeogenesis, oxaloacetate can be decarboxylated to PEP by PEP carboxykinase PckA (*pckA*) or to pyruvate by oxaloacetate decarboxylase Odx (*odx*), while the malic enzyme MalE (*malE*) decarboxylates malate to

pyruvate (Gourdon *et al.*, 2000; Jetten and Sinskey, 1993; Klaffl and Eikmanns, 2010; Riedel *et al.*, 2001). Pyc and Pck are dependent on ATP or GTP, while Ppc uses the energy of ester-bond phosphate. Odx is strictly dependent on divalent magnesium ions and MalE on $NADP^+$. The carboxylation of pyruvate by Pyc was found to be the predominant reaction of the anaplerotic node under aerobic conditions. This feature is exploited in the synthesis of lysine by expression of the pyc^{P458S} gene, encoding a deregulated carboxylase with higher activity (Georgi *et al.*, 2005; Peters-Wendisch *et al.*, 1993). Under anaerobic condition, Pyc is of subordinate importance as Ppc is the primary enzyme for the synthesis of oxaloacetate (Inui *et al.*, 2004b).

1.2.5 Tricarboxylic acid cycle

The TCA cycle serves energy generation and supplies cells with precursors for amino acids (Figure 1). 2-oxoglutarate is the precursor for amino acids of the glutamate family (glutamate, glutamine, arginine and proline) and it is irreversibly synthesized by the isocitrate dehydrogenase Icd (*icd*, cg0766). Fumarate is synthesized by the succinate dehydrogenase SdhCAB (*sdhCAB*, cg0445-0447) and oxaloacetate by the malate-quinone oxidoreductase Mqo (*mqo*, cg2192) and both compounds can serve as precursors for amino acids of the aspartate family (Eikmanns *et al.*, 1995). Succinyl-CoA can be drained off from the TCA cycle for the synthesis of lysine, or the CoA-group is transferred by the succinyl-CoA:acetate CoA-transferase Cat (*cat*, cg2840) to acetate, for the synthesis of acetyl-CoA (Veit *et al.*, 2009).

NADPH for anabolic purposes is only generated at the Icd, while energy for the respiratory chain is generated by four enzymes or protein complexes. Of these, the SdhCAB and the Mqo are membrane-bound and quinone-dependent. The SdhCAB is a trimeric flavoprotein containing a transmembrane cytochrom *b*, and oxidizes succinate to fumarate (Kurokawa and Sakamoto, 2005). The Mqo has a cytoplasmatic NAD-dependent counterpart, the malate dehydrogenase Mdh (*mdh*). During growth, Mdh and Mqo are simultaneously active and catalyze opposite reactions; Mqo oxidizes malate to oxaloacetate in a quinone-dependent manner and Mdh oxidizes NADH for the reduction of oxaloacetate to malate. Nevertheless, under aerobic conditions Mdh is dispensable for growth, whereas Mqo is essential (Molenaar *et al.*, 2000; Molenaar *et al.*, 1998).

1.3 Anaerobic conditions

The change from oxic to anoxic conditions requires several adjustments in the metabolism of *C. glutamicum*. When oxygen is limiting, the organism switches from respiration to fermentation of

succinic, lactic and acetic acid and loses its ability to grow. Therefore, *C. glutamicum* was regarded as strict aerobe until growth was detected in the presence of nitrate, which can be used as terminal electron acceptor (Nishimura *et al.*, 2007; Takeno *et al.*, 2007). The growth arrest is a major advantage for biotechnological applications, as carbon sources are not steadily invested into biomass but in the synthesis of the product of interest. The organism can be packed to high optical densities and retains stable metabolic activities for at least 360 hours (Inui *et al.*, 2004b). These features were exploited to develop processes for the anaerobic production of organic acids with *C. glutamicum* (Litsanov *et al.*, 2012a; Okino *et al.*, 2005).

Genetic profiling under anaerobic conditions revealed an up-regulation of glycolytic genes especially the *gapA-pgk-tpi-ppc* operon in addition with the genes encoding the lactate and the malate dehydrogenase (Inui *et al.*, 2007; Molenaar *et al.*, 2000). Furthermore, the genes for the oxidative arm of the TCA cycle, *aceA, gltA*, *icd*, *odhA,* and *sucCD* show a significant down-regulation under these conditions. Therefore, the fermentative metabolism based on the glycolysis and the reductive arm of the TCA cycle, which leads from oxaloacetate via malate and fumarate to succinate. The up-regulation of *gapA*, *ldhA*, *mdh* and *ppc* is crucial for carbon source consumption. The glycolytic GapA is inhibited by NADH, which is regenerated to NAD^+ either by LdhA for the synthesis of lactate, or by the Mdh during the synthesis of succinate. Furthermore, the reductive branch of the TCA cycle is fueled by Ppc. It was shown that fermentative synthesized succinate is exclusively derived from the carbon fixing reactions of Ppc and not Pyc, which is the predominant enzyme to replenish the TCA cycle under aerobic conditions (Inui *et al.*, 2004b).

1.4 The amino acid aspartate

L-Aspartate was first identified as hydrolysis product from asparagine in juice of *Asparagus officinalis*. Next to glutamate, it is the only acidic amino acid and has a negatively charged, polar side chain. L-Aspartate is non-essential in mammals and can be synthesized from oxaloacetate by transamination or from fumarate by reductive amination. The L-form of aspartate is proteinogenic but D-aspartate is found in archaeal as well as Gram-positive bacteria as part of the peptidoglycan. These organisms, like *Pyrococcus horikoshi* or *Enterococcus faecium*, possess the aspartate racemase that synthesizes the D- from the L-form (Bellais *et al.*, 2006; Liu *et al.*, 2002). In mammals, D-aspartate is a neurotransmitter and found in the nervous and endocrine system. Here, only the proteinogenic form of the amino acid is discussed, and whenever aspartate is written, the L-form is addressed.

Aspartate is a central precursor in the cell, involved in several pathways ranging from amino acid to vitamin synthesis, and it is the basis for the cell wall component diaminopimelic acid in *C. glutamicum*.

Next to these physiological functions, aspartate finds application in chemical industry. Besides the well known utilization in the low calorie sweetener aspartame, a dipeptide of aspartate and phenylalanine, new applications in the solvent and polymer market evolve. Aminated derivatives of dicarboxylic acids like amine butanediol, amine tetrahydrofuran and amine butyrolactone hold potential to replace the acids derived from petrochemical resources. Additionally, polymerized aspartate can replace polacrylic acid or polycarboxylates which find wide application as packaging material or as superabsorber in diapers. Due to the possibility of fermentative generation, aspartate was identified as a top-value added chemical from biomass (Werpy *et al.*, 2004). Industrial synthesis of aspartate is performed by enzymatic conversion of fumarate and ammonia with aspartases (or aspartate ammonia lyases) since the 1970s. For this purpose, *E. coli* cells are entrapped in matrices consisting of polyacrylamid, polyurethane foam or carrageen. In the latter a production stability of nearly two years was established, with a productivity of 200 mM aspartate per gram cells per hour. Annually, approximately 30.000 tons are synthesized (Eggeling and Sahm, 2009). A recent drawback of this enzymatic conversion is the high price for the substrate fumarate. It is synthesized chemically from maleic anhydride in a scale of 90.000 tons per year. Nevertheless, product costs are high, and fermentative routes exploiting the ability of the fungi *Rhizopus nigricans* or related species to excrete fumarate are currently in the focus of research (Roa Engel *et al.*, 2008; Roa Engel *et al.*, 2011).

1.4.1 Synthesis of aspartate under physiological conditions

Under physiological conditions, aspartate is a derivative of the TCA cycle and can be synthesized by amination of fumarate or by transamination from oxaloacetate (Figure 2). The aspartase (AspA) catalyzes the reversible amination of fumarate to aspartate and belongs to the aspartase/fumarase superfamily. The reaction of fumarase and aspartase follows the same scheme, differing only in the functional group that is leaving the molecule (Veetil *et al.*, 2012).

Several aspartases have been purified from Gram-positive and Gram-negative organisms including *Pseudomonas fluorescens*, *Bacillus subtilis* and *Bacillus* sp. YM55-1, possessing a thermostable enzyme, but the best studied is the *E. coli* aspartase AspA (*aspA*, b4139) (Cardenas-Fernandez *et al.*, 2012; Kawata *et al.*, 1999; Shi *et al.*, 1997; Sun and Setlow, 1991; Takagi *et al.*, 1986). It is a homotetramer with 478 amino acid residues each, containing three functional domains. The central domain contains the catalytic side, but due to the lack of aspartate bound crystals, not all catalytic residues were identified (Kawata *et al.*, 1999; Shi *et al.*, 1997; Weiner *et al.*, 2008). Nevertheless, engineered versions of AspA with increased catalytic properties are available (Wang *et al.*, 2000). The aspartase is characterized by a high substrate specificity, next to aspartate only aspartate semialdehyde can be deaminated resulting in an inactivation of the enzyme (Yumoto *et al.*, 1982). Activity of AspA is

depending on magnesium ions and it is allosterically activated by aspartate (Karsten *et al.*, 1986). Molecules mimicking the structure of aspartate, like malate, succinate and N-acetylaspartate were found to inhibit the aspartase (Falzone *et al.*, 1988).

Aminotransferases are categorized according to their substrates into four groups and catalyze the reversible transfer of amino groups from amino to organic acids. Although many aminotransferases show overlapping substrate specificity, glutamate is one of the most important amino group donors. In *C. glutamicum*, glutamate is synthesized in a NADP-dependent manner either from 2-oxoglutarate by the glutamate dehydrogenase Gdh (*gdh,* cg2280), or from glutamine by the glutamine synthetase GltBD (*gltBD*, cg0229-0230). However, the alanine aminotransferases AlaT and AvtA have different amino group donors. AlaT (*alaT*, cg3149) utilizes additionally to glutamate also aspartate as amino donor, while the alanine-valine aminotransferase AvtA (*avtA*, cg2877) uses valine to transaminate pyruvate to alanine (Marienhagen *et al.*, 2005). The *E. coli* aspartate aminotransferase AspC is one of the best studied pyridoxal phosphate (PLP) dependent enzymes, and its structure and reaction mechanism was studied in detail. It is homodimeric, and each monomer chain consists of a large and a small domain, with the active site located on the interface. PLP lies at the bottom of the active center and serves as acceptor molecule for the amino group (Jäger *et al.*, 1994; Okamoto *et al.*, 1994). AspC belongs to subgroup Iα of aminotransferases, where the binding of the substrate induces a conformational change that affects the large and the small domain and brings the enzyme into the closed form. Next to the biosynthesis of aspartate, *E. coli* AspC has overlapping substrate specificity and catalyzes the synthesis of tyrosine and phenylalanine (Hammes *et al.*, 2011). The aspartate aminotransferase AspB (*aspB*, cg0294) of *C. glutamicum* and shows only a weak aminotransferase motif, but the function was determined by measuring the specific activity for aspartate and α-ketoglutarate. In contrast to *E. coli*, AspB is not involved in the synthesis of other amino acids (Marienhagen *et al.*, 2005; McHardy *et al.*, 2003).

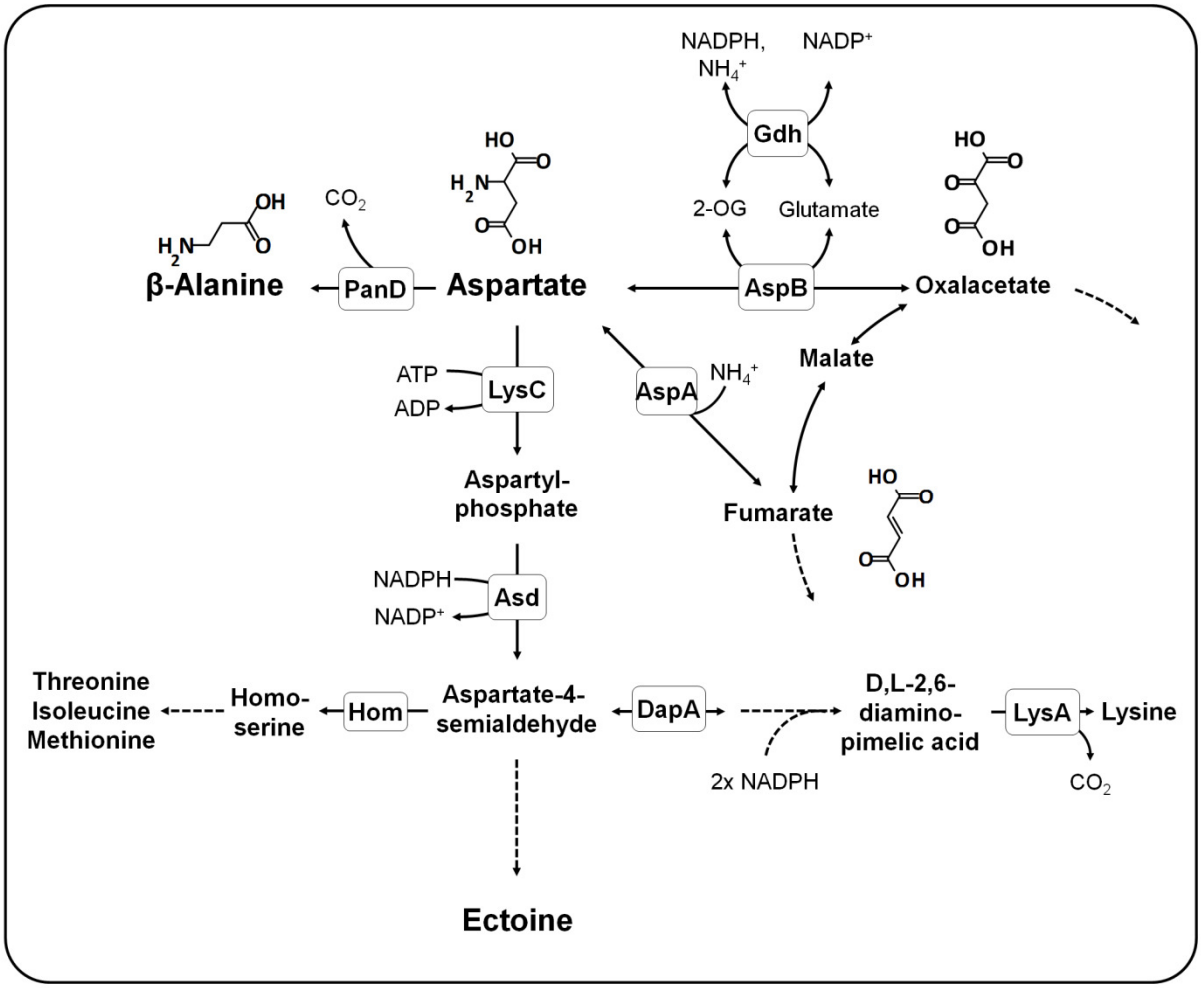

Figure 2: Synthesis of aspartate and its function as precursor molecule.
Aspartate is synthesized from the TCA cycle intermediates oxaloacetate and fumarate and serves as precursor for β-alanine, the amino acids of the aspartate family, the cell wall component diaminopimelate and the compatible solute ectoine. AspB: aspartate aminotransferase; Gdh: glutamate dehydrogenase; AspA: aspartase; PanD: aspartate-α-decarboxylase; LysC: aspartate kinase; Asd: aspartate semialdehyde dehydrogenase; Hom: homoserine dehydrogenase; DapA: dihydropicolinate synthase; LysA diaminopimelate decarboxylase.

1.4.2 Synthesis of amino acids of the aspartate family

The amino acids of the aspartate family comprise asparagine, lysine, threonine, methionine, and isoleucine. With the exception of asparagine, their synthesis is tightly regulated by the aspartokinase (*lysC*, cg0306). LysC catalyzes the initial phosphorylation of aspartate and is feedback inhibited by the amino acids lysine and threonine (Shiio and Miyajima, 1969). In the succeeding reaction, the NADP-dependent aspartate semialdehyde dehydrogenase Asd (*asd*, cg0307) converts aspartyl phosphate into aspartate semialdehyde. The dihydropicolinate synthase DapA (*dapA*, cg2161) and the homoserine dehydrogenase Hom (*hom*, cg1337) compete for aspartate semialdehyde and the molecule marks the branching point between lysine synthesis and the synthesis of threonine, isoleucine and methionine.

For the biotechnological synthesis of these amino acids, regulations at the level of aspartate and aspartate semialdehyde are applied to reach the desired fluxes. Basis for every pathway are feedback resistant variants of the aspartokinase (Kelle *et al.*, 2005). At the next branching point, reduced activity of Hom leads to lysine excretion, while increased activity of the dehydrogenase promotes synthesis of isoleucine (Cremer *et al.*, 1988; Morbach *et al.*, 1996). For example, the strain DM1729 contains only three point mutations that lead to efficient synthesis of lysine: increased activity of anaplerotic pyruvate carboxylase (*pyc*^{P458S}), feedback resistant aspartokinase (*lysC*^{T311I}) and homoserine dehydrogenase (*hom*^{V59A}) with reduced activity (Georgi *et al.*, 2005).

1.4.3 Export of amino acids

In *C. glutamicum* three transporters are known to excrete amino acids. Arginine and lysine are exported by the lysine efflux permease LysE (*lysE*, cg1424), which is characterized by a low K_M for lysine to ensure export only under excess of the amino acid (Vrljic *et al.*, 1999; Vrljic *et al.*, 1995; Vrljic *et al.*, 1996). The threonine exporter (*thrE*, cg2905) also contributes the transport of serine, while the branched chain amino acid transporter BrnFE (*brnFE*, cg0314-0315) shows the broadest substrate spectrum and facilitates the excretion of isoleucine, leucine, valine, and methionine (Kennerknecht *et al.*, 2002; Simic *et al.*, 2001). No specific transport system was identified for aspartate so far. Excretion of glutamate is still not fully understood. Several methods that induce glutamate excretion are known like the limitation of biotin or the addition of the antibiotics penicillin and ethambutol (Radmacher *et al.*, 2005; Shiio *et al.*, 1962). These methods are thought to affect membrane tension by changing the composition of the cytoplasmatic membrane or weakening the integrity of the cell wall. In turn, these changes in membrane tension activate mechanosensitive channels and lead to efflux of molecules. Mutational studies of the channel protein YggB showed that C-terminal alterations lead to constant efflux of glutamate, without the need of induction. Therefore the involvement of YggB in glutamate export is very likely (Nakamura *et al.*, 2007).

1.5 The non proteinogenic amino acid β-alanine

In *C. glutamicum*, this β-amino acid is derived by decarboxylation of aspartate by the aspartate-α-decarboxylase PanD (*panD*, cg0172) (
Figure 2). The physiological role of β-alanine in *C. glutamicum* is the synthesis of the B-vitamin pantothenate (vitamin B_5). The vitamin is a part of coenzyme A and the acyl carrier protein, and therefore, essential for enzymes in the TCA cycle and in fatty acid synthesis. Biosynthesis of

pantothenate is a split pathway involving the synthesis of D-pantoate by the α-ketopantoate hydroxymethyl transferase PanB (*panB*, cg0149) and the α-ketopantoate reductase IlvC (*ilvC*, cg1437). The D-pantothenate synthetase PanC (*panC*, cg0148) ligates β-alanine to D-pantoate and generates D-pantothenate.

Chemical synthesis of β-alanine is well known and patented since more than 70 years, but it is dependent on petrochemical resources, high pressure and rather high temperatures (Paden, 1943). In mammals, β-alanine can only be synthesized by degradation of uracil, and furthermore, it is the limiting factor for the synthesis of the dipeptide carnosine (Traut and Jones, 1996). Carnosine is mainly found in muscles and serves as physicochemical buffer. It is thought to enhance muscle performance, which explains why β-alanine is often used by athletes as food supplement (Derave *et al.*, 2007). Additionally, β-alanine can be used for the synthesis of hydroxypropionic acid, which serves as a precursor for acrylic acid (Liao *et al.*, 2010; Marx *et al.*, 2007; Straathof *et al.*, 2005). Polyacrylic acid absorbs multiple times the own weight of water and this super-absorbing property is applied, e. g. in diapers.

The decarboxylase PanD is an interesting enzyme as it undergoes a self-induced posttranslational modification. The 136 aa long protein is synthesized as an inactive pro-enzyme, and performs self-proteolysis between the amino acids Gly24 and Ser25, which leads to the formation of an α-and β-chain. Other decarboxylases like the diaminopimelate decarboxylase LysA are dependent on pyridoxal phosphate, but the self-proteolysis of PanD forms a pyruvoyl-residue located in the α-chain, which serves as the prosthetic group of the enzyme (Cui *et al.*, 2013; Schmitzberger *et al.*, 2003). The complete decarboxylase is multimeric, containing three α-and three β-chains, as well as an inactive pro-enzyme. *C. glutamicum panD* was identified by functional complementation of an *E. coli* mutant strain and was used for production of pantothenate in this organism (Dusch *et al.*, 1999a; Sahm and Eggeling, 1999). An active transport system was detected in *E. coli*, where the amino acid carrier CycA was shown to import β-alanine (Schneider *et al.*, 2004). Potential candidates from *C. glutamicum* were tested in deletion studies, but no transporter was identified so far (Radmacher *et al.*, 2002).

1.6 Ectoine

Ectoine is a compatible solute that was first discovered in *Halorhodospira halochloris,* and is widespread in γ-proteobacteria like *Halomonas* and *Chromohalobacter*, actinobacteria like *Brevibacterium* and *Streptomyces*, and firmicutes like *Bacillus* and *Marinococcus* (Galinski *et al.*, 1985; Kuhlmann and Bremer, 2002; Louis and Galinski, 1997; Onraedt *et al.*, 2005; Roberts, 2005). It is a derivative of aspartate and synthesized by five enzymatic steps. The initial synthesis of aspartate semialdehyde is similar to the synthesis of lysine and threonine and catalyzed by the aspartokinase

and the aspartate semialdehyde dehydrogenase. Glutamate is the amino group donor for the transamination of aspartate semialdehyde by diaminobutyrate transaminase (EctB) to diaminobutyrate, which is further acetylated by diaminobutyrate acetyl transferase (EctA). In the last step, acetyl diaminobutyrate is condensed to ectoine by ectoine synthase (EctC) (Figure 3). A further hydroxylation by ectoine hydroxylase (EctD) leads to hydroxyectoine, which plays an important role in heat stress protection (Garcia-Estepa *et al.*, 2006). Ectoine has several protective functions on macromolecules, cells or tissues. It was shown to improve protein folding and activity, and to decrease the melting temperature of DNA. It is used in sun blockers to quench singlet oxygen derived from ultraviolet light, and was shown to moisturize human skin (Botta *et al.*, 2008; Buenger and Driller, 2004; Heinrich *et al.*, 2007; Pastor *et al.*, 2010). The synthesis of ectoine at industrial scale is performed by a process called "bacterial milking". The natural producer *H. elongata* is continuously cultivated under high salt conditions to induce ectoine synthesis. After reaching an appropriate cell density, a hypoosmotic shock (15→3 % NaCl) is applied by reducing the culture volume and the addition of distilled water. This shock induces the release of ectoine to the culture medium. Product and cells are separated and cells are recycled in fresh medium under high salt conditions (Sauer and Galinski, 1998). Next to this a two reactor system was evolved for *C. salexigens*, that keeps cells under optimal growth conditions and constantly drains off culture to a second bioreactor, where the hypoosmotic shock is applied (Fallet *et al.*, 2010). However, these cultivation techniques have several drawbacks arising from the high salt media.

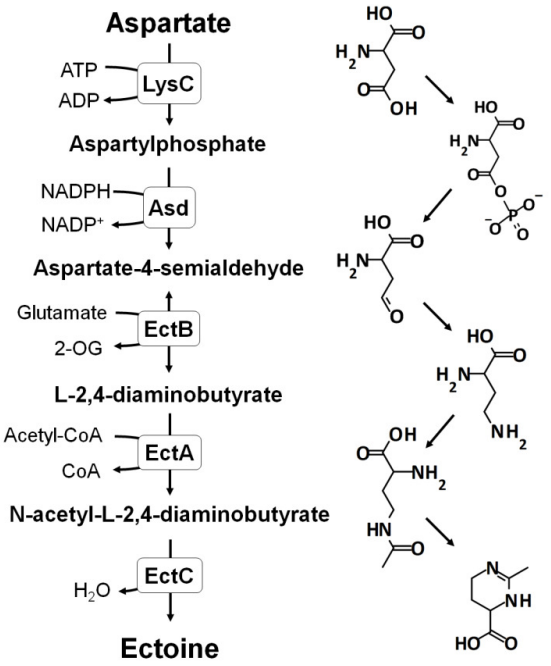

Figure 3: Biosynthesis of ectoine.
Aspartate is converted in five enzymatic steps to ectoine. Abbereviations: LysC: aspartokinase; Asd: aspartate-semialdehyde dehydrogenase; EctA: diaminobutyrate acetyl transferase; EctB: diaminobutyrate transaminase; EctC: ectoine synthase.

The biosynthesis of ectoine was studied in detail in *H. halochloris*, *Halomonas elongata* and *Chromohalobacter salexigens* (Canovas *et al.*, 1997; Ono *et al.*, 1999; Peters *et al.*, 1990). With a few exceptions, all genes for the synthesis of ectoine are organized as an operon. A gene encoding an ectoine hydroxylase is found usually in this operon, too, or it is separately encoded as in the case of *C. salexigens* (Pastor *et al.*, 2010). Transcriptional regulation of *ectABC* operon was investigated in the Gram-negative organism *C. salexigens*, and depends on several factors. Different promoters are used for induction in the stationary phase and under salt or heat stress conditions. Additionally, transcription is blocked, if ectoine or other compatible solutes can be taken up from the environment (Calderon *et al.*, 2004; Garcia-Estepa *et al.*, 2006; Rodriguez-Moya *et al.*, 2013).

The hydrostatic pressure of *H. elongata* is maintained by unspecific mechanosensitive channels, which release ectoine under hypoosmotic conditions, while no active export was discovered so far (Sauer and Galinski, 1998). Active importers belong to the betaine-carnithine-choline transporter (BCCT) superfamily like *Marinococcus halophilus* EctM and *Virgibacillus pantotheticus* EctT or to the tripartite-ATP-dependent periplasmatic transporter TRAP-T family like *H. elongata* TeaABC. The function of the BCCT-like transporters is the uptake of the compatible solute in response to increased osmolarity, while TeaABC re-imports ectoine leaking through the cell membrane (Grammann *et al.*, 2002; Kuhlmann *et al.*, 2011; Vermeulen and Kunte, 2004). *C. glutamicum* encodes members of the BCCT family, EctP and LcoP, as well as the permease ProP belonging to the major facilitator superfamily (MFS), which are known to import ectoine under hyperosmotic conditions (Peter *et al.*, 1998; Steger *et al.*, 2004). Under hypoosmotic conditions, the mechanosensitive channels MscL and YggB as well as a third yet unknown transporter are known to release compatible solutes to the environment (Nottebrock *et al.*, 2003).

1.7 Aims of this work

The aim of this work is to enable *C. glutamicum* for the bio-based production of the amino acid L-aspartate and the derivatives β-alanine and ectoine. Aspartate is valuable chemical with well-established and also new potential applications, but its large-scale production is based on petrochemical resources. To establish a glucose-based production scheme with *C. glutamicum* is one major goal of this work. The fundamental strategy is to provide cells with the precursor molecules fumarate and oxaloacetate and to produce either the aspartase or the aspartate aminotransferase which are able to convert these precursors into the product of interest. Apart from this, the drain off of aspartate must be prevented to avoid that the amino acid is metabolized intracellulary. This is either accomplished by an inactivation or deregulation of the aspartokinase, or by growth deprived

conditions, i.e. by anaerobic cultivation. After constructing a basic aspartate excreting mutant, further genetic modifications of the basic production strain target to reduce potential by-products and to optimize the supply with the aforementioned precursors, to enhance the productivity of the process. The production of the non-proteinogenic amino acid β-alanine is dependent on a sufficient supply with the substrate aspartate. Therefore, the production of an aspartate-α-decarboxylase in an aspartate producing mutant is the basis for the overproduction of β-alanine. Improvements of the process aim at by-product reduction and reduced metabolization of the β-amino acid, i.e. by blocking the pantothenate pathway. The introduction of the ectoine biosynthetic genes into *C. glutamicum* targets at the salt-independent production of the compatible solute. Furthermore, improvements in the supply with aspartate and especially aspartate semialdehyde benefit the production of ectoine. To prove the sustainability of the process, also the production from non-food carbon sources is investigated. Existing industrial processes for the production of aspartate, β-alanine, and ectoine are based on petrochemical resources or cost intensive, cyclic fermentations. This work targets to develop alternative, bio-based processes, exploiting the particular features of the host organism *C. glutamicum*.

2 Material and Methods

2.1 Chemicals

All chemicals used in this work were provided by the companies Sigma-Aldrich GmbH (Taufkirchen), Merk AG (Darmstadt), Roche Deutschland Holding GmbH (Grenzach-Wyhlen), or Carl Roth AG (Arlesheim), if not indicated otherwise.

2.2 Bacterial strains

Table 1: Strains used in this work

Strain	Genetic characteristics	Abbre-viation	Reference
E. coli			
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1		Invitrogen
S17	recA pro hsdR RP4-2-Tc::Mu-Km::Tn7		(Simon *et al.*, 1983)
C. glutamicum			
Wild type	*C. glutamicum* ATCC13032	wt	(Abe *et al.*, 1967)
DM1729	pyc^{P458S},hom^{V59A}, $lysC^{T311I}$		(Georgi *et al.*, 2005)
Δ*aspB*	Δcg0294		This work
Δ*panD*	Δcg0172		This work
Δ*fum*	Δcg1145		Dr. M. Persicke, unpublished
Δ*ldhA*	Δcg3219	L	This work
Δ*sdhCAB*	Δcg0445-0447	S	Dr. J.-W. Youn, unpublished
Δ*sdhCAB* Δ*ldhA*	Δcg0445-0447 Δcg3219	SL	Dr. J.-W. Youn, unpublished
Δ*sdhCAB* Δ*ldhA* Δ*aspB*	Δcg0445-0447 Δcg3219 Δcg0294	SLB	This work
Δ*sdhCAB* Δ*ldhA* Δ*cat*	Δcg0445-0447 Δcg3219 Δcg2840	SLC	This work
Δ*sdhCAB* Δ*ldhA* Δ*panD*	Δcg0445-0447 Δcg3219 Δcg0172	SLD	This work
Δ*sdhCAB* Δ*ldhA* Δ*panBC*	Δcg0445-0447 Δcg3219 Δcg0148-0149	SLBC	This work
Δ*sdhCAB* Δ*ldhA* Δ*mdh*	Δcg0445-0447 Δcg3219 Δcg2613	SLM	This work
Δ*sdhCAB* Δ*ldhA* Δ*alaT*	Δcg0445-0447 Δcg3219 Δcg3149	SLT	This work
Δ*sdhCAB* Δ*ldhA* Δ*avtA*	Δcg0445-0447 Δcg3219 Δcg2877	SLV	This work
Δ*sdhCAB* Δ*ldhA* Δ*alaT* Δ*avtA*	Δcg0445-0447 Δcg3219 Δcg3149 Δcg2877	SLTV	This work

2.3 Plasmids and Oligonucleotides

Table 2: Plasmids used in this work

Plasmid	Characteristics	Reference
Expression plasmids		
pEKEx3	Spec^{R}; *C. glutamicum/E. coli* shuttle vector for regulated gene expression (P_{tac} *lacI*^{Q} pBL1 *oriV*$_{Cg}$ pUC18 *oriV*$_{Ec}$)	(Stansen *et al.*, 2005)
pEKEx3-*aspA*	Expression of *C. glutamicum* cg1697	This work
pEKEx3-*aspA*$_{Bs}$	Expression of *B. subtilis* 168 *aspA* BSU23570	This work
pEKEx3-*aspA*$_{Ec}$	Expression of *E. coli* MG1655 *aspA* b4139	This work
pEKEx3-*aspB*	Expression of *C. glutamicum* cg0294	This work
pEKEx3-*aspB*$_{Bs}$	Expression of *B. subtilis* 168 *aspB* BSU22370	This work
pEKEx3-*aspB*$_{Ec}$	Expression of *E. coli* MG1655 *aspC* b0928	This work
pEKEx3-*aspDH*$_{Pa}$	Expression of *Pseudomonas aeruginosa* PAO1 *aspDH* PA3505	This work
pEKEx3-*aspDH*$_{Re}$	Expression of *Ralstonia eutropha* H16 *aspDH* H16_0736	This work
pEKEx3-*ectABC*$_{Cs}$	Expression of *C. salexigens* ectoine biosynthetic genes *ectABC* Csal_1876-1878	This work
pEKEx3-*gapA*	Expression of *C. glutamicum* cg1791	(Siedler *et al.*, 2013)
pEKEx3- *gdh*$_{Ps}$	Expression of *Peptostreptococcus asaccharolyticus gdh*$_{Ps}$	J. Schneider unpublished, according to (Marx *et al.*, 1999)
pEKEx3-*ldhA*	Expression of *C. glutamicum* cg3219	This work
pEKEx3-*lldD*	Expression of *C. glutamicum* cg3227	(Stansen *et al.*, 2005)
pEKEx3-*mdh*	Expression of *C. glutamicum* cg2613	This work
pEKEx3-*mdh*-g*apA*	Expression of *C. glutamicum* cg2613 and cg1791	This work
pEKEx3-*panD*	Expression of *C. glutamicum* cg0172	This work
pEKEx3-*panD*$_{Bs}$	Expression of *B. subtilis* 168 *panD* BSU22410	This work
pEKEx3-*panD*$_{Ec}$	Expression of *E. coli* MG1655 *panD* b0131	This work
pEKEx3-*ppc*	Expression of *C. glutamicum* cg1787	This work
pEKEx3-*ppc-mdh-gapA*	Expression of *C. glutamicum* cg1787, cg2613 and cg1791	This work
pEKEx3-*pyc*^{P458S}	Expression of deregulated *C. glutamicum* cg0791	This work
pEKEx3-*udhA*$_{Ec}$	Expression of *E. coli* MG1655 *udhA* b3962	(Lindner *et al.*, 2013)
pEKEx3-*xylA*$_{Xc}$-*xylB*	Expression of *Xanthomonas campestris* ATCC33913 *xylA* XCC1758 and *C. glutamicum xylB* cg0147	(Meiswinkel *et al.*, 2013)
pVWEx1	Kan^{R}; *C. glutamicum*/*E. coli* shuttle vector for regulated gene expression (P_{tac}, lacI^{Q}, pHM1519 *oriV*$_{Cg}$, pACYC177 *oriV*$_{Ec}$)	(Peters-Wendisch *et al.*, 2001)
pVWEx1-*araBAD*$_{Ec}$	Expression of *E. coli* MG1655 arabinose utilization genes b0061-b0063	(Schneider *et al.*, 2010)
pVWEx1-*aspA*	Expression of *C. glutamicum* cg1697	This work

Table 2 continued

pVWEx1-*aspB*	Expression of *C. glutamicum* cg0294	This work
pVWEx1-*ectABC*$_{Cs}$	Expression of *C. salexigens* ectoine biosynthetic genes *ectABC* Csal_1876-1878	This work
pVWEx1-*glpFKD*$_{Ec}$	Expression of *E. coli* MG1655 glycerol utilization genes b3926-b3927 and b3426	(Rittmann *et al.*, 2008)
pVWEx1-*pntAB*$_{Ec}$	Expression of *E. coli* MG1655 *pntAB* b1602-1603	Dr. J.-W. Youn, unpublished
Promoter probe plasmids		
pET2		(Vasicova *et al.*, 1998)
pET2-*lysC*	Carrying the intergenic region of cg0305-0306	This work
pET2-*lysC*B6	Carrying the intergenic region of cg0305-0306 with the B6 *dapA* promoter (Vasicova *et al.*, 1999)	This work
pET2-*lysC*B27	Carrying the intergenic region of cg0305-0306 with the B27 *dapA* promoter (Vasicova *et al.*, 1999)	This work
pET2-*lysC*J3	Carrying the intergenic region of cg0305-0306 with the J3 *dapA* promoter (Vasicova *et al.*, 1999)	This work
pET2-*lysC*T1C	Carrying the intergenic region of cg0305-0306 with transition of T→C position +49 upstream the start codon	This work
Deletion plasmids		
pK19*mobsacB-alaT*	For the deletion of cg3149	(Marienhagen *et al.*, 2005)
pK19*mobsacB-aspB*	For the deletion of cg0294	This work
pK19*mobsacB-avtA*	For the deletion of cg2877	(Marienhagen *et al.*, 2005)
pK19*mobsacB-ldhA*	For the deletion of cg3219	(Blombach *et al.*, 2011)
pK19*mobsacB-lldD*	For the deletion of cg3227	(Krause, 2012)
pK19*mobsacB-lysC*	For the deletion of cg0306	This work
pK19*mobsacB-mdh*	For the deletion of cg2613	(Blombach *et al.*, 2011)
pK19*mobsacB-panBC*	For the deletion of cg0148-0149	(Krause, 2012)
pK19*mobsacB-panD*	For the deletion of cg0172	This work
pK19*mobsacB-pqo*	For the deletion of cg2891	(Litsanov *et al.*, 2012b)
pK19*mobsacB-pta-ackA*	For the deletion of cg3047-3048	(Litsanov *et al.*, 2012c)
pK19*mobsacB-ramA*	For the deletion of cg2831	(Cramer *et al.*, 2006)
pK19*mobsacB-sugR*	For the deletion of cg2115	(Engels and Wendisch, 2007)

Table 3: List of oligonucleotides used in this work

Name	Sequence	Restriction site	Function
Primers for deletions			
M13 24for	CGCCAGGGTTTTCCCAGTCACGAC		Vector primer pK19mobsacB
M13 24rev	AGCGGATAACAATTTCACACAGGA		Vector primer pK19mobsacB
aspB Del A	CTCGTCGACTCAGTTTGCGGAGGATAGGG	SalI	Deletion of cg0294
aspB Del B	CCCATCCACTAAACTTAAACACAGCGAAACTGAA CTCAT		Deletion of cg0294
aspB Del C	TGTTTAAGTTTAGTGGATGGGGCGGAGCATTACG CTAACTAA		Deletion of cg0294
aspB Del D	CTCGTCGACTGTCCCGGTTGGGCATGC	SalI	Deletion of cg0294
aspB Del E	TGCAGGGACTCACCAGAG		Verfication of deletion of cg0294
aspB Del F	AGGCACGGACACGT		Verfication of deletion of cg0294
ldhA Del A	CGCCCGGGTTCGGCAACAATGACGGCGAGA	SmaI	Deletion of cg3219
ldhA Del B	CCCATCCACTAAACTTAAACAGACGGTTTCTTTC ATTTTCGATCC		Deletion of cg3219
ldhA Del C	TGTTTAAGTTTAGTGGATGGGAAGCAGTTCTTCT AAATCTTTGGCG		Deletion of cg3219
ldhA Del D	CGCCCGGGGGCATCGACGACATCTGAG	SmaI	Deletion of cg3219
ldhA Del E	CGCCCGGGGGCATCGACGACATCTGAG		Verfication of deletion of cg3219
ldhA DelF	TGATGGCACCAGTTGCGATGT		Verfication of deletion of cg3219
LysC DelA	CGAAAGCTTACATCGGTGTCATCAGAG	HinDIII	Deletion of cg0306
LysC Del B	CCCATCCACTAAACTTAAACACTGTACGACCAG GGCCAC		Deletion of cg0306
LysC Del C	TGTTTAAGTTTAGTGGATGGGTATGCAGGCAC CGGACGCTAA		Deletion of cg0306
LysC Del D	TCGGTCGACCAATAATGCCCTTGACCAGGG	SalI	Deletion of cg0306
lysC Del E	TGCCGAAACCAAGTGCTACTGT		Verfication of deletion of cg0306
LysC Del F	TACAAGACCAGCGGCATCGTGA		Verfication of deletion of cg0306
panD Del A	ATCGTCGACCGCGGTCAACGTCATC	SalI	Deletion of cg0172
panD Del B	CCCATCCACTAAACTTAAACAGGCCGGTGAAT CTTACTTCC		Deletion of cg0172
panD Del C	TGTTTAAGTTTAGTGGATGGGCCTGGATCCG GGCTT		Deletion of cg0172
panD Del D	ATCGTCGACTCGCGCCGGTGAATC	SalI	Deletion of cg0172
panD Del E	GGATGGTCTGGACTGGG		Verfication of deletion of cg0172
panD Del F	ACCAGGTAATTCTGGGTA		Verfication of deletion of cg0172
sdhCAB Del E	CATGGCGCAACTATAGCGTGA		Verfication of deletion of cg0445-0447
sdhCAB DelF	TGAAGGCTCCAGGGAACTC		Verfication of deletion of cg0445-0447
alaT Del E	CGAGGAACGGCAATAATC		Verfication of deletion of cg3149
alaT Del F	AGCAAGACCTGACATACC		Verfication of deletion of cg3149
avtA Del E	GTCTGCGTTCCTGTTGCT		Verfication of deletion of cg2877
avtA Del F	TAGCGGTTGAGCAGTTCG		Verfication of deletion of cg2877
cat actA Del E	GGAGGCAAGGTCAAAG		Verfication of deletion of cg2840
cat actA Del F	TAGGCAGGTCGATCG		Verfication of deletion of cg2840

Table 3 continued

Primers for determination of TSS and promoter strength			
pET2 FOR	CTCCCTATCACTGCATGAG		Vector primer pET2
pET2 rev	ACGGTGGTATATCCAGTG		Vector primer pET2
cg0305/0306 For	CTGCAGTTCAGGGTAGTTGACTAAAGAG	PstI	Amplification of intergenic region of cg0305 and cg0306
cg0305 0306 Rev	GGATCCCTTTGTGCACCTTTCG	BamHI	Amplification of intergenic region of cg0305 and cg0306
lysC B6 Pro 2	CGCTCACATGGTTGCCTTCCCTTTATAAACTGTG		Introduction of *dapA* promoter hexamer B6
lysC B6 Pro 3	TAAAGGGAAGGCAACCATGTGAGCGGGTAAC		Introduction of *dapA* promoter hexamer B6
lysC B27 3	TAAAGGAAACCATGAGCGGGTAACTGTC		Introduction of *dapA* promoter hexamer B27
lysC B27 2	GACAGTTACCCGCTCATGGTTTCCTTTA		Introduction of *dapA* promoter hexamer B27
lysC J3 nr 3	TAAAGGAAACCTTGAGCGGGTAACTGTC		Introduction of *dapA* promoter hexamer J3
lysC J3 Nr.2	GACAGTTACCCGCTCAAGGTTTCCTTTA		Introduction of *dapA* promoter hexamer J3
lysC T1C Nr 2	GACAGTTACCCGCTCAACTCTGCCTTTA		Introduction of *dapA* promoter hexamer T1C
lysC T1C Nr3	TAAAGGCAGAGTTGAGCGGGTAACTGTC		Introduction of *dapA* promoter hexamer T1C
lysC RACE 400	TGTCAGAACCACCACG		Determination of TSS *lysC*
lysC RACE 300	GGTGGTGAGCACACC		Determination of TSS *lysC*
lysC RACE 200	CTCACCAGCAGTCAG		Determination of TSS *lysC*
Primers for expression			
pVWEx1 for	CATCATAACGGTTCTGGC		Vector primer pVWEx1/pEKEx3
pVWEx rev	ATCTTCTCTCATCCGCCA		Vector primer pVWEx1/pEKEx3
aspA Cgl	GGAGGATCCGAAAGGAGGCCCTTCAGATGTCTAAGACGAGCAAC	BamHI	Amplification of cg1697
aspA Cgl rev	GGAGAGCTCTTAGTTCTCCAAGTAGAGCC	SacI	Amplification of cg1697
aspA Bs For	GGAGGATCCGAAAGGAGGCCCTTCAGATGTTAAACGGCCAAAAAGA	BamHI	Amplification of *aspA B. subtilis*
aspA Bs Rev	GGAGAGCTCTTATTTTTCTAATAGTTCTTTCCCTG	SacI	Amplification of *aspA B. subtilis*
aspA Ec For	GGAGGATCCGAAAGGAGGCCCTTCAGATGTCAAACAACATTCGTATCG	BamHI	Amplification of *aspA E. coli*
aspA Ec rev	GGAGAGCTCTTACTGTTCGCTTTCATCAG	SacI	Amplification of *aspA E. coli*
aspB For	CAGGTCGACGAAAGGAGGCCCTTCAGATGAGTTCAGTTTCGCTGCAG	SalI	Amplification of cg0294
aspB Rev	GTCCAGCTGTTAGTTAGCGTAATGCTCCGC	PvuII	Amplification of cg0294
aspB BSU fw	GGGTCGACGAAAGGAGGCCCTTCAGATGAAACTGGCAAAAAGAGTATCCG	SalI	Amplification of *aspB B. subtilis*
aspB BSU rv	GAGGTCGACTTAGCTATGTTTTTCTACAAAACGC	SalI	Amplification of *aspB B. subtilis*
aspC EC fw	GAGGGATCCGAAAGGAGGCCCTTCAGATGTTTGAGAACATTACCGCC	BamHI	Amplification of *aspB E. coli*
aspC EC rv	GAGGGATCCTTACAGCACTGCCACAATC	BamHI	Amplification of *aspB E. coli*
AsDH Ral fw	GAGGGATCCGAAAGGAGGCCCTTCAGATGCTGCATGTGTCCATG	BamHI	Amplification of *aspDH R. eutropha*

Table 3 continued

AsDH Ral rev	GAGGGATCCCTAGATCGATACCGCGTG	BamHI	Amplification of *aspDH R. eutropha*
AsDH Pseu fw	GAGGGATCCGAAAGGAGGCCCTTCAG ATGCTGAATATCGTCATGATCG	BamHI	Amplification of *aspDH P. aeruginosa* PAO1
AsDH Pseu rev	GAGGGATCCCTAGATCGAAATCGCGTGG	BamHI	Amplification of *aspDH P. aeruginosa* PAO1
ectA fw	CAGGGATCCGAAAGGAGGCCCTTCAGAT GACGCCTACAACCGAG	BamHI	Amplification of *ectABC C. salexigens*
ectC rev	CAGGGATCCTCAATCGACCGGTGCG	BamHI	Amplification of *ectABC C. salexigens*
ldhA for	GAGGGATCCGAAAGGAGGCCCTTCAGAT GAAAGAAACCGTCGG	BamHI	Amplification of cg3219
ldhA rev	GAGGAGCTCTTAGAAGAACTGCTTCTG	SacI	Amplification of cg3219
mdh fw	GAGGGATCCGAAAGGAGGCCCTTCAGAT GAATTCCCCGCAGAAC	BamHI	Amplification of cg0763
mdh rev	GAGGGATCCTTAGAGCAAGTCGCGC	BamHI	Amplification of cg0763
panD Fw	GAGGTCGACGAAAGGAGGCCCTTCAGAT GCTGCGCACCATCCT	SalI	Amplification of cg0172
panD rev	GAGGTCGACCGCTAAATGCTTCTCGACG	SalI	Amplification of cg0172
panD Bsu fw	CAGGGATCCGAAAGGAGGCCCTTCAGAT GTATCGAACAATGATGAGCG	BamHI	Amplification of panD *B. subtilis*
panD Bsu rev	CAGGGATCCCTACAAAATTGTACGGGCTGG	BamHI	Amplification of panD *B. subtilis*
panD Eco fw	CAGGGATCCGAAAGGAGGCCCTTCAGAT GATTCGCACGATGCTG	BamHI	Amplification of *panD E. coli*
panD Eco rev	CAGGGATCCTCAAGCAACCTGTACCGG	BamHI	Amplification of panD *E. coli*
ppc FOR	GAGGTCGACGAAAGGAGGCCCTTCAGAT GACTGATTTTTTACGCG	SalI	Amplification of cg1787
ppc REV	GAGGTCGACGAGCTAGCCGGAGTTGCGC	SalI	Amplification of cg1787
pyc FOR	GAGCCTGCAGGGAAAGGAGGCCCTTCAG GTGTCGACTCACACATC	SbfI	Amplification of cg0791
pyc REV	GAGCCTGCAGGTTAGGAAACGACGACG	SbfI	Amplification of cg0791

2.4 Cultivation and conservation of strains

2.4.1 *E. coli* strains

For the cultivation of *E. coli* DH5α and S17, lysogeny broth medium (LB) was used (Sambrook and Russell, 2001). Strains were incubated at 37 °C in baffled shaking flasks at 120 rpm or on solid agar plates (LB with 1.8 % agar). Plasmid-carrying strains were selectively incubated with appropriate antibiotics (50 µg/ml kanamycin, 100 µg/ml spectinomycin, and 100 µg/ml ampicillin)

2.4.2 *C. glutamicum* strains

C. glutamicum strains were grown aerobically at 30 °C in baffled flasks at a shaking speed of 120 rpm or on solid agar plates (BHI with 1.8 % agar). Precultivation of the *C. glutamicum* ATCC 13032 wild type strain and all deletion strains was performed in brain heart infusion (BHI). All strains harboring a deletion of the succinate dehydrogenase operon *sdhCAB* (cg0445 - cg0447) were precultured in BHI supplemented with 50 mM glucose and 100 mM 3-(N-morpholino)-propane sulfonic acid (MOPS). The pH value was adjusted to 7 with a 10 M potassium hydroxide solution (KOH). Precultures were washed

once with minimal medium and diluted to an initial optical density (OD_{600}) of 1 for the main cultivations.

Cultivation of main cultures was performed in modified CGXII minimal medium (mCGXII) developed for the ERA-IB project BioChemProBB. The medium contains 21 g/l MOPS, 5 g/l $(NH_4)_2SO_4$, 5 g/l urea, 1 g/l KH_2PO_4, 1 g/l K_2HPO_4, 0.25 g/l $MgSO_4$ x 7 H_2O, 10 mg/l $CaCl_2$ x 2 H_2O, and 0.2 mg/l biotin. The trace element solution contains 10 mg/l $FeSO_4$ x 7 H_2O, 0.1 mg/l $MnSO_4$ x H_2O, 1 mg/l $ZnSO_4$ x 7 H_2O mg, 0.2 mg/l $CuSO_4$, 0.02 mg/l $NiCl_2$ x 6 H_2O and was prepared as a stock solution (1000x). The pH value was adjusted to 7 with a 10 M KOH. Furthermore, CGXII minimal medium was used as described by Keilhauer et al. (Keilhauer *et al.*, 1993). In addition to the standard carbon source (c-source) glucose, other c-sources like maltose, arabinose, glycerol and xylose were used. Stock solutions were added to yield final concentrations of 100 - 200 mM. During media testings the concentration of ammonium sulfate and urea was modified in a range from 0 - 40 g/l. For the selection of recombinant strains, the medium was supplemented with appropiate antibiotics (100 µg/ml ampicillin, 100 µg/ml spectinomycin, and 25 µg/ml kanamycin). Induction of expression plasmids was performed by the addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG).

2.4.2.1 Anaerobic cultivation of *C. glutamicum* strains

For anaerobic cultivations, Hungate culture tubes (Dunn Labortechnik, Asbach) with screw caps and butyl rubber septum were used. Strains were precultivated in BHI-MOPS to reach the mid-exponential growth phase, harvested, and washed once with minimal medium. In a volume of 10 ml, main cultures were routinely incubated at 30 °C in a roller system with an OD_{600} of 30. Minimal medium was supplemented with different c-source- concentrations ranging from 25 to 100 mM. No selective pressure was applied with antibiotics, as anaerobic conditions prevent cell division, and therefore, a loss of recombinant plasmids. Furthermore, different bicarbonates ($NaHCO_3$, $Ca(HCO_3)_2$, and NH_4HCO_3) were added solid in concentrations ranging from 0 to 200 mM. An additional supplement to the cultures was potassium nitrate (0 - 60 mM). Samples of 1 ml were taken with syringes via the rubber septum. All figures in the results section show data of two independently grown cultures.

2.4.2.2 Cultivation of *C. glutamicum* in the Biolector

Cultivation of *C. glutamicum* in the Biolector was performed in 48 well flowerplates. Overnight cultures were washed once in minimal medium and used to inoculate the main cultures to an OD_{600} of 1. Cells were cultivated in 1 ml CGXII minimal medium supplemented with appropriate c-source (100-200 mM) at 30 °C and a shaking speed of 1100 rpm. The scattered light was detected with optodes with a filterset appropriate to measure high cell densities (gain of 20), which implies that low optical densities (below OD_{600}=1) are measured with decreased accuracy. The biomass (OD_{600}) was calculated automatically based on a calibration experiment for the flowerplates with a slope of 0.0525 and offset of -1.9481. The specific growth rate was determined in the logarithmic growth phase from the

calculated OD_{600} (gain 20) with an average of 10 data points. All graphs depicted in the results section show data from three independently grown cultures.

2.4.3 Conservation of strains

Strains were grown over night in 5 ml BHI. 700 µl of culture were mixed with 300 µl of sterile 87 % glycerol and stored at –80 °C.

2.4.4 Determination of cell dry weight

A 100 ml culture was inoculated in minimal medium supplemented with 50 mM glucose and 100 mM sodium bicarbonate to OD_{600} of 30. The experiment was performed in 100 ml Schott flask with screw cap. After 0, 24, 48 and 72 h the optical density was measured, and 20 ml of culture were sampled in 50 ml Falcon tubes, washed twice with 0.9 % sodium chloride solution and resuspended in 5 ml. As a blank, Falcon tubes were loaded with 5 ml of the solution. All samples were dried at 60 °C for three days and weight twice. All used Falcons tubes were kept at 60 °C for two days and weighed prior to use.

2.5 Generation of competent cells

2.5.1 *E. coli* DH5α and S-17 cells

1 ml overnight culture was used to inoculate 50 ml LB medium. The culture was grown as described above to an OD_{600} of 0.6. After centrifugation (7 min, 4000 rpm, 4 °C) the cells were washed three times with 25 ml RF1-solution and resuspended in fresh RF1-solution. In between every washing step cells were and stored on ice for 10 min. The final pellet was resuspended in 2 ml RF2 buffer and 500 µl aliquots were stored at –80 °C. The RF1 buffer contains 12 g/l rubidium chloride, 9.9 g/l $MgCl_2$ x 4 H_2O, 2.9 g/l potassium acetate, 1.5 g/l $CaCl_2$ x2 H_2O, and 150 g/l glycerol (87 %). The pH-value was adjusted to 5.8 with 0.2 N acetic acid. The RF2 solution contains 2.1 g/l MOPS, 1.2 g/l rubidium chloride, 11 g/l $CaCl_2$ x2 H_2O, and 15 g/l glycerol (87 %). The pH-value was adjusted to 6.8 with 0.4 M NaOH. Both buffers were sterile filtered and stored at 4 °C.

2.5.2 *C. glutamicum* cells

1 ml overnight culture was used to inoculate 50 ml BHIS medium. The culture was grown as described above. At an OD_{600} of 0.6, 15 µl ampicillin (5 mg/ml) was added and cells were incubated for another 1.5 h. Cells were harvested (4000 rpm, 7 min, 4 °C) and washed three times in 30 ml EPB1 buffer. The final pellet was resuspended in 1.5 ml EPB2 buffer and 150 µl aliquots were stored at –80 °C.

The EPB1 buffer contains of 40 ml/l 0.5 M HEPES, and 50 ml/l glycerol (100 %) ad 1 l dH_2O. Buffer EPB2 contains 2 ml 0.5 M HEPES, 30 ml glycerol (100 %), in a final volume of 200 ml dH_2O. The 0.5 M HEPES solution was adjusted to pH=7.2. All buffers were autoclaved and stored at 4 °C.

2.6 Transformation of bacterial cells

2.6.1 *E. coli* DH5α and S-17 cells

For the transformation of *E. coli* cells, 150 µl of competent cells were thawed on ice and mixed with 50-250 ng of plasmid DNA or 2-5 µl of ligation mix. After incubation on ice for 15 min, a heat shock at 42 °C was applied for 90 sec. Cells were mixed with 1 ml of LB medium and incubated for 45-60 min at 37 °C and 500 rpm in a Thermomixer (Eppendorf, Hamburg). Afterwards, cells were pelleted, resuspended in 100 µl LB medium and plated selectively on appropriate agar plates.

2.6.2 *C. glutamicum* cells

For the electroporation of *C. glutamicum* cells, an aliquot of competent cells was thawed on ice and mixed in a sterile electroporation cuvette with 100-300 ng of plasmid DNA. For transformation of suicide vectors like pK19*mobsacB*, a higher amount of DNA was used (1 – 20 µg). The electroporation was performed with a BioRad Gene Pulser Xcell (Hercules, USA) at 2.5 kV, a parallel resistance of 200 Ω, and a capacitance of 25 µF. The cell suspension was transferred in a sterile Eppendorf cup filled with 1 ml preheated BHI medium, and incubated for 6 min at 46 °C. The heat shock was applied to increase transformation efficiency, by inhibiting the restriction system of *C. glutamicum* (30). Afterwards 4 ml BHI was added, cells were incubated in a roller system at 30 °C for 1 h and finally plated on selective agar plates.

2.6.3 Conjugation (*E. coli* S-17/*C. glutamicum*)

For the conjugation of plasmid DNA a two-organism system was used including *E. coli* S17 as donor organism and *C. glutamicum* as the recipient of the plasmid. First, a 50 ml culture of *C. glutamicum* is inoculated. A 50 ml LB culture with 50 µg/ml kanamycin of the *E. coli* S17 donor strain is inoculated two hours later, so that both strains reach the desired OD_{600} of 1-1.5 at the same time. Of the recipient culture 30 ml were harvested (4000 rpm, 7min, RT) and resuspended in 3 ml LB medium. Aliquots of 800 µl were treated for 9 min at 50 °C, and afterwards, kept on ice for 5 min. Of the donor culture 10 ml were harvested (4000 rpm, 10 min, RT) and resuspended in 1 ml LB medium. 200 µl of donor cells were mixed with 800 µl of heat treated recipient cells and centrifuged for 10 min at 3000 rpm and RT. Subsequently, the pellet was resuspended in 100 µl BHI medium and transferred to a sterile nitrocellulose membrane, placed on a BHI plate without selection marker. After incubation for 20 h at 30 °C, the membrane is transferred to a test tube. Cells are removed from the membrane by adding

2 ml BHI medium and vortexing. Dilutions of the cells were plated on BHI containing kanamycin (25 µg/ml) and nalidixin (50 µg/ml). Nalidixin is used as a marker against Gram-negative species, and is therefore inhibiting growth the *E. coli* donor strain. Growing recipient cells are streaked out on fresh BHI kanamycin 25 (µg/ml)/nalidixin 50 (µg/ml) plates.

2.7 Generation of deletion strains

All deletions in this work were performed in-frame by a two step homologous recombination procedure (Eggeling and Bott, 2005). In this method, the non-replicative vector pK19*mobsacB* was used, which contains two selection markers. A kanamycin resistance cassette is used for the first step of homologous recombination, and serves thereby as a control for the integration of the vector into the chromosome. The second marker is the *sacB* gene, originated from *B. subtilis*, encoding a levan sucrase, which inhibits growth on sucrose (Jäger *et al.*, 1992). Both flanking regions of the gene of interest, each spanning at least 400 bp, were amplified and combined in a second PCR to one fragment with the help of a linker sequence. This fragment was cloned in the vector pK19*mobsacB* and the construct was transformed into *C. glutamicum*. To initiate the second step of recombination, the sensitivity to sucrose was utilized. Cells growing on sucrose lack the levan sucrase, either by disintegration of the vector from the chromosome, or due to the low stability of the *sacB* gene.
Two different strategies were used for the disintegration. First, positive clones were streaked on BHI Suc10 plates containing 10 % sucrose. If this method failed to obtain the deletion, 5 ml of an overnight culture were washed three times with CgXII medium, lacking all nitrogen- and phosphorus-sources. The culture (5 ml) was incubated at 30 °C with 25 mM glucose in the same medium for 6 h, and three dilutions (10^{-2}, 10^{-3}, 10^{-4}) were plated on BHI Suc10 agar.
Growing cells were replica-plated on BHI and BHI_{Kan25} plates. In kanamycin sensitive cells the second step of recombination, the disintegration of the vector occurred. These positive clones were tested by colony PCR for the chromosomal deletion of the gene of interest.

2.8 Isolation and purification of nucleic acids

2.8.1 Isolation of genomic DNA

Genomic DNA was isolated according to Eikmanns et al. (Eikmanns *et al.*, 1994). Cells were disrupted with SDS (10 %) and lysozyme (10 mg/ml). Proteins were denatured by Proteinase K treatment and residual RNA was degraded with RNaseA (Fermentas, Sankt Leon Roth). Residual cell debris was salted out with 6 M sodium chloride and removed by centrifugation (30 min, 5000 rpm). DNA was precipitated with ethanol and resuspended in TE-buffer (10 mM Tris/HCl, pH 8; 0,5 mM EDTA).

2.8.2 Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* with the Qiaprep Spin MiniPrep kit from Qiagen (Hilden) according to the manufacturer's protocol. In case of *C. glutamicum*, cell walls were disrupted by incubation with lysozyme (10 mg/ml lysozyme) for 2 hs prior to kit use.

2.8.3 Isolation of total RNA

Cells were cultivated under the desired conditions and grown to exponential phase (OD_{MAX}=5) and 20 ml of culture where centrifuged in 50 ml falcon tubes filled with 25 g of ice. The cells were resuspended in 350 µl RLT buffer from the RNeasy Mini Kit (Qiagen), mixed with 250 mg silica beads (d= 0.5 mm) and disrupted for 2 x 30 sec with the Silamat S6 (Vivadent, Ellwangen). After centrifugation (3 min, 16000 rpm, 4 °C) RNA was purified from the supernatant with RNeasy Mini Kit according to the manufacturer's protocol.

2.8.4 Purification of DNA

PCR products were purified with the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's recommendations. For ligations, restricted DNA (vector and insert) was purified with the "Qiaquick MinElute purification kit" (Qiagen).

2.8.5 Determination of nucleic acid concentrations

The extinction was measured spectrophotometrically at 260 nm with the Nanodrop Spectrophotometer ND-1000 from Peqlab (Erlangen) to determine the concentration of DNA. Impurities caused by proteins were determined by the factor of E260/E280 (1.8 – 2.0) , while contaminations of carbohydrates were determined with the factor of E260/E230 (> 2.0) (Sambrook and Russell, 2001).

2.9 Polymerase chain reaction (PCR) methods

2.9.1 PCR

Amplification of DNA fragments was performed as described by Saiki and Tyndall (Saiki *et al.*, 1988; Tindall and Kunkel, 1988). Taq-DNA polymerase (NEB, Frankfurt/Main) was used in analytical PCRs, for the verification of ligations or deletions. For preparative PCRs, the proofreading KOD Hot Start DNA polymerase (Novagen; San Diego, USA) was used. PCR protocols were carried out as recommended by the manufacturer. Primers were provided by Eurofins MWG operon (Ebersberg) and Metabion (Martinsried/München). The annealing temperature of the used primers was calculated with

CloneManager Suite9. Genomic DNA, plasmid DNA or lysed bacteria served as template for the PCR reactions.

2.9.2 Rapid amplification of cDNA ends with PCR (RACE-PCR)

The 5' RACE-PCR was performed to determine the transcriptional start point of the gene *lysC*. 2 µg (ad dH_2O 12 µl) of total RNA were mixed with 1 µl random primer (10 pmol/µl), and incubated at 65 °C for 5 min. After a short incubation on ice (1 min), 1 µl of a 10 mM dNTP mix, 4 µl of 5x SuperScriptII buffer, and 2 µl 100 mM DTT were added and the mix was incubated for 2 min at room temperature (RT). After the addition of 1 µl SuperScriptII (200 U), the mixture was kept for 10 min at RT, and was incubated for 50 min at 42 °C. The enzyme was inactivated for 10 min at 70 °C. For removal of residual RNA, 2.3 µl of RNAseH buffer and 1 µl RNAseH were added and incubated for 10 min at 37 °C. The cDNA was purified with the Qiagen PCR purification kit and eluted in 50 µl. For poly (cA) or poly (cT) tailing, 15 µl of cDNA were mixed with 14 µl dH2O, 8 µl of 5x tdT buffer, and 1 µl of dATP or dCTP (10 mM), respectively. The solution was heated for 3 min at 94 °C and cooled on ice. 2 µl of terminal deoxynucleotide transferase (tdT, 20 U/µl, Thermo scientific, Waltham, USA) were added and incubated for 25 min at 37 °C. The enzyme was heat inactivated for 10 min at 70 °C. Afterwards, PCRs were performed with oligo(dT) or oligo (dG) promers and the primer lysC RACE 400; the product purified, splitted, and used in another PCR with oligo(dT) or oligo(dG) and lysC RACE 300. The two products, one with polyC tail, one with polyA tail, were sequenced with lysC RACE 200. The first base after each tail resembles the transcriptional start point of *lysC*.

2.9.3 Agarose gel electrophoresis

Separation of DNA fragments was performed in agarose gels with TAE-buffer (40 mM Tris, 1 mM EDTA, 0.35 % (v/v) acetic acid, pH=8) (Sambrook and Russell, 2001). According to the size of the DNA fragments, the concentration of agarose varied between 0.8 and 2 %. DNA fragments were mixed with 6x loading buffer (0.25 % bromophenol blue (Sigma), 40 % (v/v) glycerol) and separated in an electric field (80 – 140 V). Staining of DNA fragments was performed with ethidium bromide (1 mg/l H_20) for 5 – 15 min. As DNA standards served the 1 kb DNA ladder (New England Biolabs) or the 100 bp marker (Invitrogen).

2.10 Restriction, modification and ligation of DNA

2.10.1 Restriction

Restriction of nucleic acids was performed with restriction endonucleases from Fermentas (Sankt Leon-Roth). DNA was mixed with nuclease-free water, buffer and enzyme according to the manufacturer's protocol and incubated for at least 2 h at the given temperatures.

2.10.2 Dephosphorylation of vector DNA

To prevent religation of restricted vector DNA, blunt or sticky 5'ends of DNA were dephosphorylated with the help of rapid alkaline phosphatase (Rapid DNA Dephos and Ligation Kit, Roche). The enzyme was used according to the manufacturer's protocol. However, incubation time of the dephosphorylation was exceeded to 2 h.

2.10.3 Phosphorylation of nucleic acids

Prior to blunt-end ligation, all PCR products were phosphorylated with the T4-Polynucleotide kinase from Thermo Scientific (Schwerte) according to the manufacturer's protocol.

2.10.4 Ligation

Restricted and dephosphorylated vector DNA (50-100 ng) was mixed with insert DNA in a molar ratio between 1:3 and 1:20. Ligation was accomplished by the T4-ligase of the Rapid DNA Dephos and Ligation kit from Roche (Mannheim).

2.11 Protein-biochemical methods

2.11.1 Preparation of crude protein extracts

For enzymatic assays, cells were cultivated in complex media (LB or, if necessary, BHI) to an OD_{600}=4. The cultures were harvested (10 min, 4000 rpm, 4 °C) and stored at -20 °C. Pelleted cells were washed once in the appropriate assay buffer (4 °C) and resuspended in 1 ml. The disruption of the cell wall and membrane was either achieved by sonication or bead milling. The resuspended cells were sonicated for 9 min with Hielscher (Teltow) UP200S, 24 kHz (amplitude: 50 %, pulse: 50%) in an ice bath. Bead milling was performed with Silamat S6 (ivoclar vivadent, Ellwangen), with 0.5 mm silica beads three times for 30 sec. Cell debris was removed by centrifugation (1 h, 16000 rpm, 4 °C). For discontinuous assays based on quantification by HPLC, crude protein extracts were purified with PD-10 columns (GE Healthcare, Munich), to remove residual salts and amino acids.

2.11.2 Determination of protein concentration according to Bradford

Protein concentration was determined spectrophotometrically at 595 nm (Shimadzu Spectrophotometer UV1800) using Bradford reagent (Sigma, Seelze) (Bradford, 1976). 50 µl of sample were mixed with 1.5 ml Bradford reagent and incubated for 20 to 60 min at RT. A standard curve was prepared accordingly in a range of 0.1 – 0.8 mg/ml with bovine serum albumin. All standards were prepared at least in duplicates; the protein samples were measured in at least two different dilutions.

2.12 Enzymatic assays

2.12.1 Aspartate aminotransferase (AspB) activity assay

The activity of the aminotransferase was determined by quantifying the amount of formed aspartate during the conversion of glutamate and the amino-acceptor oxaloacetate (modified from Marienhagen (Marienhagen *et al.*, 2005)). The assay was performed in 200 mM Tris-HCl buffer (pH 8) at 30 °C in a final volume of 2 ml. The assay mixture contained 50 mM L-glutamate, 0.2 mM pyridoxal-phosphate and crude protein extract (50, 100 and 200 µl purified with PD-10 columns). The reaction was started with the addition of 10 mM oxaloacetate. 350 µl samples were taken at 0, 0.5, 1, 2, and 24 h and mixed immediately with 350 µl 3 % perchloric acid to stop the reaction. After centrifugation (6 min, 14680 rpm, RT), 600 µl of the solution were mixed with 300 µl 1.5 M Tris-HCl buffer (pH 8). The produced amount of aspartate was quantified by HPLC with 200 µM asparagine as internal standard. The samples at 0 h were used to calculate the basal amount of glutamate in the crude extracts.

2.12.2 Aspartate ammonium lyase (AspA) activity assay

The aspartase catalyzes the amination of fumarate to aspartate. In a discontinuous assay, the amination of fumarate to aspartate was quantified by HPLC analysis (modified from Karsten et al. (Karsten *et al.*, 1986)). The assay was performed at 30 °C in 0.1 M Tris-HCl buffer (pH 8.4) with a final volume of 2 ml and contained 50 mM fumarate, 2 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM DTT and purified crude protein extract. 150 µl samples were taken at 0, 0.5, 1, 2, and 24 h and mixed immediately with 150 µl of 3 % perchloric acid to stop the reaction. After centrifugation (6 min, 14680 rpm, RT), 200 µl of the solution were mixed with 100 µl 1.5 M 1.5 M Tris-HCl buffer (pH 8). Aspartate concentrations were calculated according to the internal standard (200 µM asparagines). For the basal amount of aspartate, the samples at 0 h were taken in account. The deamination of aspartate was followed spectrophotometrically by the liberation of fumarate at 240 nm (ε= 2,255 $mM^{-1}*cm^{-1}$) (Kawata *et al.*, 1999). The assay was performed in 50 mM TAPS-NaOH (N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid) buffer, pH=8.5 and contained crude protein extract, 1 mM DTT, and 2 mM $MgCl_2$. The basal absorption was measured for 3 min and the reaction was started by addition of 100 mM aspartate.

2.12.3 Aspartate decarboxylase (PanD) activity assay

The activity of the aspartate decarboxylases of *C. glutamicum*, *E. coli* MG1655 and *B. subtilis* 168 were determined by measuring the conversion of aspartate to β-alanine via HPLC-analysis, modified from Dusch (Dusch *et al.*, 1999a). The reaction was kept at 30 °C, containing 5 mM aspartate, extraction buffer (0.1 M potassium phosphate, 0.5 mM dithiothreitol, pH 6.8) and dilutions of the crude extract in a final volume of 2 ml. All crude protein extracts were purified with PD-10 columns (GE Healthcare, Munich). 350 µl samples were taken at 0, 1, 2, and 24 h and mixed immediately with 350 µl of a 3 %

perchloric acid solution to stop the reaction. After centrifugation of precipitated proteins (6 min, 14680 rpm, RT), 600 µl of the supernatant were mixed with 300 µl of 1.5 M Tris-HCl buffer (pH 8) to neutralize the solution. Adequate dilutions of the mixture were measured by HPLC with 200 µM of asparagine as an internal standard. The samples at 0 h were taken as t_0 for the calculation of the specific activity.

2.12.4 Aspartate dehydrogenase (AspDH) activity assay

The assay was performed as described by Li (Li *et al.*, 2011) with crude protein extracts from a malate dehydrogenase deficient strain (Δ*sdhCAB*Δ*ldhA*Δ*mdh*). The amination of oxaloacetate was measured indirectly by detection of the oxidation of NADH at 340 nm (ε= 6.22 $mM^{-1}*cm^{-1}$) and at 30 °C. The assay mixture had a final volume of 1 ml and contained 50 µl 2 M NH_4Cl, 50 µl 4 mM NADH and 100 mM Tris-HCl buffer (pH 8). The basal absorption was measured for 3 min and the reaction was started with 50 µl of 80 mM oxaloacetate. The deamination of aspartate was monitored by measuring the reduction of NAD+. The reaction was performed in a final volume of 1 ml and contained crude protein extract, 50 µl 2.5 mM NAD^+, and 100 mM glycine NaOH buffer (pH 9.8). The reaction was started by adding 50 µl of a 1 M aspartate solution.

2.12.5 Chloramphenicol acetyl-CoA (CAT) transferase assay

The chloramphenicol acetyl-CoA transferase assay was used to determine the promoter strength of the *lysC* gene and variants thereof. The promoter probe vector pET2 carries a promoterless reporter gene for the chloramphenicol acetyl-CoA transferase. The enzyme catalyses the transfer of the acetyl group from acetyl-CoA to chloramphenicol. The free CoA-group converts the reagent 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) to yellowish 2-nitro-5-thiobenzoeic acid (TNB) which can be quantified at 412 nm (ε=13.6 $mM^{-1}*cm^{-1}$). The amount of hydrolyzed acetyl-CoA is proportional to the generated TNB. The assay contained 50 µl 0.9 M Tris-HCl (pH 7.8), 50 µl 0.9 mM acetyl-CoA, 50 µl DTNB [3.6 mg/ml dissolved in 100 mM Tris-HCl (pH=7.6)], water, and crude extract in a final volume of 500 µl. Basal absorption was measured spectrophotometrically at 412 nm for 3 min at 37 °C after addition of 50 µl 2.5 mM chloramphenicol.

2.12.6 Diaminobutyrate acetylase (EctA) assay

The acylation of diaminobutyrate with acetyl-CoA was performed at 30 °C as described by Schubert (Schubert *et al.*, 2007). The hydrolysis of acetyl-CoA was measured indirectly by the formation of 2-nitro-5-thiobenzoeic acid, which can be detected spectrophotometrically at 412 nm (ε=13.6 $mM^{-1}*cm^{-1}$). The assay mixture contains 60 mM Tris-HCl buffer (pH 8.5), 0.3 mM DTNB, 0.4 mM NaCl, and 1 mM acetyl-CoA. The basal absorption was measured for 3 min, and the reaction was induced with the addition of 30 mM diaminobutyrate.

2.12.7 Diaminobutyrate transaminase (EctB) assay

For the determination of the activity of the diaminobutyrate transaminase, the formation of glutamate was measured by HPLC analysis (Ikai and Yamamoto, 1997). In this reaction L-2, 4-diaminobutyrate and α-ketoglutarate are converted to L-aspartate-semialdehyde and glutamate. The assay mixture was kept at 30 °C and containing 100 mM Tris-HCL (pH 8.5), 10 mM L-2, 4-diaminobutyrate, 10 mM α-ketoglutarate and 0.2 mM pyridoxal phosphate in a final volume of 2 ml. The crude extracts were purified with PD-10 columns. 150 µl samples were taken at 0, 0.5, 1, 2, and 24 h and mixed immediately with 150 µl of 3 % perchloric acid to stop the reaction. After centrifugation (6 min, 14680 rpm, RT), 200 µl of the solution were mixed with 100 µl 1.5 M Tris-HCl buffer (pH 8). The generated glutamate was quantified by HPLC with 200 µM asparagine as internal standard. The samples at 0 h were used to quantify the basal amount of glutamate in the crude extracts.

2.12.8 Glyceraldehyde-3-phosphate dehydrogenase (GapA) assay

The assay was performed using a modified version from Omumasaba (Omumasaba *et al.*, 2004). Phosphorylation of glyceraldehyde-3-phosphate (GAP) to 1, 3-bisphosphoglycerate is measured spectrophotometrically by formation of NADH at 340 nm (ε= 6.22 $mM^{-1}*cm^{-1}$). The assay contained 25 µl 1 M sodium hydrogen phosphate, 25 µl 4 mM EDTA, 25 µl 1 mM NAD^+, and 50 µl 0.5 M triethanolamine hydrochloride (pH 8.5), and water in a total volume of 500 µl. The basal absorption was followed for 3 min; the reaction was started with the addition of 2.5 mM GAP.

2.12.9 Malate dehydrogenase (Mdh) activity assay

The activity of the malate dehydrogenase was performed with crude protein extracts from a malate dehydrogenase deficient strain (Δ*sdhCAB*Δ*ldhA*Δ*mdh*). The reduction of oxaloacetate to malate was measured indirectly by the oxidation of NADH at 340 nm (ε= 6.22 $mM^{-1}*cm^{-1}$) and 30 °C. The assay was performed in 100 mM Tris-HCl buffer (pH 8.2) and contained 0.2 mM NADH. The basal absorption was measured for 3 min and the formation of NADH was induced by the addition of 4 mM oxaloacetate.

2.13 Quantification of amino acids and other organic acids

2.13.1 Determination of pyruvate in culture supernatants

Due to low stability in cell free culture supernatants, pyruvate was quantified enzymatically directly after sampling. The conversion of pyruvate to lactate by the L-lactate dehydrogenase was followed photometrically by measuring the decrease of absorption of NADH at 340 nm. The standard curve covered pyruvate concentrations ranging from 1 – 100 µM. 100 µl of sample was mixed with 500 µl 100 mM Tris-HCl buffer (pH 7.4), 290 µl dH_20, and 100 µl 1.5 mM NADH. After incubation for 5 min at 37 °C, the absorption at t_0 at 340 nm was determined. The enzymatic reaction was started by addition

of 1.2 U L-lactate dehydrogenase and incubated for another 60 min at 37 °C. The amount of converted NADH is proportional to the amount of pyruvate in the supernatants and was calculated by determining the final absorption at 340 nm.

2.13.2 High performance liquid chromatography (HPLC)

For the determination of acids, sugars and amino acid quantities, an "Agilent 1200 series"-system was used. It was equipped with a diode array detector (DAD), a refractive index detector and a fluorescence detector. Columns were provided by CS-Chromatographie Service (Langerwehe). Cell free culture samples were obtained by centrifugation (6.5 min, 146800 rpm, RT).

Sugars and organic acids were isocratically detected with the RID, separated on an organic acid resin (300x8 mm) column in 5 mM sulfuric acid with a flow rate of 1 ml/min at 70 °C for 14.5 min. Standards were obtained from Sigma (Seelze), and were measured in a range from 1 to 200 mM.

The detection of ectoine was performed isocratically in 80 % acetonitrile with a flow rate of 1 ml/min with the DAD detector at 210 nm on a Multospher APS-HP 5 µ Hilic (125x4 mm) column for 16 min (Kuhlmann and Bremer, 2002). 200 µl of sample or standard was mixed with 800 µl of 100 % acetonitrile. The standards were solved in CGXII medium and covered a concentration range of 1 to 25 mM ectoine.

Amino acids were detected by the FLD (excitation at 230 nm; emission at 450 nm) by automated precolumn derivatization with *ortho*-phthaldialdehyde (OPA) and reversed-phase high performance liquid chromatography (RP-HPLC) on a LiChrosphere 100RP 18 EC 5 µ (125x4 mm) at 40 °C. For the detection of aspartate, glutamate, α- and β-alanine the program OPA1G8quick (15.5 min) was used, for lysine the program OPA1G8 (22.5 min). The programs differ in their concentration gradients, but are based on the same solvents; methanol and 0.1 M trisodium acetate buffer (pH 7.2) supplemented with 0.03 % sodium azide. For the quantification of amino acids, the quality of the derivatization was taken into account by normalizing the peak areas with the quotient of external and internal asparagine standard. Standards were obtained from Sigma (Seelze), and were measured in a range of 10 to 300 µM, depending on the respective amino acid.

Table 4: RP-HPLC program OPA1G8

Time (min)	Buffer (%)	Methanol (%)	Flow rate (ml/min)
1.0	75	25	0.35
3.0	55	45	0.60
7.0	35	65	0.60
13.0	30	70	0.60
13.2	20	80	0.60
13.4	15	85	0.60
14.0	80	20	0.60
17.0	80	20	0.60

Table 5: RP-HPLC program OPA1G8quick

Time (min)	Buffer (%)	Methanol (%)	Flow rate (ml/min)
0.0	75	25	0.35
0.5	55	45	0.60
4.0	35	65	0.60
7.0	30	70	0.60
7.2	20	80	0.60
7.4	15	85	0.60
8.0	80	20	0.60
10.6	80	20	0.60

3 Results

3.1 Production of aspartate under aerobic conditions

A major aim of this study was the rational development of *C. glutamicum* strains that overproduce aspartate. Aspartate is synthesized either by direct amination of fumarate by aspartase (AspA) or by transamination of oxaloacetate by aspartate aminotransferase (AspB). As aspartate is not only used for protein synthesis, but at the same time serves as precursor of the other amino acids of the aspartate family, i.e. asparagine, lysine, threonine, methionine, and isoleucine, and of the cell wall precursor diaminopimelate. Therefore, it occupies an important role in metabolism as both an end product and an intermediate. As a consequence, metabolic engineering of aspartate producers has to consider not only the enhancement of its biosynthesis, but also the consequences for the metabolites derived from it.

3.1.1 Aspartate does not serve as a carbon or nitrogen source in *C. glutamicum*

Prior to constructing a strain producing aspartate, it was essential to investigate the ability of *C. glutamicum* to utilize aspartate as sole carbon and nitrogen source. Therefore, the *C. glutamicum* wild type was cultivated either in CGXII medium supplemented with glucose and 200 mM aspartate as sole nitrogen source or in standard CGXII medium containing 200 mM aspartate as sole carbon source.

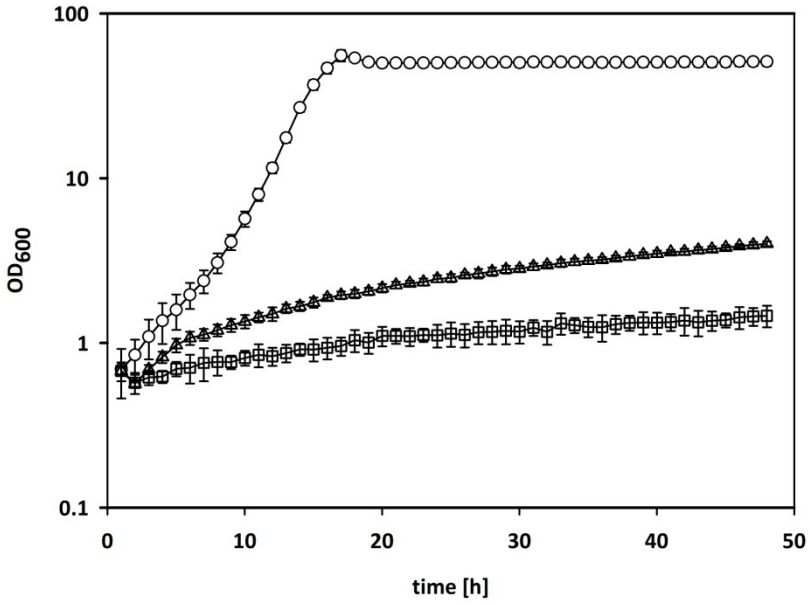

Figure 4: Growth of *C. glutamicum* pEKEx3 in CGXII containing different carbon and nitrogen sources.
Dots: Standard CGXII supplemented with 200 mM glucose; triangles: CGXII without standard nitrogen sources supplemented with 200 mM aspartate and 200 mM glucose; squares: CGXII medium supplemented with 200 mM aspartate as carbon source. All cultures were induced with IPTG. The cells were cultivated in the BioLector microfermenter at 30 °C in microflower plates at 1100 rpm in a total volume of 1 ml.

The *C. glutamicum* wild type shows exponential growth in standard medium supplemented with glucose. In contrast, only poor growth was detected with aspartate as sole nitrogen source (Figure 4). In comparison to the standard sources urea and ammonium sulfate, utilization of aspartate as nitrogen source is possible, but inefficient. Negligible growth was detected when aspartate served as a carbon source. Therefore, *C. glutamicum* fulfills basic requirements as a host organism for the synthesis of aspartate.

3.1.2 Expression of aspartase or aspartate aminotransferase is not sufficient for aspartate production

The aspartase from *E. coli* was shown to promote lysine formation in *C. glutamicum*, when cells are fed with additional fumarate (Menkel *et al.*, 1989), and engineered versions of this enzyme are used in industrial synthesis of aspartate since decades (Chibata *et al.*, 1974; Jayasekera *et al.*, 1997). To investigate whether the overexpression of the genes encoding the aspartase AspA (*aspA*, b4139) from *E. coli* or the aspartate aminotransferase AspB (*aspB*, cg0294) from *C. glutamicum* leads to the overproduction of aspartate in the wild type, both genes were cloned in the inducible expression vector pEKEx3. Strains carrying either pEKEx3-*aspA*$_{Ec}$ or pEKEx3-*aspB* were cultivated in CGXII medium supplemented with 200 mM glucose. In comparison to the wild type carrying pEKEx3, both strains showed comparable growth behavior with rates of 0.36 ± 0.00 h^{-1} (AspA) and 0.38 ± 0.03 h^{-1} (AspB) and similar biomass formation. Analysis of the supernatants after 48 hours by HPLC analysis revealed neither aspartate nor any other amino acids or organic acids.

3.1.3 Deletion of the fumarase gene *fum* and expression of *aspA*$_{Ec}$ is not sufficient for aspartate production

The fumarase Fum (*fum*, cg1145) catalyzes the hydration of fumarate to malate. A deletion of *fum* interrupts the TCA cycle and presumably leads to an accumulation of fumarate, which serves as precursor for the amination reaction by AspA to aspartate. Therefore, a wild type derivative with a deleted *fum* gene was transformed with the plasmid pEKEx3-*aspA*$_{Ec}$ and cultivated in CGXII medium supplemented with 200 mM glucose.

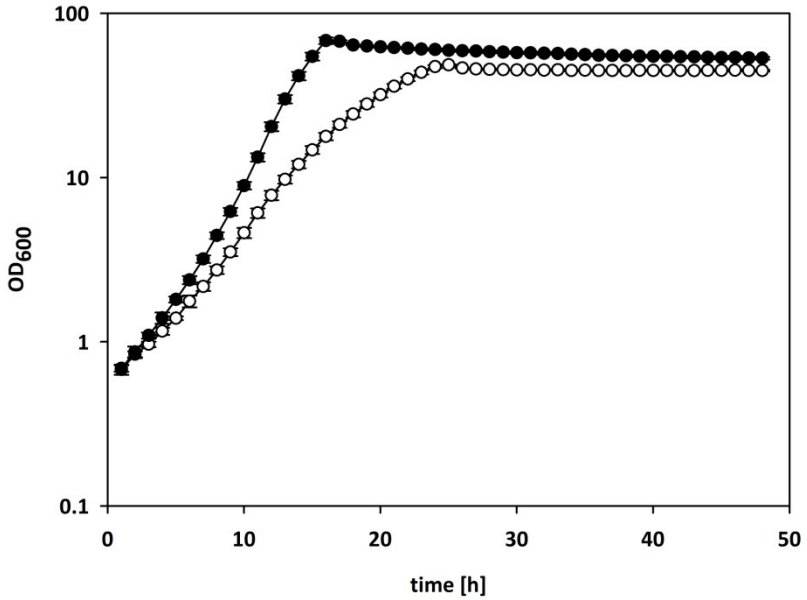

Figure 5: Growth of Δ*fum* in CGXII medium supplemented with 200 mM glucose.
Open dots: Δ*fum* pEKEx3; filled dots: Δ*fum* pEKEx3-*aspA*$_{Ec.}$ All cultures were induced with IPTG. The cells were cultivated in the BioLector microfermenter at 30 °C in microflower plates at 1100 rpm in a total volume of 1 ml.

Interestingly, the Δ*fum* strain expressing *aspA*$_{Ec}$ showed an increased growth rate compared to the control strain (0.38 ± 0.00 h^{-1} and 0.25 ± 0.00 h^{-1}, respectively) and reaches a 28 % increased biomass of 14.6 ± 0.3 gCDW*l^{-1} (Figure 5). However, the supernatants did not contain significant amounts of aspartate. Instead 3.4 ± 0.1 mM of alanine and 30.0 ± 1.9 mM of acetate were detected by HPLC analysis after 48 hours.

3.1.4 Aspartate aminotransferase versus aspartase: a futile cycle?

In *C. glutamicum*, the endogenous aspartate aminotransferase AspB (*aspB*, cg0294) transfers the amino group from glutamate to oxaloacetate. To prevent a futile cycle between the amination of fumarate by the plasmid encoded aspartase and the deamination of aspartate by the aspartate aminotransferase, a deletion of the *aspB* gene was generated. The deletion was introduced with the plasmid pK19*mobsacB-aspB* in the wild type as well as into a strain with deleted succinate dehydrogenase and lactate dehydrogenase (Δ*sdhCAB*, cg0445-cg0447 and Δ*ldhA*, cg3219). The strains were transformed with the inducible expression vectors pEKEx3-*aspA*$_{Ec}$ and pEKEx3-*aspB*$_{Cg}$, and cultivated in CGXII medium supplemented with 200 mM glucose.

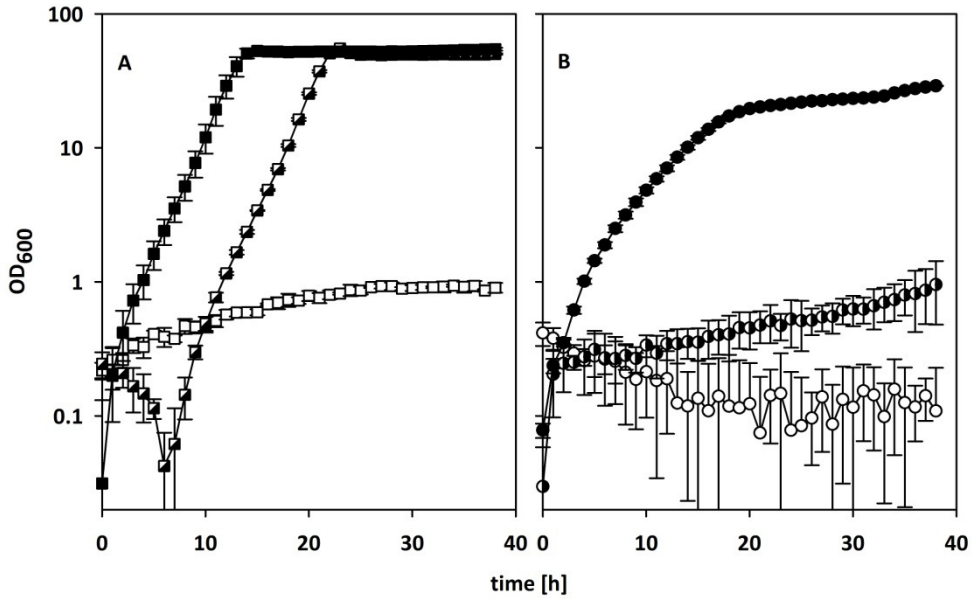

Figure 6: Growth of strains with deleted aspartate aminotransferase in CGXII supplemented with 200 mM glucose.
A: Δ*aspB*; B: Δ*sdhCAB* Δ*ldhA* Δ*aspB*. Open symbols: pEKEx3; semi-filled symbols: pEKEx3-$aspA_{Ec}$; filled symbols: pEKEx3-$aspB_{Cg}$. All cultures were induced with IPTG. The cells were cultivated in the BioLector microfermenter at 30 °C in microflower plates at 1100 rpm in a total volume of 1 ml.

Both mutants could be cultivated in complex medium without any detectable *aspB*-dependent growth defect (data not shown). In contrast, the Δ*aspB* strain is characterized by a severe growth defect in glucose minimal medium, which could be rescued by the expression of a plasmid encoded *aspB* gene (Figure 6A, open and closed squares). Furthermore, the addition of 100 mM aspartate to a culture of Δ*aspB* pEKEx3 did not enable growth, indicating the lack of an aspartate specific uptake system (data not shown). When transformed with the plasmid pEKEx3-$aspA_{Ec}$ (Figure 6A, semi-filled symbols), the Δ*aspB* strain showed a prolonged lag-phase of six hours and then resumed growth with a rate (0.41 h^{-1}) comparable to that of strain Δ*aspB* pEKEx3-$aspB_{Cg}$ (0.42 h^{-1}). To differentiate if this growth behavior is due to a mutation or an adaptation, the cultures of strain Δ*aspB* pEKEx3-$aspA_{Ec}$ were inoculated a second time under the same growth conditions, including a precultivation in complex medium. Cultures immediately started growing without a lag-phase, implying that one or more mutations had occurred that rescue the observed phenotype (data not shown). Growth of this strain was strictly dependent on the expression of the aspartase gene.

The Δ*sdhCAB*Δ*ldhA* background is characterized by an interruption of the oxidative branch of the TCA cycle at the level of succinate, and a deletion of the *aspB* gene leads to a different growth behavior (Figure 6B). In this case, the severe growth defect caused by the deletion of *aspB* could only be complemented by plasmid-encoded AspB whereas plasmid-encoded $AspA_{Ec}$ only allowed very slow growth. Taken together, this suggests that the mutation allowing growth of strain

Δ*aspB* pEKEx3-*aspA*$_{Ec}$ could be in the fumarase gene, causing a reduced activity and thus improved conversion to aspartate by the aspartase.

A futile cycle between the synthesis of aspartate by AspA and its deamination by AspB can therefore be excluded. *E. coli* AspA cannot rescue the deletion of the *aspB* gene without further mutations, which implies that the enzyme is not able to synthesize aspartate under the tested conditions. Interestingly, the endogenous aspartase AspA of *C. glutamicum* (*aspA*, cg1697) is either not activated as a back-up system or not able to rescue the *aspB* deletion in the control strains.

3.1.5 Deletion of *panD* does not improve the anaplerotic flux towards oxaloacetate

The aspartate-α-decarboxylase PanD (*panD*, cg0172) generates the precursor β-alanine for the synthesis of pantothenate, which is a crucial part of coenzyme A. A deletion of the *panD* gene impairs the synthesis of acetyl-CoA in the pyruvate dehydrogenase complex thereby blocking the oxidative branch of the TCA cycle. This leads to an accumulation of pyruvate, which can be carboxylated to oxaloacetate by the anaplerotic enzyme pyruvate carboxylase Pyc (*pyc*, cg0791). Furthermore, PanD uses aspartate as substrate and irreversibly decarboxylates it to β-alanine.

A deletion of the *panD* gene was obtained in the wild type with the vector pK19*mobsacB-panD*, which deletes 339 bp of 411 bp. The Δ*panD* strain was transformed with the expression vector pEKEx3-*aspB*, and cultivated in CGXII medium (Figure 7). In contrast to previous results from Dusch *et al.*, all strains were able to grow in glucose minimal medium without supplementation of pantothenate (Dusch *et al.*, 1999b). Cells overexpressing the *aspB* gene showed improved growth compared to the control strain with rates of 0.37 ± 0.00 h^{-1} (Δ*panD* pEKEx3-aspB) and 0.27 ± 0.01 (Δ*panD* pEKEx3), respectively. The Δ*panD* pEKEx3-*aspB* cultures excreted alanine (20.6 ± 3.1 mM) and valine (14.1 ± 3.2 mM) as main fermentation products, but no aspartate was detected in the supernatants.

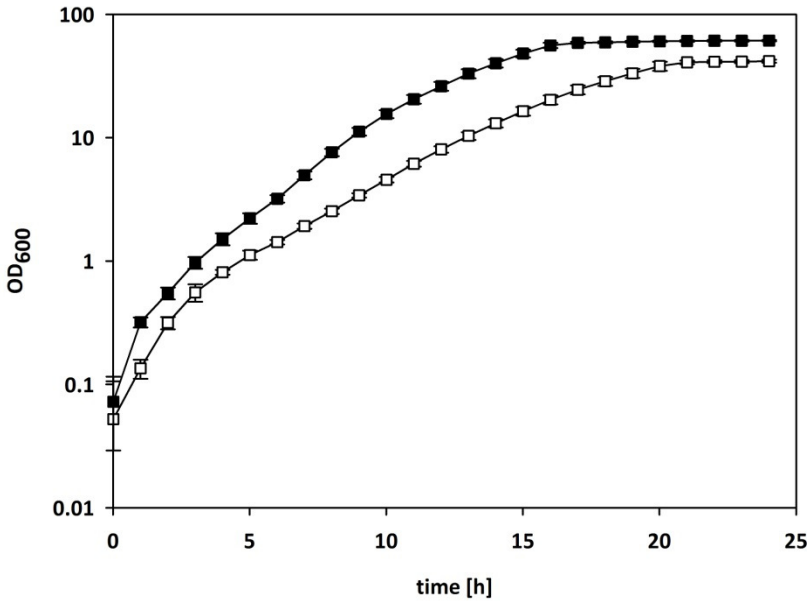

Figure 7: Growth of *C. glutamicum* Δ*panD* in CGXII medium supplemented with 200 mM glucose.
Open squares: Δ*panD* pEKEx3; filled squares: Δ*panD* pEKEx3-*aspB.* All cultures were induced with IPTG. The cells were cultivated in the BioLector microfermenter at 30 °C in microflower plates at 1100 rpm in a total volume of 1 ml.

3.1.6 The aspartokinase LysC

The aspartokinase LysC (*lysC*, cg0306) catalyzes the first step in the synthesis of amino acids derived from aspartate. It transfers the terminal phosphate from ATP to aspartate and activates the molecule for subsequent reactions. In the *C. glutamicum* wild type, LysC is feedback inhibited by lysine and threonine, for which reason feedback-resistant variants of the enzyme are used in industrial amino acid synthesis (Shiio and Miyajima, 1969).

3.1.6.1 The aspartokinase is an essential enzyme of *C. glutamicum*

The aspartokinase constitutes one of the main targets for constructing a strain overproducing aspartate, as it marks the entry point for biosynthetic pathways based on this amino acid. An inhibition of this reaction reduces the conversion of aspartate to other amino acids and diaminopimelate and should lead to an accumulation of the compound. In order to inactivate the aspartokinase, the deletion vector pK19*mobsacB-lysC* was constructed. In case of a successful deletion, a LysC´-peptide of 20 amino acids is synthesized. The plasmid was transformed into *C. glutamicum* and the kanamycin-resistant integration mutants obtained by the first homologous recombination event were exposed to sucrose to induce a second homologous recombination event. In total, twelve integration mutants were obtained, 2000 clones were replica plated, and approximately 300 colony-PCRs were performed. However, none of the tested clones showed the desired *lysC* deletion. All kanamycin-sensitive and

sucrose-resistant clones showed the wild-type genotype, strongly indicating that *lysC* is an essential gene in *C. glutamicum* and cannot be deleted.

3.1.6.2 Analysis of promoter variants for the downregulation of *lysC*

To gain insight into the expression of the *lysC-asd* operon, the transcriptional start site (TSS) of *lysC* was mapped by 5´RACE-PCR. The TSS is located 36 bp upstream of the start codon GTG. In front of the TSS, a common σ^A binding motif with the sequence TAGAGT was found, which is also present in other promoter regions and matches the -10 consensus sequence **TA**NAN**T** for *C. glutamicum* closely (Barreiro *et al.*, 2004; Follettie *et al.*, 1993; Jungwirth *et al.*, 2008).

To identify appropriate substitutes that reduce the expression of the aspartokinase, the strength of the native promoter was determined. The best studied promoter of *C. glutamicum* is the *dapA* promoter, where the exchange of all bases of the -10 hexamer showed a clear influence on the strength of *dapA* expression. The first and the last thymine corresponding to the positions -14 and -9, are the most important bases for functional expression (Vasicova *et al.*, 1999). Three promoters of the *dapA* study, which lead to a weak expression of the gene, were chosen to replace the native promoter of *lysC*. Additionally, a fourth promoter sequence was used, in which the first base was changed from thymine to cytosine (T1C) (Figure 8).

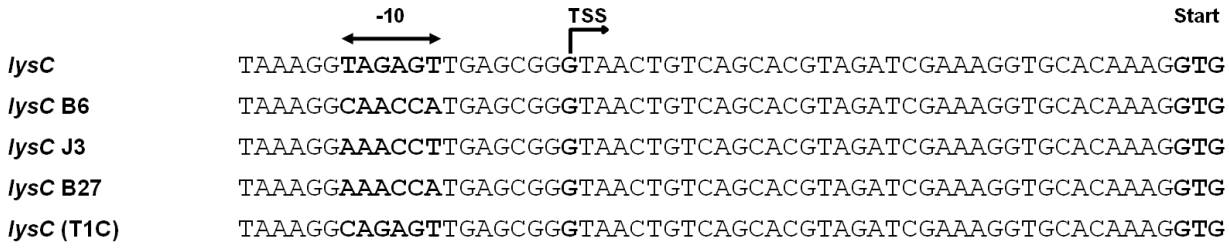

Figure 8: Promoter region of *lysC* and variants thereof.
The -10 region, transcriptional start point (TSS), and the start codon are indicated. The hexamers B6, J3 and B27 are -10 variants of the *dapA* promoter. In the T1C hexamer, the first base of the *lysC* promoter was exchanged (T→C).

The promoter variants depicted in Figure 8 were introduced in the intergenic region of cg0305 and *lysC* and the complete intergenic region was cloned in the promoter probe vector pET2, which contains a promoterless chloramphenicol acetyl transferase gene. The corresponding pET2 derivatives were transferred into *C. glutamicum* wild type and expression of the reporter gene was measured in a photometric assay.

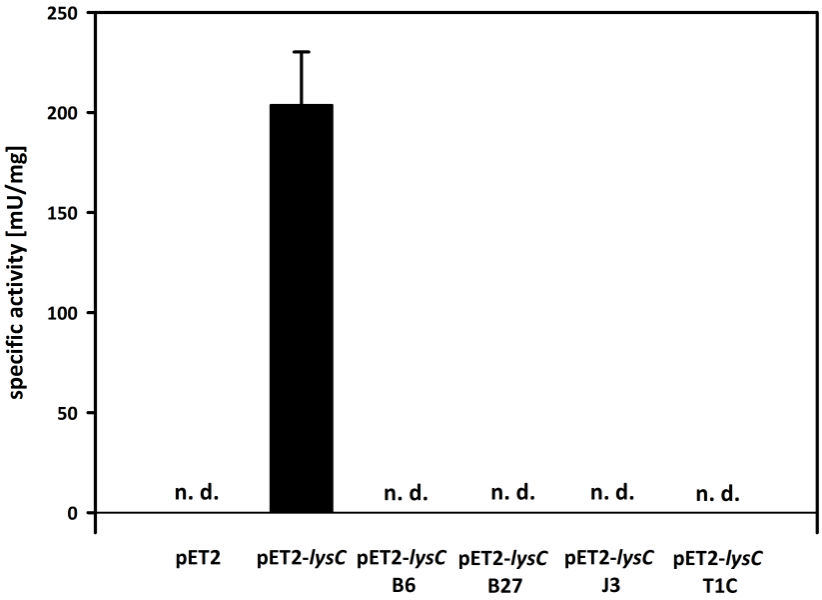

Figure 9: Specific chloramphenicol acetyltransferase (CAT) activity of *C. glutamicum* wild type carrying the indicated pET-plasmids.
The CAT activity correlates with the strength of the promoter. N. d.: not detected.

For the wild type promoter an activity of 203.7 ± 26.5 mU*mg^{-1} was detected (Figure 9). For the hexamers B27, J3 and B6 specific activities between 20 to 40 mU*mg^{-1} were reported by Vasicova (Vasicova *et al.*, 1999). In contrast, no activity of the reporter enzyme was detected in the experiment described here. The exchange of the first base of the σ^A core hexamer TANANT from T to C leads to an inactivation of the promoter, as no activity was detected in the CAT-assay (Figure 9). As the complete exchange of the promoter and the single nucleotide exchange hamper the expression of the *cat* gene, no attempt was performed to use these fragments *in vivo* for a down regulation of the *lysC-asd* operon. A reduction of the activity by controlling the expression level of the *lysC* gene was not possible with the tested promoters.

3.2 Production of aspartate under anaerobic conditions

As an alternative approach for aspartate overproduction, anaerobic instead of aerobic conditions were chosen. *C. glutamicum* does not grow under these conditions, but glucose is metabolized to lactate, acetate and succinate. Succinate is formed in the reductive branch of the TCA cycle via oxaloacetate, malate and fumarate. As the cells do not grow, aspartate formed under anaerobic conditions from oxaloacetate or fumarate is presumably not used as precursor for other amino acids and diaminopimelate. In this way, anaerobic conditions may mimic a *lysC* deletion. For anaerobic aspartate production, *C. glutamicum* Δ*sdhCAB* Δ*ldhA* (SL) is used, which harbors in-frame deletions of genes for the succinate dehydrogenase (*sdhCAB*, cg0445-0447) and the L-lactate dehydrogenase (*ldhA*, cg3219).

These deletions ensure reduction of the by-products lactate and succinate and interrupt the reductive branch of the TCA cycle at the level of fumarate. However, *C. glutamicum* is unable to proliferate under anaerobic conditions without a terminal electron acceptor. Therefore, the cells for anaerobic aspartate production have to be precultured aerobically and then packed at high optical densities for the anaerobic process.

3.2.1 Aspartate is produced under anaerobic conditions

A culture of SL pEKEx3 was inoculated in anaerobic test tubes to an OD_{600}=30 and incubated at 30 °C on a rolling shaker. The CGXII minimal medium was supplemented with 50 mM glucose and contains ammonium sulfate ($(NH_4)_2SO_4$) and urea (CH_4N_2O) as nitrogen sources. After 72 hours, the supernatants of the cultures were analyzed by HPLC for the presence of amino acids and organic acids, as well as residual glucose (Figure 10). In contrast to aerobic cultivations, the production of aspartate is possible when cells are cultivated under anaerobic conditions. Within 72 hours, 25.5 mM aspartate were detected in the supernatants, corresponding a molar yield of 0.55 mol/mol glucose. During the fermentation, glutamate, alanine, succinate, lactate, and acetate accumulate as by-products. The amino acid alanine (20.5 mM) is the main by-product, while the excretion of succinate and lactate is not completely prevented by the deletion of the succinate dehydrogenase and the lactate dehydrogenase genes.

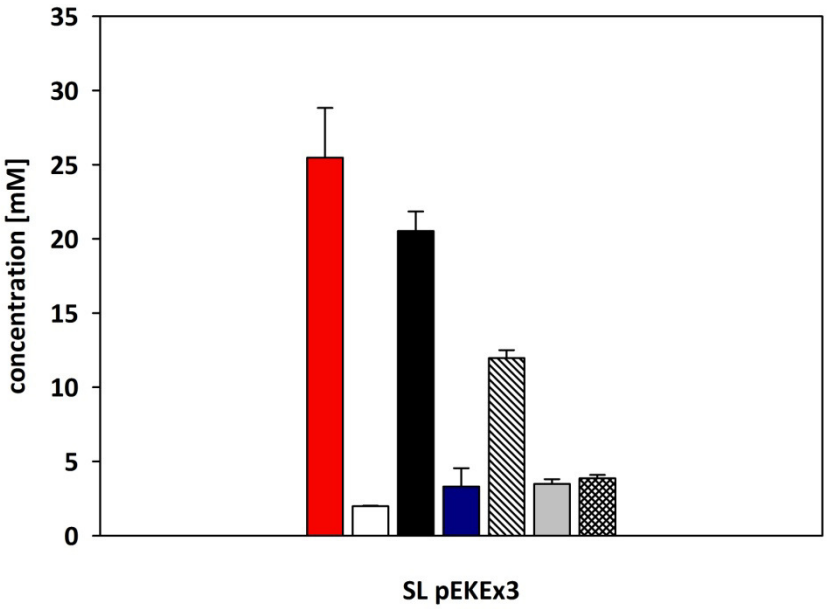

Figure 10: Production of aspartate by SL pEKEx3 under anaerobic conditions.
The strain was inoculated in CGXII medium supplemented with 50 mM glucose. Accumulation of amino acids, organic acids and residual glucose in supernatants is depicted after 72 h. Red: aspartate; white: glutamate; black: alanine; blue: glucose; striped: succinate; grey: lactate.

3.2.2 Optimization of cultivation conditions

3.2.2.1 Optimization of precultivation conditions promotes growth of SL

The precultivation conditions need to be adapted to the special needs of the strain SL. The deletion of the succinate dehydrogenase led to decreased growth in lysogeny broth (LB) medium so that precultivation is best performed in BHI medium, which contains 2 % glucose. In this medium, the strain is able to grow to an optical density of OD_{600}~5 while the wild type is able to grow to OD_{600}~12. The Δ*sdhCAB* strain synthesizes 40 mM succinate from 200 mM glucose in CGXII, which causes acidification of the medium (Litsanov *et al.*, 2012b). Hence, the BHI medium was buffered with 100 mM MOPS and the pH was adjusted to pH 7. Still the growth of SL ceases at OD_{600}~5 (Figure 11). To further increase cell density of the preculture, 50 mM glucose was added to the BHI-MOPS medium. This enables growth to an optical density of OD_{600}~20 and delivers a reasonable amount of cells for inoculation of the main cultures and prevents acidification of the medium.

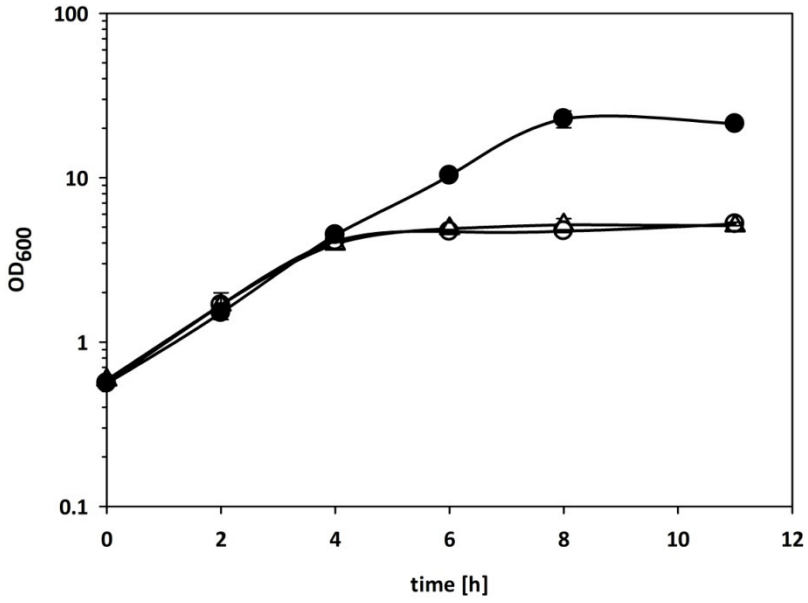

Figure 11: Growth of SL in complex medium.
Open triangles: growth in BHI; open dots: growth in BHIMOPS; filled dots: growth in BHIMOPS supplemented 50 mM glucose.

3.2.2.2 Addition of sodium hydrogen carbonate improves the production of aspartate under anaerobic conditions

The standard CGXII medium is optimized for the aerobic synthesis of amino acids and requires adaptations for anaerobic cultivations of SL. Under these conditions, the reductive branch of the TCA cycle is active and fueled by the carbon dioxide fixing reactions of phosphoenol pyruvate carboxylase and pyruvate carboxylase. To promote these anaplerotic reactions, the strain SL pEKEx3-*aspB* is

incubated with 50 mM glucose and increasing concentrations of sodium hydrogen carbonate ($NaHCO_3$). Samples were taken after 72 hours and supernatants were analyzed by HPLC for formation of amino and organic acids.

With increasing concentrations of $NaHCO_3$, the aspartate titer elevates from 26.7 mM to 41.7 mM (Figure 12). This is an improvement of 56 % compared with the standard medium. A positive side effect of $NaHCO_3$ utilization is the reduction of alanine in the supernatants. It still constitutes the most prominent by-product, but the amount is lowered by 40 % to 16.7 mM. A further increase of $NaHCO_3$ above 2mol/mol glucose had no beneficial effect on aspartate concentration. However, the amount of alanine was further reduced to 12.1 mM when 200 mM was added. Consequently, the concentration of two mol bicarbonate per mol glucose was used for all further experiments.

Organic acids where quantified by HPLC, but the amounts of succinic and lactic acid are not influenced by the addition of $NaHCO_3$. An accumulation of fumarate was not detected. Due to the instability of pyruvate in supernatants it was tested enzymatically directly after the sampling of the probes, but the precursor for alanine synthesis was not detected.

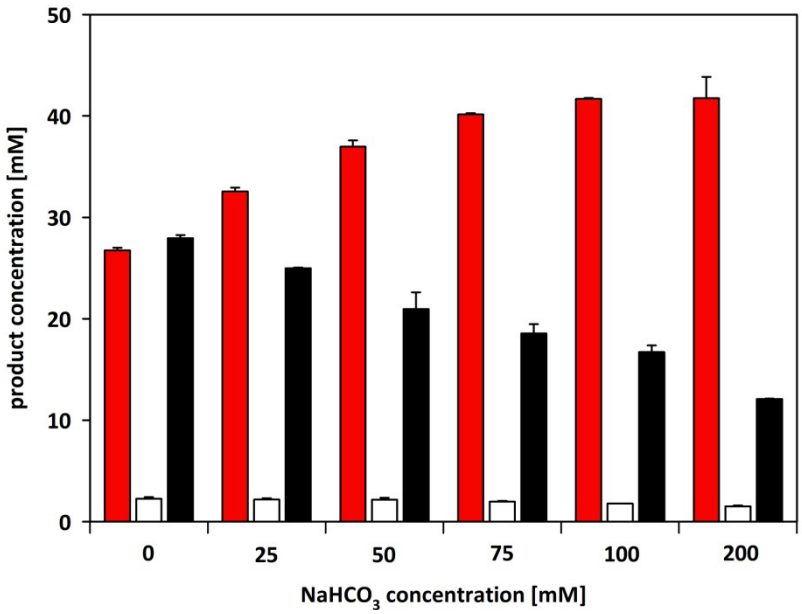

Figure 12: Aspartate synthesis positively correlates with increasing NaHCO3 concentrations.
The strain SL pEKEx3-*aspB* was incubated for 72 h with 50 mM glucose and $NaHCO_3$ (0-200 mM). Red: aspartate; white: glutamate; black: alanine; dark blue: glucose.

Besides $NaHCO_3$, also potassium ($KHCO_3$) and ammonium (NH_4HCO_3) hydrogen carbonate were tested as supplements with concentrations of 2 mol/mol glucose. The latter is of special interest, because it may also increase the input of ammonium to the medium. Compared to $NaHCO_3$, both tested

carbonates had no beneficial effects. The use of $KHCO_3$ reduced aspartate formation by 22 %, while NH_4HCO_3 shows results similar to $NaHCO_3$ (data not shown).

3.2.2.3 Addition of nitrate promotes glucose consumption and inhibits aspartate production

To substitute oxygen during the cultivation, potassium nitrate was used in the standard medium as terminal electron acceptor. The strain SL is impaired in the fermentation of lactic and succinic acid and shows a slow glucose consumption which is presumably improved by the addition of nitrate.

Cultures of SL pEKEx3 were supplemented with concentrations of 0, 30, or 60 mM nitrate, and the influence on glucose consumption was determined. After 0, 2.5, 6, 8, 10 and 24 hours supernatants were sampled and tested for the presence of glucose as well as amino and organic acids.

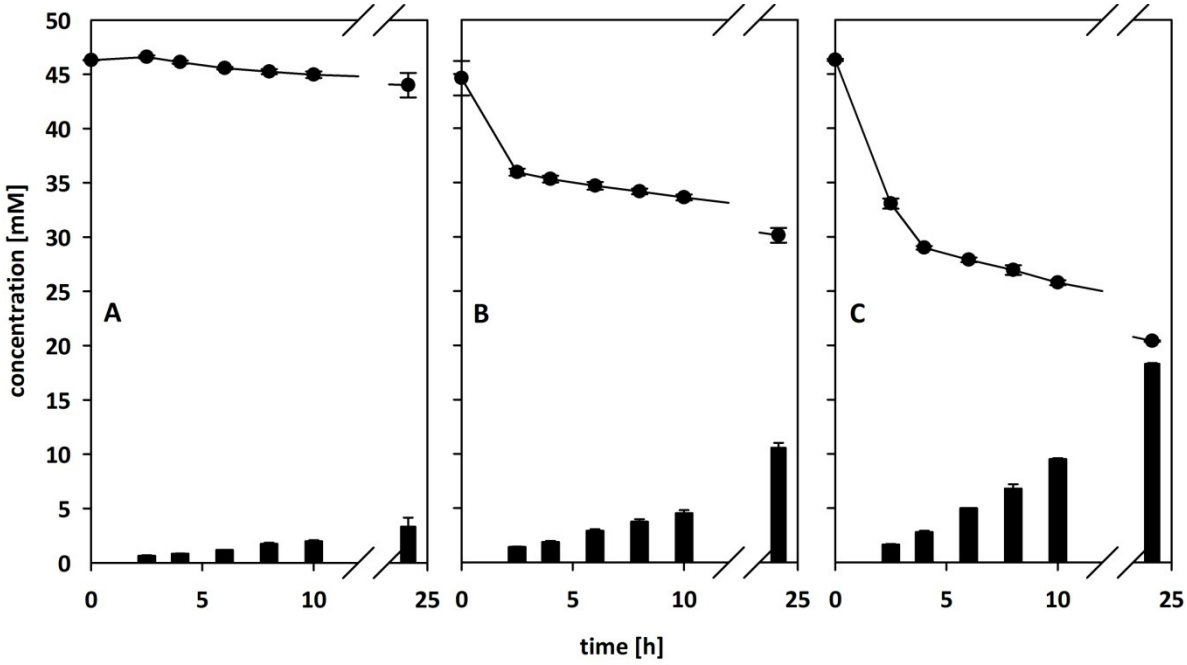

Figure 13: Anaerobic glucose consumption of SL depending on the nitrate concentration.
Cultures were inoculated to OD_{600}=30 in CGXII minimal medium supplemented with 50 mM glucose and with the indicated concentrations of potassium nitrate. A: 0 mM nitrate; B: 30 mM nitrate; C: 60 mM nitrate. Dots: glucose concentration; bars: alanine.

Addition of nitrate leads to a bi-phasic consumption of glucose (Figure 13). Depending on the concentration, the first phase is visible in the first four hours of incubation. The second phase shows a linear decrease of glucose. During this phase, consumption of glucose is 0.19 $mM*h^{-1}$ without nitrate, 0.28 $mM*h^{-1}$ with 30 mM nitrate, and 0.53 $mM*h^{-1}$ with 60 mM nitrate. The main fermentation product is the amino acid alanine.

To enable complete glucose consumption by the control strain without nitrate, the incubation time was prolonged. Samples were taken after 24, 48 and 72 hours and amino acid composition was determined (Figure 14). Without nitrate, the concentrations of aspartate and alanine increase over time reaching 16.9 mM and 15.1 mM within 72 hours, respectively. In contrast, the low amount of glutamate in the supernatants is not influenced by nitrate concentrations. However, the synthesis of aspartate is diminished by nitrate and alanine formation is promoted up to a concentration of 32.6 mM. Furthermore, alanine synthesis is accelerated by increasing the nitrate concentration to 60 mM.

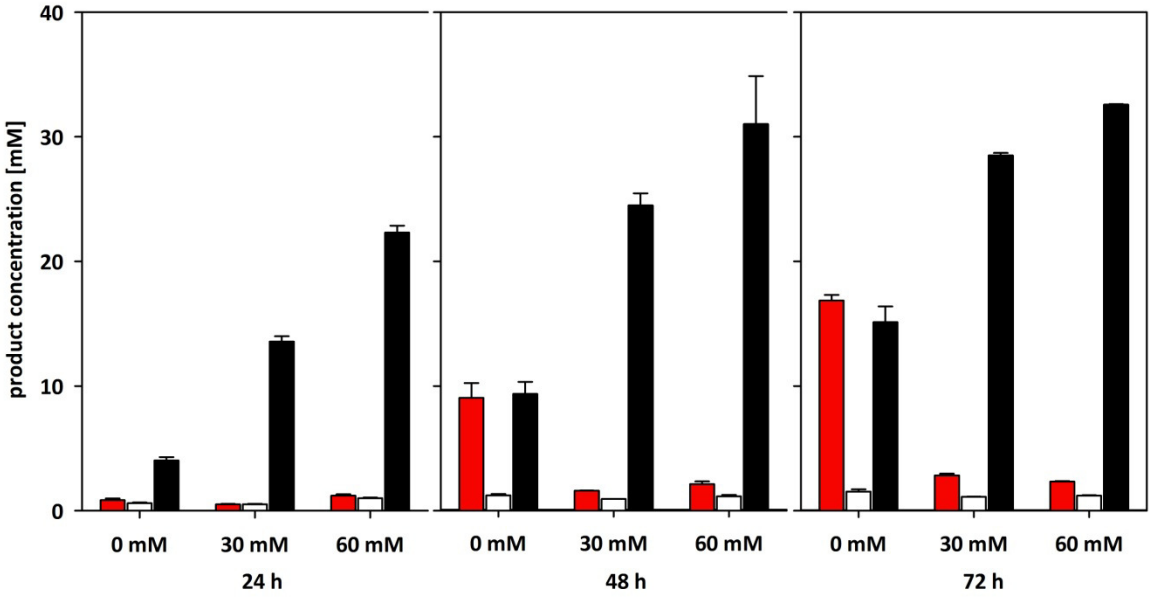

Figure 14: Anaerobic amino acid synthesis of SL pEKEx3 depending on nitrate concentration.
Depicted is the amino acid composition of supernatants at the indicated time points. Cultures inoculated to OD_{600}=30 in minimal supplemented with 50 mM glucose and 0, 30, or 60 mM potassium nitrate. Red: aspartate; white: glutamate; black: alanine.

3.2.2.4 Biomass is stable under anaerobic conditions

In production experiments, all tested cultures are packed to an OD_{600} of 30, but showed a significant decrease to OD_{600} of about 20 within the first 24 hours of cultivation. To test whether this decline was caused by cell death, the cell dry weight (CDW) of cultures was determined and compared to the optical density (Figure 15). After 24 hours, a drop in the OD_{600} from 30.8 to 19.1 was observed. Over time, the OD stabilizes at this level and did not decrease any further. Even though the CDW of the cultures decreased as well, this decline did not correlate with the drop in OD. The CDW is reduced from 7.43 to 6.27 g/l which resembles a change of OD_{600} of approximately 4. Biomass is therefore lost to a small extent (~13 %) in the beginning of anaerobic cultivation, but is generally stable over time.

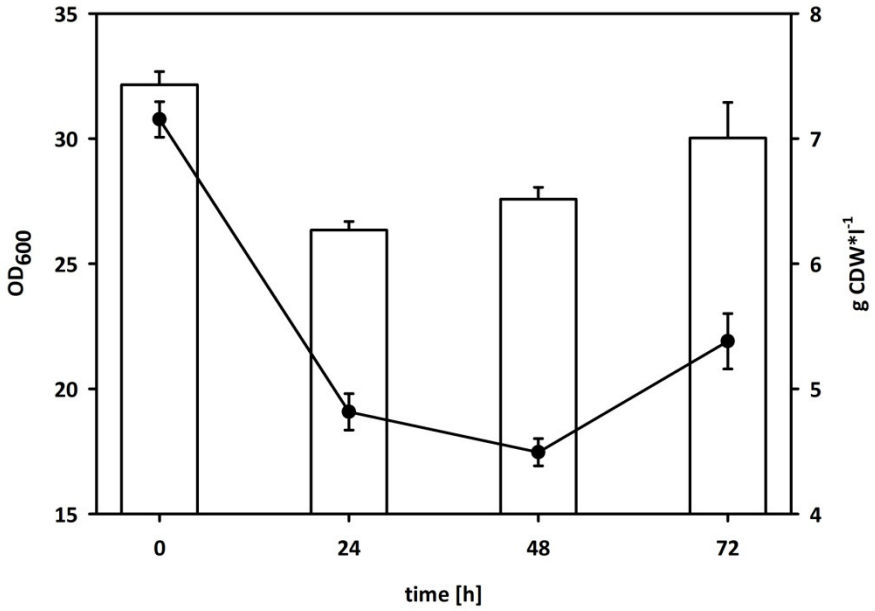

Figure 15: Comparison of optical density and cell dry weight in the strain SL pEKEx3.
Circles: OD_{600}; bars: gram cell dry weight (gCDW*l-1).

3.2.3 Synthesis of aspartate from the TCA intermediates fumarate and oxaloacetate

3.2.3.1 Aspartases favor degradation of aspartate over amination of fumarate

The deletion of the *sdhCAB* genes interrupts the reductive branch of the TCA cycle at the level of fumarate, which can then be aminated to aspartate by aspartase (EC 4.3.1.1). To find the best suited enzyme for production, the endogenous AspA (cg1697, *aspA*) as well as homologous enzymes from *B. subtilis* (BSU23570, *aspA*) and *E. coli* (b0928, *aspC*) were tested. The activity of the enzymes was tested in both directions, i.e. for the amination and the deamination reaction. The corresponding genes were expressed with 1 mM IPTG with the expression vector pEKEx3 and crude extracts obtained from cultures cultivated in complex medium were used for measurement of the aspartase activity.

Table 6: Specific activities for aspartate synthesis and degradation of aspartases of *C. glutamicum*, *B. subtilis*, and *E. coli*.
The corresponding genes were overexpressed in *C. glutamicum* wild type using the IPTG-inducible vector pEKEx3. Cell-free extracts were obtained from cells cultured aerobically in BHI medium with 1 mM IPTG to OD_{600} of 4, and used for the enzymatic assay. Abbreviations: *Bs*: *B. subtilis*; *Cg*: *C. glutamicum*; *Ec*: *E. coli.*

	Amination [mU/mg]	Deamination [mU/mg]
***C. glutamicum* pEKEx3**	7.9 ± 1.7	34.9 ± 20.0
***C. glutamicum* pEKEx3-*aspA*$_{Bs}$**	120.2 ± 19.6	1273.0 ± 283.9
***C. glutamicum* pEKEx3-*aspA*$_{Cg}$**	55.7 ± 7.9	72.5 ± 16.6
***C. glutamicum* pEKEx3-*aspA*$_{Ec}$**	105.4 ± 18.6	693.9 ± 122.5

The specific activities for the amination reaction of AspA from *B. subtilis* and *E. coli* are in a similar range, although the highest activity was measured for $AspA_{Bs}$ (Table 6). Interestingly, the native enzyme of *C. glutamicum* has a more than 50 % reduced activity.

In comparison to the amination reaction, much higher activities were measured for the deamination of aspartate. $AspA_{Bs}$ showed a tenfold increase in activity (1273 mU/mg). The activity of $AspA_{Ec}$ is also increased, but is approximately 46 % lower than the latter (694 mU/mg). Lowest activity was detected for the native AspA, which is comparable to the activity of the amination reaction.

In conclusion, the preference for the degradation of aspartate is prominent in all tested enzymes. Although the native enzyme showed the lowest activity for the deaminating reaction, it was not chosen for the production of aspartate, caused by the low activity in the aminating direction. Overall, the enzymes of *B. subtilis* and *E. coli* were much more active. The difference in activity between amination and degradation is lower for $AspA_{Ec}$ than for $AspA_{Bs}$, leaving it as the most suitable enzyme for aspartate synthesis.

3.2.3.2 Expression of $aspA_{Ec}$ reduces aspartate formation

In order to produce aspartate directly from fumarate under anaerobic conditions, the strain SL was transformed with the expression vector pEKEx3-$aspA_{Ec}$ and used in production experiments. Supernatants were taken after 144 hours of anaerobic incubation in glucose-bicarbonate medium and analyzed by HPLC (Figure 16). In both strains a significant formation of aspartate was detected, but the expression of the aspartase gene from *E. coli* decreased aspartate synthesis by 26 % to 0.53 mol/mol glucose. Moreover, the strain SL pEKEx3-$aspA_{Ec}$ excreted 40 % more alanine than the control strain.

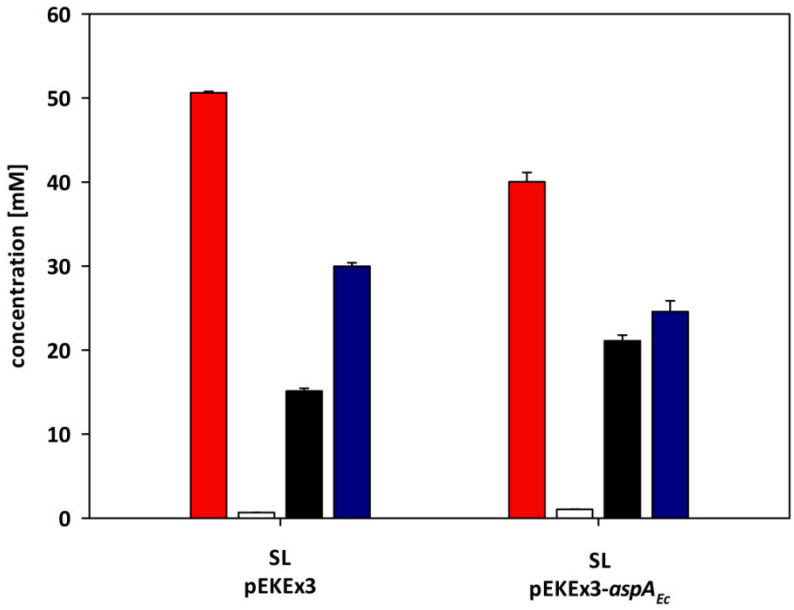

Figure 16: Production of aspartate with strains SL pEKEx3 and SL pEKEx3-*aspA*$_{Ec}$.
Strains were inoculated in CgXII medium supplemented with 100 mM glucose and 200 mM sodium bicarbonate. Accumulation of amino acids and residual glucose is shown in supernatants of cultures after 144 h. Red: aspartate; white: glutamate; black: alanine; blue: glucose.

3.2.3.3 Expression of *aspA* cannot rescue the phenotype of *aspB* deletion

Aspartate is connected to the TCA cycle by the intermediates fumarate and oxaloacetate. A disruption of the TCA at the level of Δ*sdhCAB* is thought to lead to an accumulation of fumarate, which can be aminated to aspartate by the aspartase. The aspartate aminotransferase can deaminate aspartate to oxaloacetate, and hence reverse the amination of aspartase. To test this hypothesis, a deletion of the *aspB* gene was introduced into SL. The strain Δ*sdhCAB* Δ*ldhA* Δ*aspB* (SLB) was transformed with expression vectors for the genes of the aspartases from *B. subtilis*, *E. coli* and *C. glutamicum*, and used in anaerobic production experiments.

In agreement with the results of the aerobic cultivations, the deletion of the *aspB* gene had a severe effect on aspartate overproduction (Figure 17). The control strain with intact AspB amino transferase showed significant excretion of aspartate, while the deletion strains utilized only a small proportion of the glucose within 72 hours and did not excrete aspartate. Expression of the different aspartase genes was not sufficient to compensate this phenotype. Therefore, the aspartate produced under anaerobic conditions is most likely synthesized from the transamination of oxaloacetate via AspB.

The decreased aspartate content observed in the supernatants of SL pEKEx3-*aspA*$_{Ec}$ showed that AspA$_{Ec}$ deaminates aspartate to fumarate under the tested conditions (Figure 16). This is in agreement with the results for the specific activities of the aspartases (Table 6). A sufficient supply with fumarate for the aspartase-based overproduction of aspartate is not created by deletion of the succinate

dehydrogenase. Whether this deletion leads to an accumulation of fumarate is not known, as measurements of the intracellular metabolite pool were not performed.

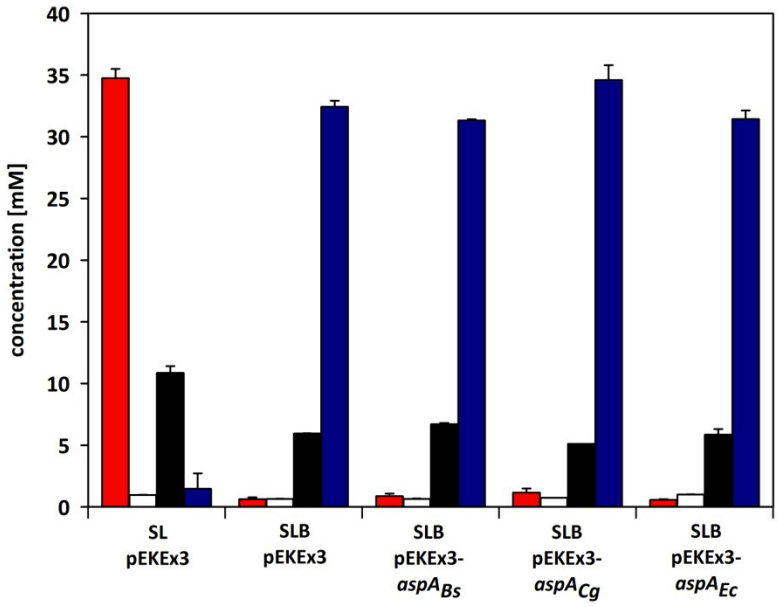

Figure 17: Aspartate formation and glucose consumption of SLB expressing AspA.
Strains were transformed with $AspA_{Bs}$, $AspA_{Cg}$, and $AspA_{Ec}$ and cultivated in optimized medium with 50 mM glucose. Accumulation of amino acids and residual glucose is shown in supernatants of cultures after 72 h. Abbreviations: SL: Δ*sdhCAB* Δ*ldhA*; SLB: Δ*sdhCAB* Δ*ldhA* Δ*aspB; Bs*: *B. subtilis*; *Cg*: *C. glutamicum*; *Ec*: *E. coli.* Red: aspartate; white: glutamate; black: alanine; dark blue: glucose.

3.2.3.4 Overexpression of *aspB* improves aspartate formation

The endogenous aspartate aminotransferase (*aspB*, cg0294) was identified in a bioinformatic approach by screening for aminotransferases in *C. glutamicum* and was found to be specific for aspartate and 2-oxoglutarate (Marienhagen *et al.*, 2005; McHardy *et al.*, 2003). Under production conditions, plasmid-borne expression of the *aspB* gene in the strain SL increases aspartate formation by 18 % to 40.9 mM (Figure 18), resembling a molar yield for aspartate of 0.83 mol/mol glucose. Altogether, the molar amounts of the product aspartate and the by-products glutamate, alanine, succinate, lactate, and acetate correspond 89.9 % of the invested molar carbon of glucose, while 10.1 % is lost to unknown by-products.

Additionally, the heterologous aspartate aminotransferases of *B. subtilis* (*aspB*, BSU22370) and *E. coli* (*aspC*, b0928) were tested for aspartate production. Both genes were cloned into the expression vector pEKEx3 and compared to the native enzyme. All strains expressing the aminotransferase showed increased aspartate formation compared to the control strain carrying the empty vector, but do not exceed the aspartate levels obtained with the endogenous enzyme (data not shown).

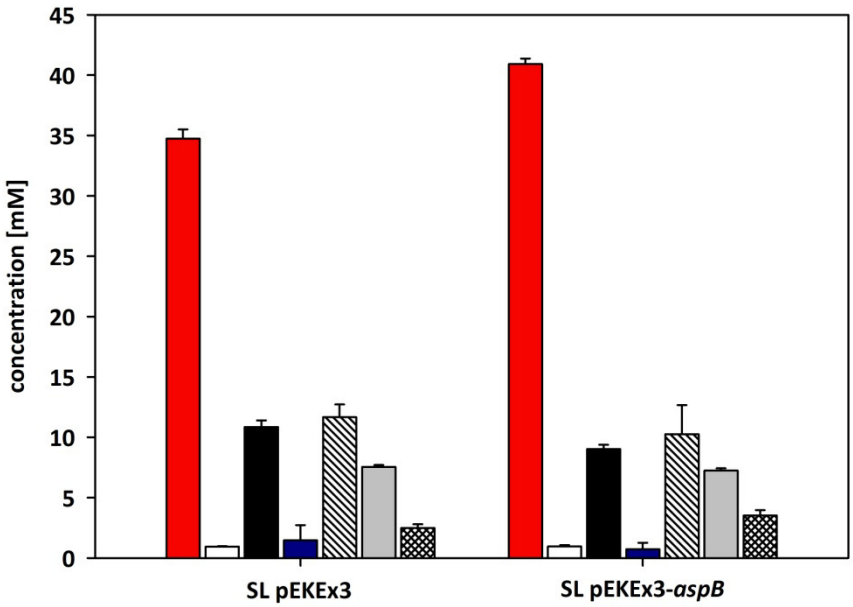

Figure 18: Production of aspartate with plasmid-borne expression of the endogenous *aspB*.
SL was transformed with the expression vector pEKEx3-*aspB* and cultivated in CGXII medium supplemented with 50 mM glucose and 100 mM $NaHCO_3$. Accumulation of amino and organic acids as well as residual glucose is shown in supernatants of cultures after 72 h. Red: aspartate; white: glutamate; black: alanine; dark blue: glucose; striped: succinate; grey: lactate; checked: acetate.

3.2.3.5 Reductive amination by aspartate dehydrogenases

The synthesis of aspartate from fumarate leads to NADH formation of up to two mol/mol glucose. These can be regenerated by the malate dehydrogenase Mdh (*mdh*, cg2613) to NAD^+ when all PEP or pyruvate is carboxylated at the anaplerotic node. If oxaloacetate is directly converted to aspartate and not reduced to malate, a surplus of NADH is generated inhibiting several enzymes of the glycolysis. Aspartate dehydrogenases (AspDH) synthesizing aspartate from oxaloacetate in a NAD-dependent manner can circumvent this problem. AspDH are a newly discovered group of amino acid dehydrogenases with high substrate specificity. They are clustered into three different groups according to their origin, the hyperthermophilic, the archeal and the protobacterial cluster (Li *et al.*, 2012).

Due to their mesophilic origin the AspDH of *Pseudomonas aeruginosa* PAO1 (*aspDH*$_{Pa}$, PA3505) and *Ralstonia eutropha* H16 (*aspDH*$_{Re}$, H16_B0736) were selected and cloned into the expression vector pEKEx3. To determine the specific activities of the AspDH, the Δ*sdhCAB* Δ*ldhA* Δ*mdh* (SLM) background was used, as the NAD-dependent malate dehydrogenase is competitive for the substrate oxaloacetate and NADH in the assay. However, for none of the recombinant strains carrying an *aspDH* expression plasmid, aspartate dehydrogenase activity was detected, neither for the amination nor for the deamination reaction. According to these results, the expression of the aspartate dehydrogenases in SL had no beneficial effect under production conditions. Strains expressing the *aspDH* genes excrete

aspartate on the level of the control strains, and glucose consumption was unchanged (data not shown).

3.2.4 Overexpression of *ppc* and feedback resistant *pyc* does not improve aspartate overproduction

Addition of $NaHCO_3$ to the medium was shown to enhance aspartate synthesis by improving anaplerosis (Figure 12). To further improve the supply with oxaloacetate for the transamination by AspB, the genes coding for phosphoenolpyruvate carboxylase Ppc (*ppc*) and a feedback resistant version of the pyruvate carboxylase Pyc^{P458S} (*pyc*^{P458S}) were introduced into *C. glutamicum* SL pVWEx1-*aspB* (Figure 19). Cultures were inoculated anaerobically, but the simultaneous overexpression of *ppc* or *pyc*^{P458S} plus *aspB* did not improve production of aspartate. Instead the accumulation of the amino acid in supernatants was in both cases decreased by around 13 % compared to the control strains.

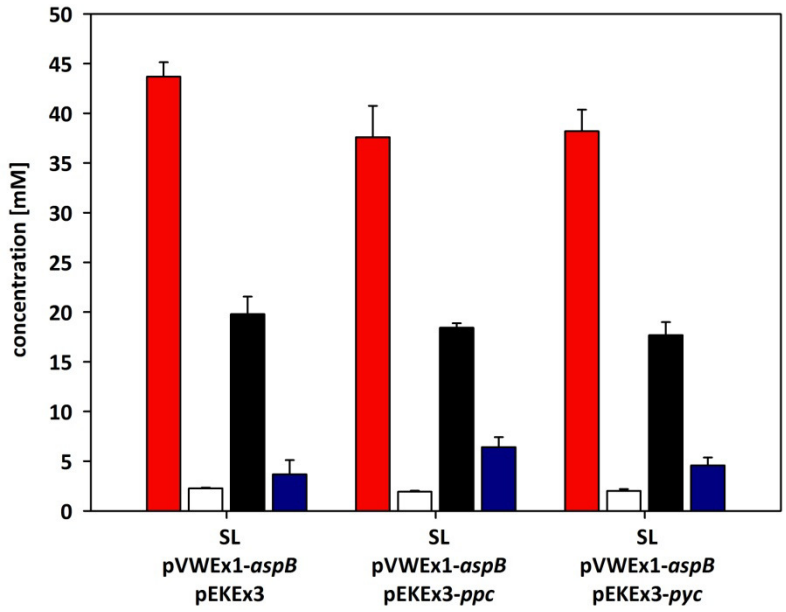

Figure 19: Aspartate production in SL pVWEx1-*aspB* expressing *ppc* and *pyc*^{P458S}.
Strains were inoculated in CGXII medium and supplemented with 50 mM glucose and 100 mM $NaHCO_3$. Accumulation of amino acids and residual glucose is shown in supernatants of cultures after 72 h. Red: aspartate, white: glutamate; black: alanine; blue: glucose.

3.2.5 Deletion of the aspartate-α-decarboxylase gene *panD* does not improve aspartate excretion

Another approach to increase flux through the anaplerotic node is the deletion of the gene encoding the aspartate-α-decarboxylase PanD (cg0172, *panD*). PanD is a part of the pantothenate synthesis pathway and a deletion of the gene should inhibit the activity of the pyruvate dehydrogenase complex by depletion of coenzyme A. Thereby, pyruvate should accumulate and is available for carboxylation at

the anaplerotic node. The plasmid pK19*mobsacB-panD* was used to introduce the deletion of *panD* in the SL strain and led to Δ*sdhCAB* Δ*ldhA* Δ*panD* (SLD). The influence of the deletion on aspartate synthesis was tested under anaerobic conditions with and without *aspB* overexpression. Compared to the control strains, the supernatants of SLD displayed no variations in the amount or the composition of the excreted compounds (data not shown).

3.2.6 Reducing by-product formation during aspartate production

3.2.6.1 Deletion of *cat* decreases succinate concentration but negatively affects aspartate formation

In the wild type expressing *aspB*, the organic acids succinate, lactate, and acetate are the main fermentation products and a reduction of these by-products is needed to enable the production of aspartate. Both single deletions of *ldhA* and *sdhCAB* reduce the content of lactate (grey) and succinate (striped), and promote the formation of the other; e.g. the deletion of *ldhA* promotes excretion of succinate (Figure 20). Still, residual amounts of succinate can be found in Δ*sdhCAB* as well as lactate in Δ*ldhA*. Acetate is found in all supernatants, but its accumulation is reduced with the deletion of the *sdhCAB* genes. Interestingly, the deletion of *sdhCAB* led to the formation of a small amount of aspartate (9 mM). The additional deletion of the *ldhA* gene in Δ*sdhCAB* further decreased the excretion of organic acids and promotes amino acid production. SL pEKEx3-*aspB* excretes mainly aspartate, alanine, and succinate, and small proportions of lactate and acetate. However, compared to the strain Δ*sdhCAB*, the content of succinate is again increased in the strain SL. For a further reduction of succinate, the succinyl-CoA:acetate CoA transferase Cat (*cat*, cg2840) was deleted in SL to yield Δ*sdhCAB* Δ*ldhA* Δ*cat* (SLC). Cat transfers the prosthetic group from succinyl-CoA to acetate and releases succinate and acetyl-CoA. In fact, the deletion of the *cat* gene reduces succinate in the supernatants but simultaneously caused a decrease of aspartate and an increase of alanine synthesis. The aspartate concentration was reduced by 32 % to 22.5 mM and the alanine content was almost doubled (20.9 mM) compared to the strain SL.

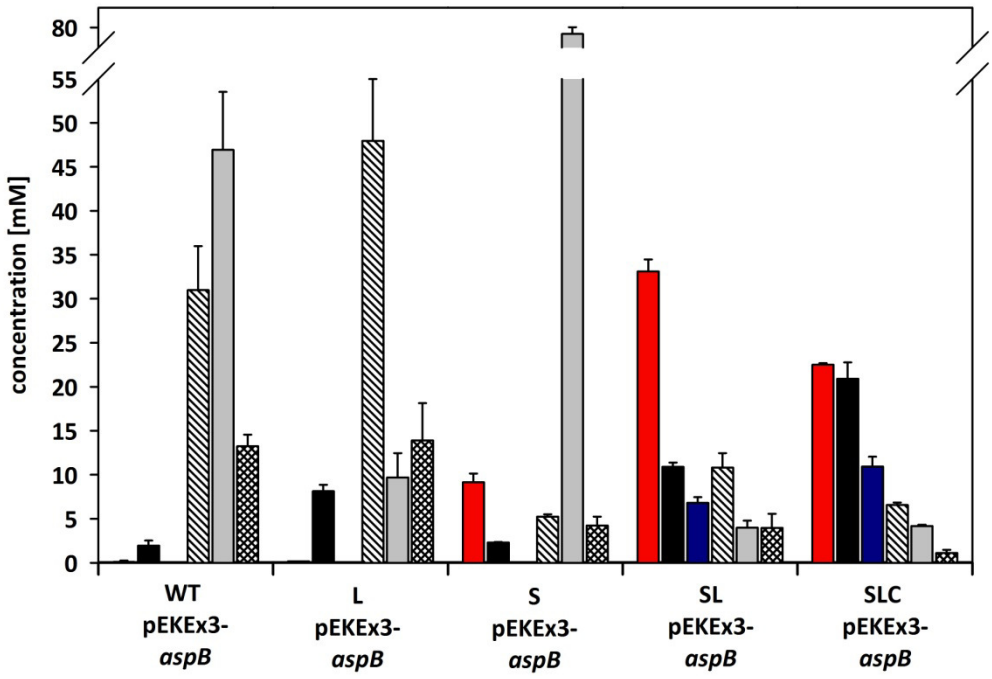

Figure 20: Reduction of the by-product succinate by deletion of succinate dehydrogenase and butyryl-CoA:acetate CoA transferase.
Strains were inoculated in CGXII medium and supplemented with 50 mM glucose and 100 mM NaHCO3. Accumulation of amino acids, organic acids, and residual glucose is shown in supernatants of cultures after 72 h. Red: aspartate; black: alanine; dark blue: glucose; striped: succinate; grey: lactate; checked: acetate. Abbreviations: WT: wild type; L: ΔldhA; S: ΔsdhCAB; SL: ΔsdhCAB ΔldhA; SLC: ΔsdhCAB ΔldhA Δcat.

3.2.6.2 Deletion of alanine-valine aminotransferase gene *avtA* improves the synthesis of aspartate

The aminotransferases AlaT and AvtA were identified to provide cells with alanine in minimal medium and are therefore the main targets for a reduction of this by-product (Marienhagen and Eggeling, 2008). Both single and a double deletion in the strain SL were obtained and named according to their deletions; SLT for Δ*sdhCAB* Δ*ldhA* Δ*alaT*, SLV for Δ*sdhCAB* Δ*ldhA* Δ*avtA* and SLTV for the double deletion. To determine the influence of the deletions on alanine formation, the strains were cultivated anaerobically. Furthermore, the effect of the expression of the *aspB* gene was determined. Supernatants were analyzed by HPLC after 72 hours and the concentrations of the amino acids and residual glucose are depicted in Figure 21.

The control strain SL pEKEx3 shows significant accumulation of aspartate and alanine (Figure 21 A). In contrast to their proposed functions, the single deletions of *alaT* and *avtA* resulted in an increased concentration of alanine in the supernatants. Excretion of aspartate was also affected negatively. The double deletion of these genes did not eliminate alanine excretion completely, but reduced it to 5.2 mM. In contrast, the aspartate concentration stays on the same level of the control strain SL.

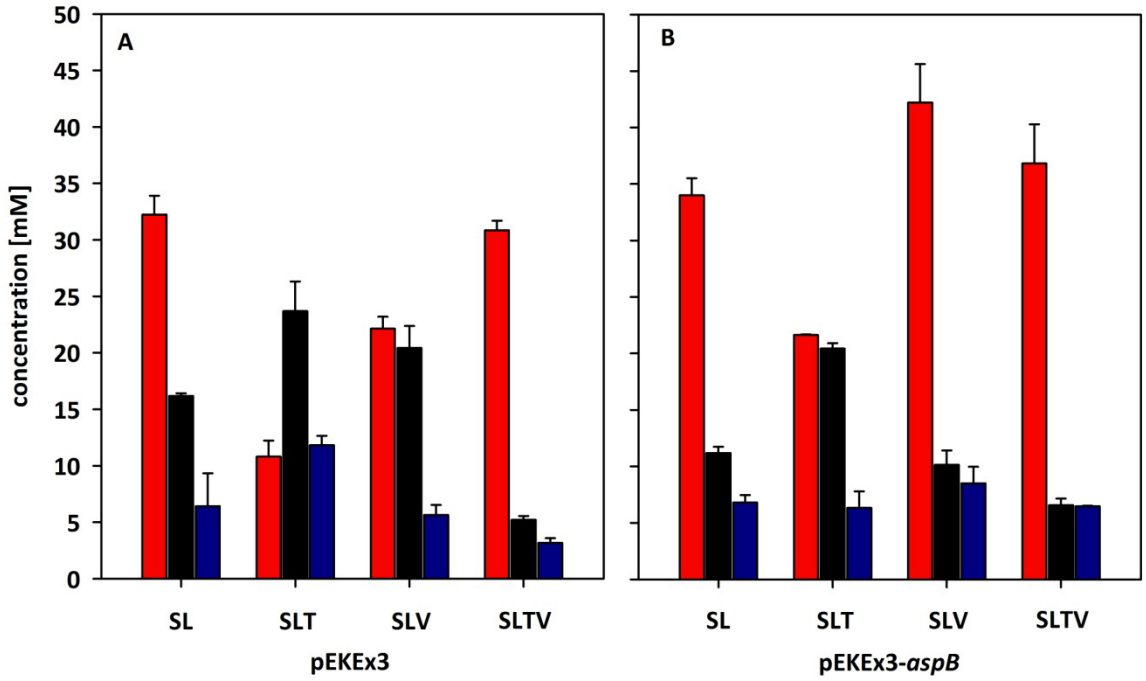

Figure 21: Deletions of alanine transaminases *alaT* and *avtA*.
The single and double deletions were compared to the initial SL strain with and without expression of the aspartate transaminase. Strains were inoculated in CGXII medium and supplemented with 50 mM glucose and 100 mM $NaHCO_3$. Accumulation of amino acids and residual glucose is shown in supernatants of cultures after 72 h. Red: aspartate; black: alanine; dark blue: glucose; Abbreviations: SL: Δ*sdhCAB* Δ*ldhA;* SLT: Δ*sdhCAB* Δ*ldhA* Δ*alaT;* SLV: Δ*sdhCAB* Δ*ldhA* Δ*avtA*; SLTV: Δ*sdhCAB* Δ*ldhA* Δ*alaT* Δ*avtA*.

In general, the overexpression of *aspB* improves aspartate synthesis and reduces alanine formation (Figure 21 B). The highest concentration of aspartate was found in supernatants of SLV pEKEx3-*aspB* corresponding to an increase of 24 % compared to SL pEKEx3-*aspB*. The overexpression of *aspB* in the SLT background results in lower amounts of aspartate, which were at the level of the alanine concentration. Aspartate synthesis in the double deletion is also increased, but did not exceed the amount formed by strain SLV pEKEx3-*aspB*, whereas alanine formation was further reduced. Surprisingly, the deletion of both transaminases did not prevent accumulation of alanine as a residual amount of ~6 mM were detected. This is in contrast to the finding, that AlaT and AvtA are the only aminotransferases able to synthesize alanine (Marienhagen and Eggeling, 2008). For further investigation SL, SLTV, and the single deletions were cultivated aerobically in minimal medium. All strains reached similar biomass, but the strain SLTV showed a growth defect with a prolonged lag phase, which could be compensated by the addition of 1 mM alanine (data not shown). In summary, the by-product alanine can be reduced by the deletion of both transaminases. The strain SLV pEKEx3-*aspB* shows with 1.02 mol/mol glucose the highest aspartate yield, and was therefore chosen for further genetic improvements.

3.2.7 Regeneration of reduction equivalents

Considering the overall reactions of the production route, the regeneration of reduction equivalents is of major concern. In glycolysis, two moles of NADH per mol glucose are generated by the glyceraldehyde-3-phosphate dehydrogenase GapA (cg1791, *gapA*). The regeneration of NAD^+ can be performed by the malate dehydrogenase Mdh (cg2613, *mdh*), which reduces oxaloacetate to malate and thereby competes with the aspartate aminotransferase AspB for the substrate oxaloacetate. Both, *gapA* and *mdh* are upregulated on a transcriptional level under anaerobic conditions. Additionally, GapA was shown to be regulated by the NAD^+/NADH ratio and ATP, and thereby controlling the glycolytic flux (Inui *et al.*, 2004b; Omumasaba *et al.*, 2004). Therefore, both enzymes were further investigated to determine their influence on aspartate synthesis.

3.2.7.1 The enzymes GapA and Mdh are highly active in crude extracts of *C. glutamicum*

The specific activity of GapA was tested in crude extracts of the strain SLV harboring the expression vectors pEKEx3-*gapA* or pEKEx3-*mdh-gapA* (Figure 22). All strains were cultivated in BHI medium supplemented with 1 mM IPTG. Basal expression of *gapA* was tested with the empty vector and resulted in a specific activity of 0.345 U/mg. Plasmid borne *gapA* expression increased the activity to 0.513 U/mg. In strain carrying pEKEx3-*mdh-gapA* the GapA activity was similar to the control strain carrying pEKEx3.

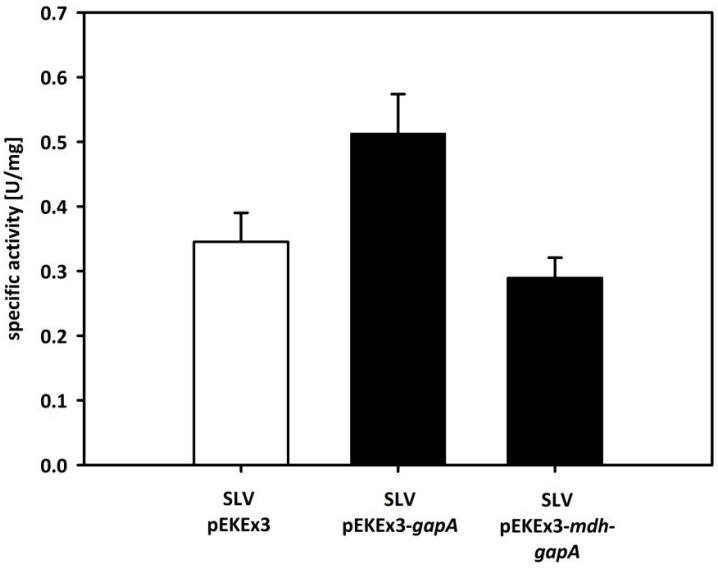

Figure 22: Specific activity of GapA in crude extracts of SLV.
Cultures were grown aerobically in BHI medium and the plasmids were induced with 1 mM IPTG.

The Mdh is encoded on pEKEx3-*mdh* and was first tested in the strain SL. As the empty vector already showed a specific activity of 1.37 ± 0.25 U/mg, the plasmid was transformed into the strain Δ*sdhCAB* Δ*ldhA* Δ*mdh* (SLM, see section 3.2.7.3). The plasmid borne expression of the gene led to an

activity of 1.63 ± 0.22 U/mg, which is exceeding the basal activity by 18 %. In the empty vector control (SLM pEKEx3) no residual Mdh specific activity was detected.

3.2.7.2 Overxpression of *gapA* increases velocity of aspartate formation

To determine the influence of *gapA* and *mdh* overexpression on aspartate formation, the strain SLV pVWEx1-*aspB* was transformed with pEKEx3-*gapA*, pEKEx3-*mdh,* pEKEx3-*mdh-gapA* and the empty vector as a control. Cultures were supplemented with 50 mM glucose and 100 mM $NaHCO_3$ and supernatants were analyzed by HPLC after 24, 48, 72, and 144 hours of incubation.

The effect of overexpression of *gapA* or *mdh* either alone or in combination on the total aspartate yield was rather low. The titers of aspartate and the by-products vary only insignificantly after complete consumption of glucose. But the overexpression clearly influenced the velocity of the process (Figure 23). After 48 hours, an accumulation of 23.5 mM of aspartate was detected in the supernatants of the control strain SLV pVWEx1-*aspB* pEKEx3. At the same time point, the strains overexpressing *mdh* had formed 26.2 mM aspartate, while strains expressing *gapA* had formed 30.9 mM. Glucose consumption was improved in this strain, while similar amounts of by-products are formed. The combined overexpression of *mdh* and *gapA* did not improve the process further. The supernatants showed comparable results to cultures overexpressing *mdh*. Expression of the gene combinations was also tested in the SL strain as well as in SLTV. A similar pattern was observed in all strains.

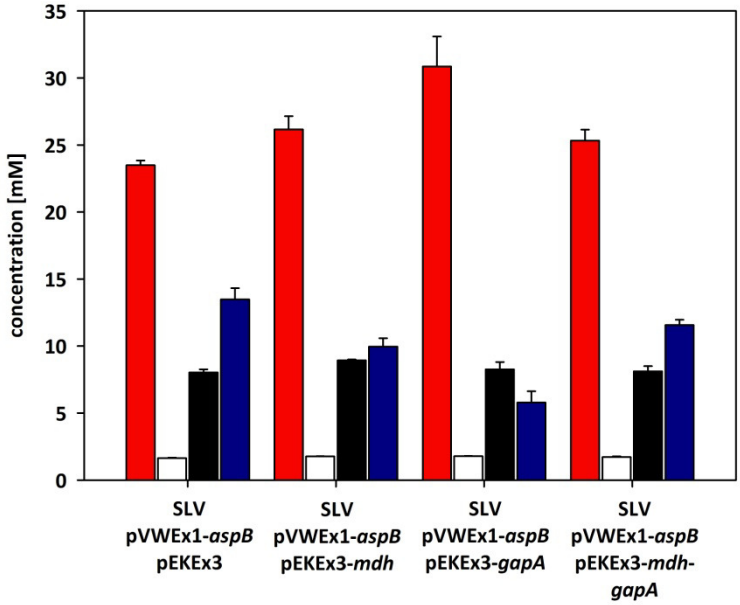

Figure 23: Influence of *gapA* and *mdh* overexpression in the strain SLV on aspartate production.
Cultures were inoculated in CGXII medium supplemented with 50 mM glucose and 100 mM $NaHCO_3$. Accumulation of amino acids and residual glucose in supernatants of cultures after 48 h is depicted. Red: aspartate, white: glutamate; black: alanine; blue: glucose.

3.2.7.3 Deletion of malate dehydrogenase impairs glucose consumption

Mdh is a competitor of AspB, drawing off oxaloacetate to malate on the reductive branch of the TCA cycle, thereby regenerating NAD^+. To further investigate the role of the enzyme for aspartate production an in-frame deletion was introduced into the SL strain with the vector pK19*mobsacB-mdh*. The strain Δ*sdhCAB* Δ*ldhA* Δ*mdh* (SLM) was transformed with pEKEx3-*aspB* and tested under aspartate production conditions. Deletion of the dehydrogenase severely effects consumption of the carbon source. More than 40 % of the glucose was left after 72 hours and synthesis of aspartate was completely blocked. This clearly indicates the importance of the enzyme for the regeneration of NAD^+ and for aspartate production.

3.2.7.4 NAD-dependent Gdh_{Pa} restores glucose consumption in the strain SLM but is not suitable for aspartate production

To find a substitute for NAD^+ regeneration in the strain SLM, the glutamate dehydrogenase (Gdh_{Pa}) of *Peptostreptococcus asaccharolyticus* was tested under aspartate production conditions. This enzyme catalyzes the reductive amination of 2-oxoglutarate in a NAD-dependent manner, while the endogenous enzyme is NADP-dependent. As glutamate is the amino donor for the transamination of oxaloacetate to aspartate, the enzyme is also advantageous for precursor supply.

The expression of gdh_{Pa} was tested in SL and SLM carrying pVWEx1-*aspB*. SL pVWEx1-*aspB* pEKEx3 serves as control strain, as SLM expressing *aspB* is not able to utilize glucose properly (3.2.7.3). The expression of gdh_{Pa} disturbed the production of aspartate in the SL strain (Figure 24). It was reduced by almost 50 % to 20.1 mM and this was accompanied by a significant increase in alanine excretion. In the SLM background, expression of gdh_{Pa} only partially compensated the *mdh* deletion. Glucose consumption is restored in this strain, but as in SL, aspartate formation was reduced and alanine formation increased. Consequently, restoring glucose consumption of an *mdh* deletion mutant by expression of gdh_{Pa} is possible, but not applicable for aspartate overproduction.

Next to Gdh_{Pa}, also the aspartate dehydrogenases of *R. eutropha* and *P. aeruginosa* were tested in the SLM background. These enzymes catalyze the NAD-dependent amination of oxaloacetate to aspartate, and can therefore couple NAD^+ regeneration and product formation. However, in accordance with no detectable enzymatic activity, their expression did not promote the formation of aspartate.

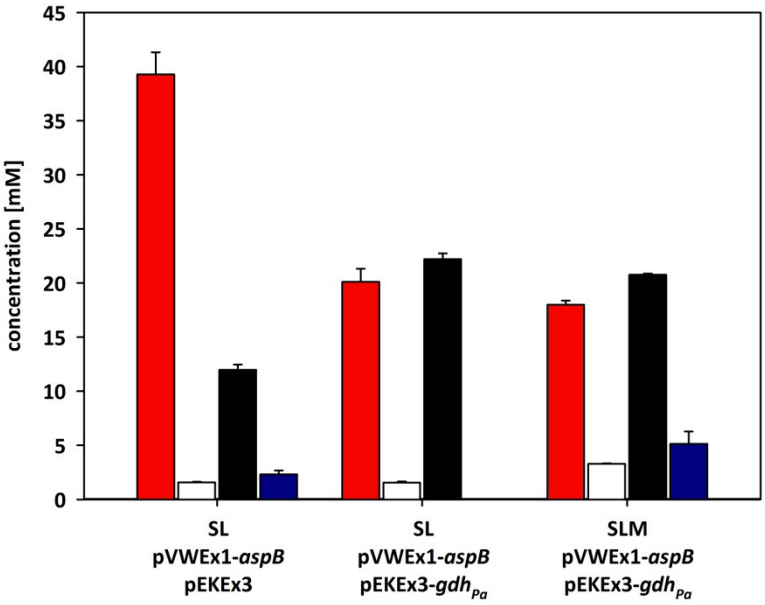

Figure 24: Expression of Gdh_{Pa} from *Peptostreptococcus asaccharolyticus* in SL and SLM.
Cultures were inoculated in CGXII medium supplemented with 50 mM glucose and 100 mM $NaHCO_3$. Accumulation of amino acids and residual glucose in supernatants of cultures after 72 h is depicted. Red: aspartate, white: glutamate; black: alanine; blue: glucose.

3.2.7.5 Expression of the transhydrogenases genes *udhA* and *pntAB* does not improve aspartate formation

Another approach to improve NAD^+-regeneration was the expression of transhydrogenase genes. These enzymes catalyze the transfer of electrons between different reduction equivalents. $NADP^+$ can be reduced to NADPH with the electrons from NADH and used in the cell for reductive amination of 2-oxoglutarate to glutamate via the endogenous glutamate dehydrogenase. The aminotransferase AspB requires glutamate as amino donor, and therefore the transhydrogenase activity can improve aspartate synthesis. Two enzymes of *E. coli* were tested. $PntAB_{Ec}$ is a membrane-integral enzyme and reduces $NADP^+$ with NADH. This reaction is driven by the proton-motive force and one proton is transported into the cell for each NADPH formed. $UdhA_{Ec}$ is a soluble enzyme and transfers electrons between NAD^+ and $NADP^+$ depending on their concentrations. The expression of the enzymes was performed in SL pEKEx3-*aspB* during aspartate synthesis. Both enzymes reduced the accumulation of aspartate and promoted alanine formation. The expression of $pntAB_{Ec}$ reduced the aspartate concentration by more than 60 % and glucose is consumed incompletely. The effect of $udhA_{Ec}$ expression was not that distinct, but still reduced the aspartate excretion significantly by 38 %.

3.2.8 Utilization of maltose improves aspartate formation

As industrial fermentations are not only performed with glucose, but molasses and other mixed sugars, additional carbon sources were tested for the production of aspartate. Next to glucose, the utilization of fructose and maltose was tested. To balance the molar ratio of carbon, the concentration of the disaccharide maltose was reduced to 25 mM. Supernatants from SL pEKEx3-*aspB* cultures incubated for 72 hours with 100 mM $NaHCO_3$ were sampled and analyzed by HPLC. The utilization of fructose was diminished in the strain SL pEKEx3-*aspB* under anaerobic conditions (Figure 25). After 72 h, less than 50 % of the sugar was consumed, along with reduced excretion of aspartate. However, the utilization of maltose was beneficial for the production of aspartate and led to a 13 % increase.

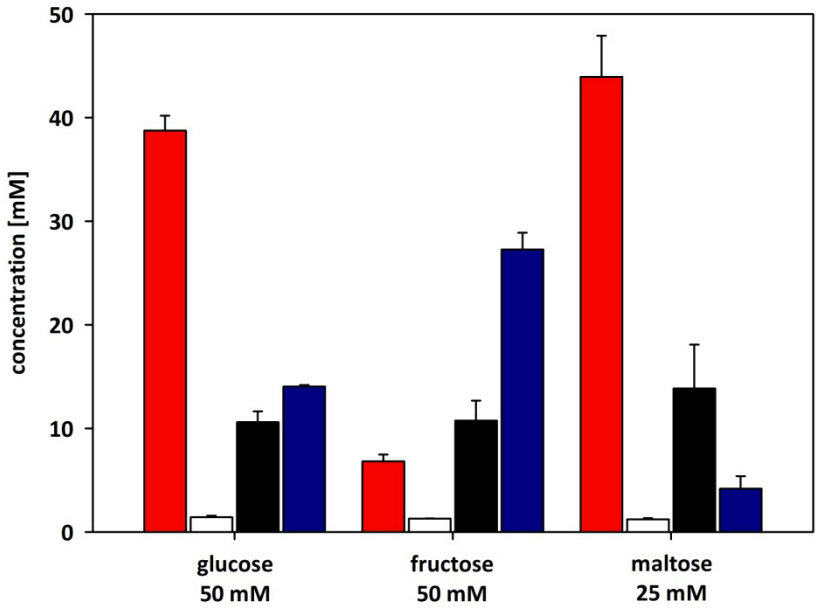

Figure 25: Production of aspartate in SL pEKEx3-*aspB* from alternative carbon sources.
Cultures were inoculated in CGXII medium supplemented with 100 mM $NaHCO_3$ and 50 mM glucose, 50 mM fructose or 25 mM maltose. Accumulation of amino acids and residual carbon source in supernatants of cultures after 72 h is depicted. Red: aspartate, white: glutamate; black: alanine; blue: carbon source.

Next to these food carbon sources, also non-food carbon sources were tested. The hemicellulosic sugars arabinose and xylose are waste products derived from lignocellulosic biomass. Utilization of arabinose is enabled in *C. glutamicum* by plasmid borne expression of the *araBAD* operon of *E. coli*. For xylose utilization, the plasmid encoding the xylose isomerase from *Xanthomonas campestris* ($xylA_{Xc}$) and the endogous xylulose kinase (*xylB*, cg0147) were used. Glycerol is a waste product of biodiesel synthesis and utilization was enabled by the *glpFKD* operon of *E. coli*. The strain SL was transformed with the plasmids and tested with 60 mM of the pentose sugars and 100 mM of the C3 compound, to balance the ratios of molar carbon. Nevertheless, the tested carbon sources were not

consumed properly. After 72 hours, the glycerol concentration was unchanged. Furthermore, only 35 % of the arabinose and 50 % of the xylose were consumed. None of these alternative carbon sources promoted aspartate synthesis.

3.2.9 Velocity of aspartate production is increased by GapA and utilization of maltose

The production rate of aspartate synthesis was determined depending on the carbon source and the genetic background of the strain. The strains SL and SLV overexpressing either *aspB* or *aspB* plus *gapA* were supplemented with 100 mM $NaHCO_3$ and 50 mM glucose or 25 mM maltose. Sampling of supernatants was performed thrice a day in four hour steps for 72 hours. During the experiment, a clear production phase appears between 20 and 52 h. The production rate is calculated only for this phase and covers a time of 24 h for each strain (Table 7).

Table 7: Aspartate production rate, carbon utilization and product yield of SL and SLV expressing *aspB* or *aspB* plus *gapA*.
Strains were cultivated in CGXII medium supplemented with 100 mM $NaHCO_3$ and 50 mM glucose or 25 mM maltose. Rates were calculated for the production phase between 20 and 52 h and cover a range of 24 h for each strain. In order to compare the carbon consumption of the mono- and disaccharide, values are depicted in molC/h*gCDW. Product yield was determined after complete consumption of the carbon source. Abbreviations: x: pVWEx1-*aspB* pEKEx3; xG: pVWEx1-*aspB* pEKEx3-*gapA.*

		Aspartate production rate [mM/h*gCDW]	Carbon consumption rate [molC/h*gCDW]	Aspartate yield $Y_{P/S}$ [g/g]	Alanine yield $Y_{P/S}$ [g/g]
Glucose	**SLx**	0.091 ± 0.002	0.679 ± 0.012	0.51 ± 0.02	0.18 ± 0.00
	SLxG	0.156 ± 0.008	0.959 ± 0.034	0.49 ± 0.05	0.20 ± 0.00
Glucose	**SLVx**	0.100 ± 0.007	0.553 ± 0.017	0.59 ± 0.07	0.11 ± 0.00
	SLVxG	0.154 ± 0.006	0.763 ± 0.025	0.59 ± 0.02	0.12 ± 0.00
Maltose	**SLVx**	0.153 ± 0.012	0.631 ± 0.040	0.80 ± 0.07	0.10 ± 0.01
	SLVxG	0.176 ± 0.018	0.664 ± 0.023	0.80 ± 0.02	0.10 ± 0.00

The effects of the deletion of *avtA*, the overexpression of *gapA* and the used carbon source were determined for the aspartate production rate, the carbon utilization and the yields of aspartate and alanine. The deletion of *avtA* affects the carbon utilization and the formation of the by-product alanine. The strains SLxG and SLVxG had similar aspartate production rates, but SLxG showed a by 20 % increased carbon consumption. Compared to SLxG, the additional deletion of *avtA* increased the aspartate yield and decreased the alanine formation in SLVxG. In all tested strains, the expression of *gapA* had positive effects on aspartate production and carbon consumption, but did not affect the total aspartate yields. On glucose, the overexpression of *gapA* in SL and SLV increased carbon

consumption by i.e. 40 %, which also positively affects the aspartate production rate. On maltose, the highest aspartate production rate and the highest yields were achieved. However, the *gapA* overexpression promoted the aspartate production rate and the carbon utilization to a lesser extent than on glucose. However, the utilization of the disaccharide raised the yield by 35 % compared to glucose.

3.2.10 Aspartate is constantly produced from glucose and maltose

To show the stability of the process, SLV pVWEx1-*aspB* pEKEx3-*gapA* was incubated with the standard (Figure 26 A) and the doubled (Figure 26 B) amounts of glucose and maltose. Samples were taken after 72 and 144 hours and analyzed by HPLC. In all cultures, the same pattern can be observed, a doubling of carbon source and time results in a doubled amount of the product. Cells supplemented with 100 mM glucose synthesize 69.8 mM aspartate, but a residual amount of almost 20 mM of the carbon source is still present in supernatants. From 25 mM maltose, 46.3 mM aspartate is synthesized, which is almost doubled up to 90.4 mM from 50 mM maltose within 144 hours.

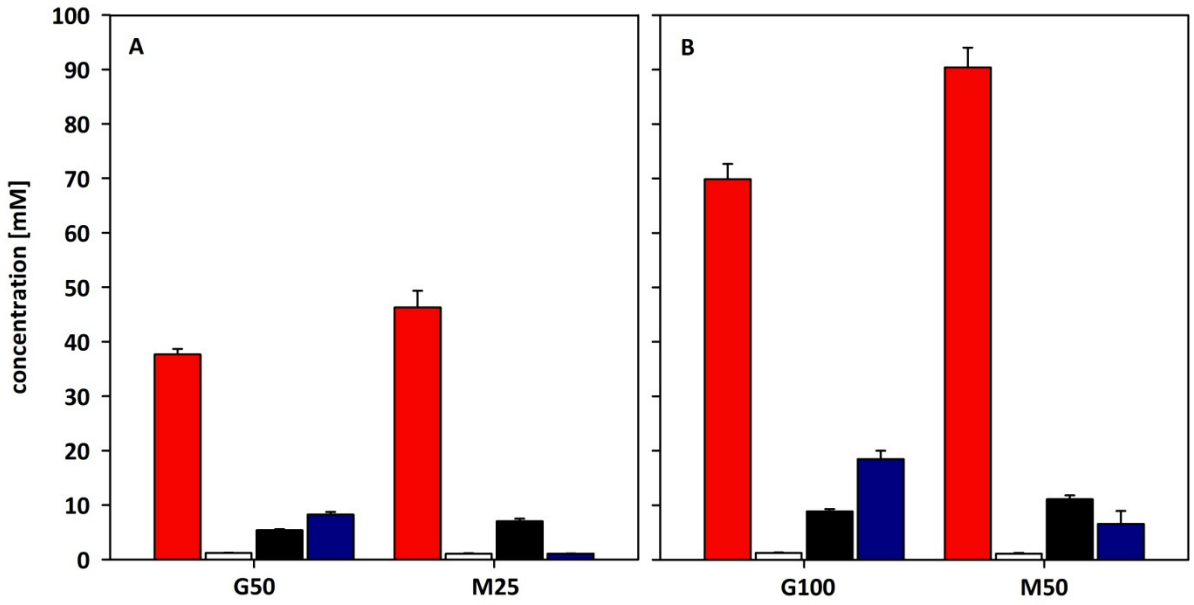

Figure 26: Constant production of aspartate in SLV pVWEx1-*aspB* pEKEx3-*gapA* with (A) standard and (B) doubled amounts of glucose and maltose.
Cultures were inoculated in CGXII medium supplemented with the indicated amounts of carbon sources and $NaHCO_3$ (100 mM $NaHCO_3$ for 50 mM glucose and 25 mM maltose or 200 mM $NaHCO_3$ for cultures supplemented with 100 mM glucose and 50 mM maltose). A: 72 h; B: 144 h. Abbreviations: G: glucose; M: maltose. Numbers indicate carbon source concentrations in mM. Red: aspartate; white: glutamate; black: alanine; blue: carbon source.

3.3 Conclusion of aspartate production

In order to develop a strain for the production of aspartate, two general strategies were followed. Under aerobic conditions, growth and production phase are coupled. As aspartate is metabolized during growth, it was of major concern to reduce aspartate consuming reactions. However, a deletion of the aspartokinase, the main aspartate catabolizing enzyme, was not possible. Furthermore, the expression of the aspartate aminotransferase and the aspartase in the *C. glutamicum* wild type or Δ*fum* was not sufficient to enable aspartate overproduction. However, the decoupling of growth and production phase enabled overproduction of aspartate. Under anaerobic conditions, the strain SL excreted aspartate with a yield of 0.55 mol/mol glucose in minimal medium and formed the by-products alanine and succinate. The initial aspartate titer was increased by adaptations of the medium and improvements of the production strain. The addition of bicarbonate was beneficial for product formation and increased the yield to 0.83 mol/mol glucose. As a side effect, the content of alanine was reduced in the supernatants. Nitrate as terminal electron acceptor improved glucose consumption but inhibited the production of aspartate. Furthermore, the utilization of maltose as carbon source was beneficial for aspartate production. In the strain SL, the transamination of oxaloacetate by the aspartate aminotransferase proved to be more efficient than the amination of fumarate to aspartate. A reduction of the by-product succinate by the deletion of the *cat* gene was coupled to reduced aspartate production, while the reduction of alanine by the deletion of the alanine valine transaminase *avtA* promoted aspartate formation to 1.02 mol/mol glucose. The velocity of the production with glucose was enhanced 1.5-fold by the overexpression the glyceraldehyde dehydrogenase and led to the production of 0.154 mM/h*gCDW in the production phase. The highest yield was achieved with maltose (0.80 g/g) and the velocity of aspartate production was further enhanced to 0.176 mM/h*gCDW. Overall, an efficient growth decoupled production scheme was developed that enables aspartate synthesis based on glucose and maltose.

3.4 Anaerobic production of β-alanine

Under physiological conditions, β-alanine is crucial for the synthesis of pantothenate. The non-proteinogenic β-amino acid is derived from aspartate by decarboxylation by the aspartate-α-decarboxylase PanD (*panD*, cg0172). Overexpression of *panD* in an aspartate producing strain of *C. glutamicum* was used to enable the overproduction of β-alanine.

3.4.1 The aspartate-α-decarboxylase PanD of *C. glutamicum* shows the highest specific activity

To define the best suited enzyme for β-alanine production heterologous enzymes were tested for their activity. Next to the endogenous PanD protein, the enzymes of *E. coli* (*panD*, b0131) and *B. subtilis* (*panD*, BSU22410) were investigated. The corresponding genes were cloned in the expression vector pEKEx3, and specific activity was determined in the SL background with an HPLC based assay. For the empty vector control, no aspartate specific decarboxylase activity was detected, while $PanD_{Cg}$ demonstrated the highest specific activity with 55.4 ± 6.7 mU/mg. For $PanD_{Bs}$, an activity of 15.4 ± 3.9 mU/mg was measured, but the decarboxylase of *E. coli* was inactive in crude extracts of the strain SL. Therefore, the aspartate-α-decarboxylase of *C. glutamicum* represents the best suited enzyme for β-alanine production.

3.4.2 Production of β-alanine by $PanD_{Cg}$ is possible but inefficient

Synthesis of β-alanine was performed in the aspartate producing strain SL expressing the plasmid encoded *panD* genes of *B. subtilis*, *E. coli*, and the endogenous gene. Cultures were inoculated anaerobically in medium optimized for aspartate synthesis and supplemented with 50 mM glucose and 100 mM $NaHCO_3$. Sampling was performed after 72 hours, and the amino acid composition of the supernatants was analyzed by HPLC.

In general, the decarboxylation of aspartate is inefficient, as more than 50 % of total aspartate is not converted to β-alanine (Figure 27). The determined specific activities of the decarbocxylases in crude extracts correlated with the results obtained for synthesis of β-alanine. The maximal titer of 15.3 mM was obtained with the strain that overexpressed the endogenous enzyme. Expression of $panD_{Bs}$ led to an excretion of 2.7 mM, while no β-alanine was detected in the supernatant of the strain expressing $panD_{Ec}$. The main by-products of the cultivation are aspartate and α-alanine. Consequently, the endogenous enzyme (referred as *panD* in the following) was chosen for all further experiments. Despite this inefficient conversion of aspartate to β-alanine, the activity of the enzymes was not improved in this work.

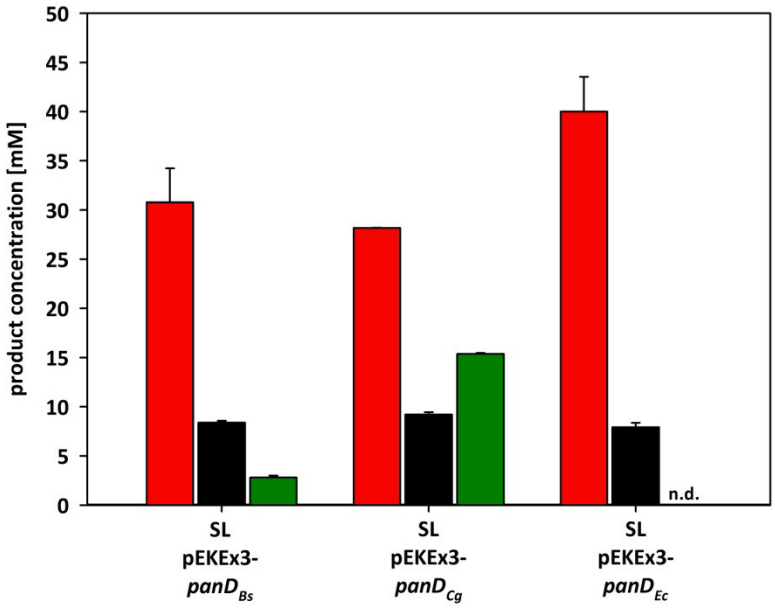

Figure 27: Production of β-alanine in the strain SL expressing the plasmid encoded *panD* genes of *B. subtilis*, *C. glutamicum,* and *E. coli*.
Cultures were inoculated in CGXII medium supplemented with 50 mM glucose and 100 mM $NaHCO_3$. Accumulation of amino acids in supernatants of cultures after 72 h is depicted. Red: aspartate; black: alanine; green: β-alanine. N.d.: not detected; *Bs: B. subtilis*; *Cg*: *C. glutamicum*; *Ec*: *E. coli.*

3.4.3 The overexpression of *aspB* improves synthesis of β-alanine

Next to improving the decarboxylation reaction, improvements in the metabolism of the production strain can be performed. Physiologically, β-alanine is the precursor for the synthesis of the B-vitamin pantothenate. To prevent flux in this pathway, a deletion of the *panBC* genes was introduced into the strain SL that led to Δ*sdhCAB* Δ*ldhA* Δ*panBC* (SLBC). Furthermore, the synthesis of β-alanine was tested in the strains SLV and SLTV carrying deletions in the genes *avtA* and *alaT,* encoding the two known α-alanine synthesizing transaminases of *C. glutamicum.* Moreover, the expression of *panD* was combined with the expression of the aspartate aminotransferase *aspB*.

In general, the production of β-alanine is dependent on the overexpression of *panD* (Figure 28). The control strain SL pEKEx3 excretes only aspartate (35.7 mM) and α-alanine (Figure 28 A). The overexpression of *panD* led to the excretion of 15.8 mM β-alanine into the supernatant. Deletion of the α-alanine transaminases did not decrease the amount of the by-product α-alanine. Instead deletion of the aminotransferase *avtA* decreases β-alanine synthesis, and its production was completely prevented in the *avtA-alaT* double mutant SLTV. Furthermore, the deletion of the *panBC* genes was not beneficial for β-alanine production, since the titer was lower compared to SL pEKEx3-*panD* (Figure 28 B).

Although high amounts of residual aspartate were detected in all supernatants, the expression of *panD* with *aspB* was tested (Figure 28 C). This strategy improved the production of β-alanine from 13.2 mM to 20.8 mM, which corresponds a yield of 0.24 g/g glucose.

In summary, the introduced deletions in the transaminases as well as the deletion of *panBC* did not further promote the synthesis of β-alanine and only the overexpression of *aspB* proved to be beneficial.

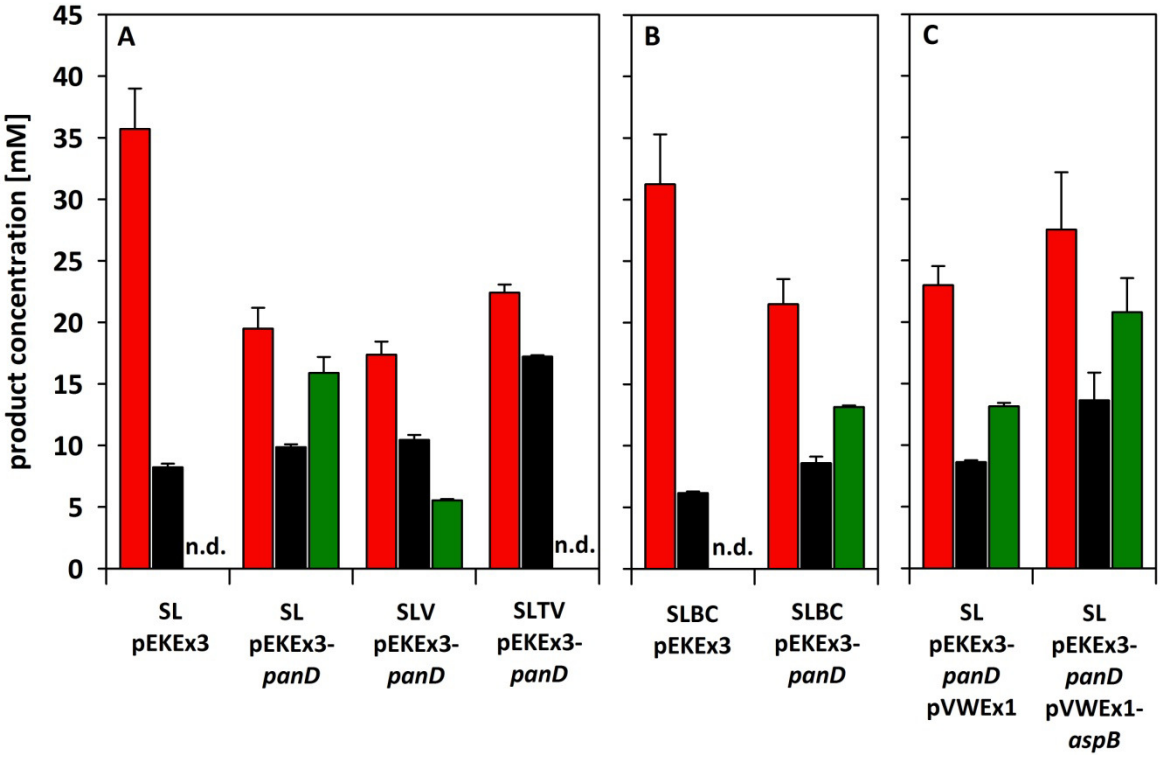

Figure 28: Production of β-alanine in the strains SL, SLV, SLTV and SLBC overexpressing *panD*.
A: Deletion of the α-alanine transaminases *alaT* and *avtA* (SLV and SLTV). B: Deletion of the genes coding for the pantothenate synthetic pathway (*panBC*); C: Combined overexpression of *aspB* and *panD*. Cultures were inoculated in CGXII medium supplemented with 50 mM glucose and 100 mM $NaHCO_3$. Accumulation of amino acids in supernatants of cultures after 72 h is depicted. Red: aspartate; black: α-alanine, green: β-alanine.

3.5 Aerobic production of ectoine

Ectoine is synthesized from aspartate via the lysine pathway and branches off from the intermediate aspartate-β-semialdehyde. The ectoine biosynthetic genes consisting of the diaminobutyrate transaminase (*ectB*), the diaminobutyrate acetyltransferase (*ectA*), and the ectoine synthase (*ectC*) are encoded in the *ectABC* operon and sequences from several organisms are available.

To find a suitable gene cluster that enables ectoine synthesis in *C. glutamicum*, alignments of all enzymes were performed using the amino acid sequences of the three native ectoine producers *Chromohalobacter salexigens, Halomonas elongata*, and *Mycobacterium smegmatis.* Overall, large homologies were found between *H. elongata* and *C. salexigens*, while the maximal identities of homologous *M. smegmatis* proteins stayed below 50 %. The largest differences were found for EctA, where $EctA_{Cs}$ and $EctA_{He}$ share 84 % sequence identity, whereas $EctA_{Ms}$ shares only 40 % identity with $EctA_{Cs}$. Important to note is an amino acid substitution in the coenzyme A (CoA) binding pocket of $EctA_{Ms}$ form arginine to serine, which might influence the binding of CoA (red box, Figure 29). As a consequence, the ectoine biosynthetic genes of *C. salexigens* were used for further experiments.

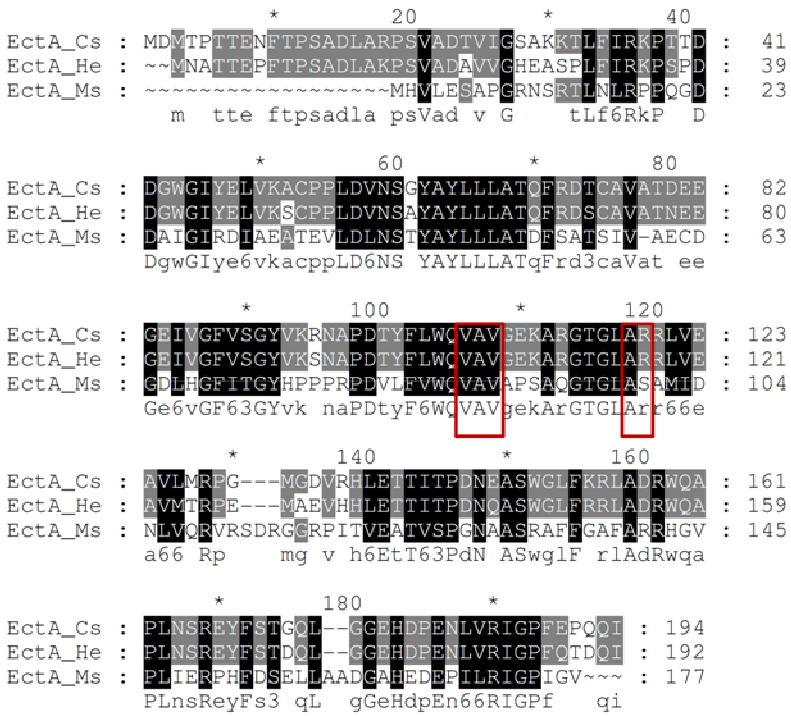

Figure 29: Multiple sequence alignment of amino acid sequences of EctA from *C. salexigens* (EctA_Cs), *H. elongata* (EctA_He) and *M. smegmatis* (EctA_Ms).
Conserved regions in all three enzymes are shown in black. Grey color depicts conserved regions in two of the three enzymes. The red boxes indicate the CoA binding motif.

3.5.1 *C. salexigens* EctA and EctB are active in *C. glutamicum* DM1729

To test the specific activities of the enzymes, the *ectABC* operon of *C. salexigens* was cloned into pEKEx3. The resulting plasmid was transferred into the lysine producing strain DM1729. EctB catalyzes the reversible transfer of the γ-amino group from diaminobutyrate to 2-oxoglutarate and the resulting glutamate was measured discontinuously via HPLC. The specific activity of EctB was 54.3 ± 17.1 mU/mg, while the control strain carrying pEKEx3 shows no diaminobutyrate transaminase activity. The acetyl-CoA transferase EctA catalyzes the addition of an acetyl group to diaminobutyrate, thereby releasing CoA, which can be detected spectrophotometrically with Ellmann´s reagent at 412 nm. A specific activity of 26.0 ± 5.6 mU/mg was detected in crude extracts of DM1729 carrying pEKEx3-*ectABC*, whereas no activity was found in the control strain DM1729 pEKEx3.

3.5.2 Expression of *C. salexigens ectABC* enables efficient ectoine synthesis in the wild type and DM1729

To enable salt-independent production of ectoine, the *C. salexigens ectABC* operon is expressed from the IPTG inducible promoter P_{tac}. The aerobic production of ectoine was performed in the wild type and in DM1729, which has three point mutations (*pyc*^{P458S}, *hom*^{V59A}, *lysC*^{T311I}) that enable lysine production and potentially enhance ectoine synthesis, too (Figure 30). Cells were cultivated in CGXII minimal medium supplemented with 200 mM glucose in the presence of IPTG.

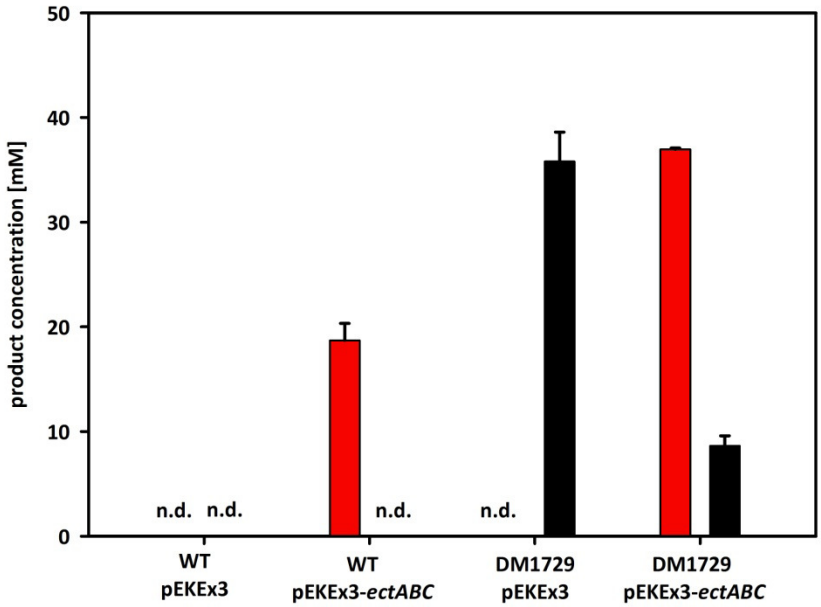

Figure 30: Aerobic production of ectoine in *C. glutamicum* wild type and DM1729 by expression of *C. salexigens ectABC*.
Cells were cultivated in CGXII medium supplemented with 200 mM glucose for 24 h. Red: ectoine [mM]; black: lysine [mM]; n. d.: not detected.

Production of ectoine is clearly dependent on the expression of the *C. salexigens ectABC* operon. In the wild type, a titer of 18.1 mM ectoine was achieved after 24 hours, while no ectoine was detectable in the control strains (Figure 30, red bars). In DM1729 expressing *ectABC*, lysine production was reduced by 76 % to 8.6 mM compared to the reference strain carrying pEKEx3, which accumulated 35.7 mM lysine in the supernatant. The expression of *ectABC* in DM1729 led to an accumulation of 36.9 mM ectoine. In summary, expression of *ectABC* in the genetic background of DM1729 enabled efficient production of ectoine with yields comparable to lysine overproduction with this strain.

3.5.3 Production of ectoine is promoted in fed-batch cultivation

Fed-batch cultivation was performed with DM1729 pEKEx3-*ectABC* to determine the stability of the process. Cells were inoculated with 200 mM glucose and fed with the same amount of substrate after 24 hours. The production of lysine and ectoine is similar within the first 24 hours, but a notable higher OD_{600} is achieved in the control strain carrying pEKEx3 (Figure 31). The additional feeding of glucose is used differently in both strains. The control culture increased the amount of lysine in the supernatant to 76.3 mM, and the OD_{600} was increased only by 16 %. In contrast, the feeding of glucose to cultures expressing the *ectABC* operon increased the ectoine titer only by 26 % to 44.1 mM, but caused a doubling of biomass. Taken together, the additional feeding of glucose doubled the optical density of the ectoine production strain, but not the concentration of the product.

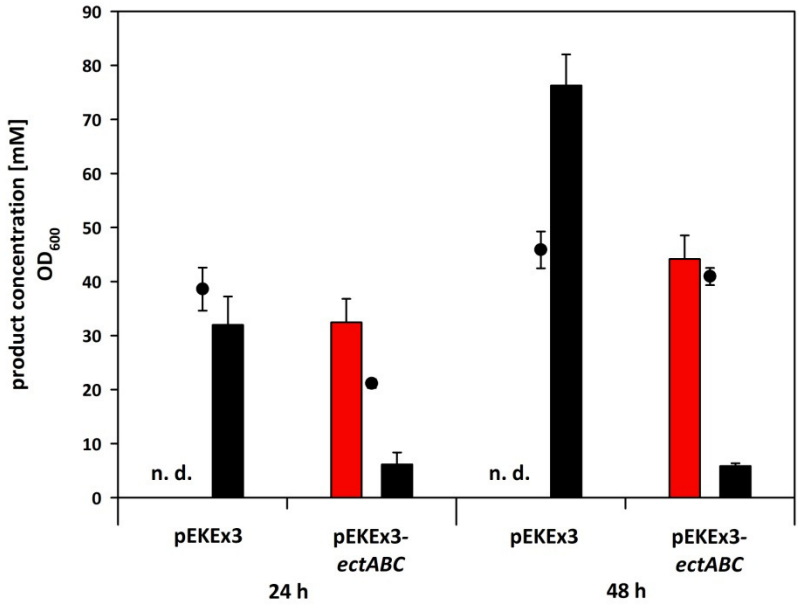

Figure 31: Production of ectoine and lysine of the strains DM1729 pEKEx3 and DM1729 pEKEx3-*ectABC* after 24 and 48 hours.
Cells were fed batch cultivated with additional 200 mM glucose after 24 hours. Red: ectoine, black: lysine; n. d.: not detected. The dots indicate the OD_{600}.

3.5.4 Use of alternative carbon sources

3.5.4.1 Growth in glycerol is faster than in arabinose and xylose

The use of waste products and non-food carbon sources is of major interest for the synthesis of compounds of biotechnological relevance. Therefore, DM1729 pEKEx3-*ectABC* was combined with pVWEx1-*araBAD* or pVWEx1-*glpFKD,* encoding the biosynthetic genes of *E. coli* for the utilization of arabinose or glycerol, respectively. The genes for the utilization of xylose are encoded on pEKEx3-*xylA*$_{Xc}$*B*$_{Cg}$, which was transferred into DM1729 pVWEx1-*ectABC*. The respective plasmid combinations without the ectoine biosynthetic genes served as controls in the experiments.

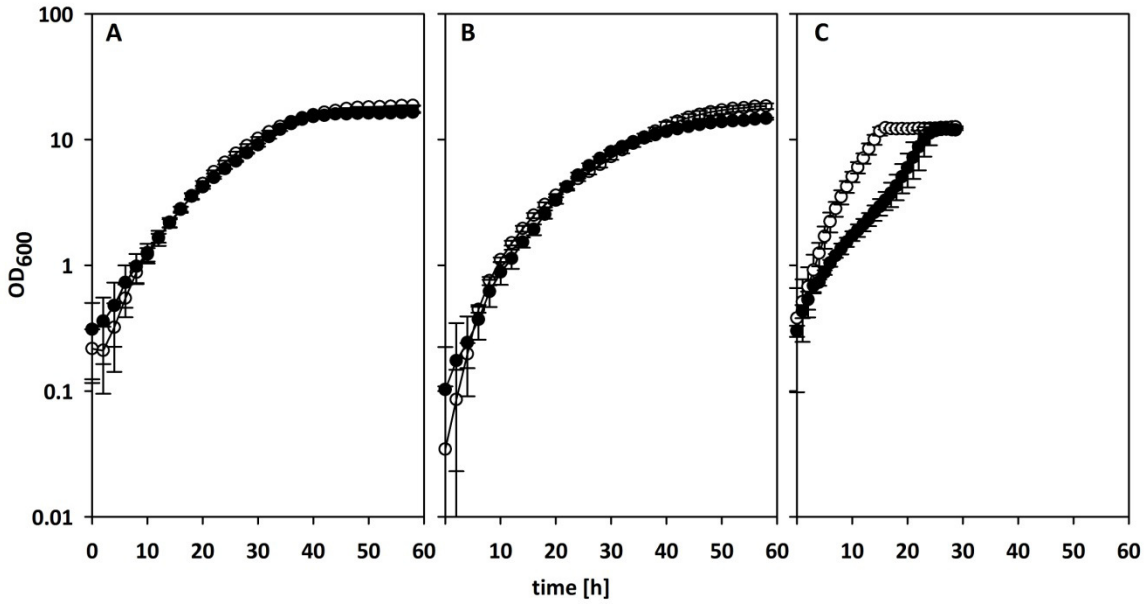

Figure 32: Growth of DM1729 in CgXII medium with alternative carbon sources.
A: Growth with 120 mM arabinose, DM1729 pVWEx1-*araBAD* combined with: open circles: pEKEx3, filled circles: pEKEx3-*ectABC*; B: Growth with 120 mM xylose, DM1729 pEKEx3-*xylA*$_{Xc}$*B*$_{Cg}$ combined with: open circles: pVWEx1, filled circles: pVWEx1-*ectABC*; C: Growth with 200 mM glycerol, DM1729 pVWEx1-*glpFKD* combined with: open circles: pEKEx3, filled circles: pEKEx3-*ectABC*.

Cultures grown with arabinose and xylose enter the stationary phase after 40 and 44 hours, respectively (Figure 32 A and B). In contrast, growth on glycerol is significantly faster. Cells enter the stationary phase after 25 hours (Figure 32 C). Additionally, only minimal differences in the growth rates on arabinose and xylose can be detected between the control strains and the strains expressing the *ectABC* genes. On glycerol, expression of the operon reduced the growth rate from 0.20 to 0.14 h^{-1}. Nevertheless, all carbon sources are consumed properly.

3.5.4.2 Production of ectoine is enabled by arabinose, xylose, and glycerol

To test the utilization of the non-food carbon sources for ectoine synthesis, the appropriate strains were cultivated in CGXII media supplemented with arabinose, xylose or glycerol. The pentoses were used in a concentration of 240 mM while glycerol were fed stepwise, as it shows an inhibitory effect in higher concentrations (Rittmann *et al.*, 2008). The cultures were inoculated with 200 mM glycerol and fed with the same amount of substrate after 24 hours. The different concentrations of the pentoses arabinose and xylose and the C-3 compound glycerol were chosen to balance the amount of molar carbon used in the experiments.

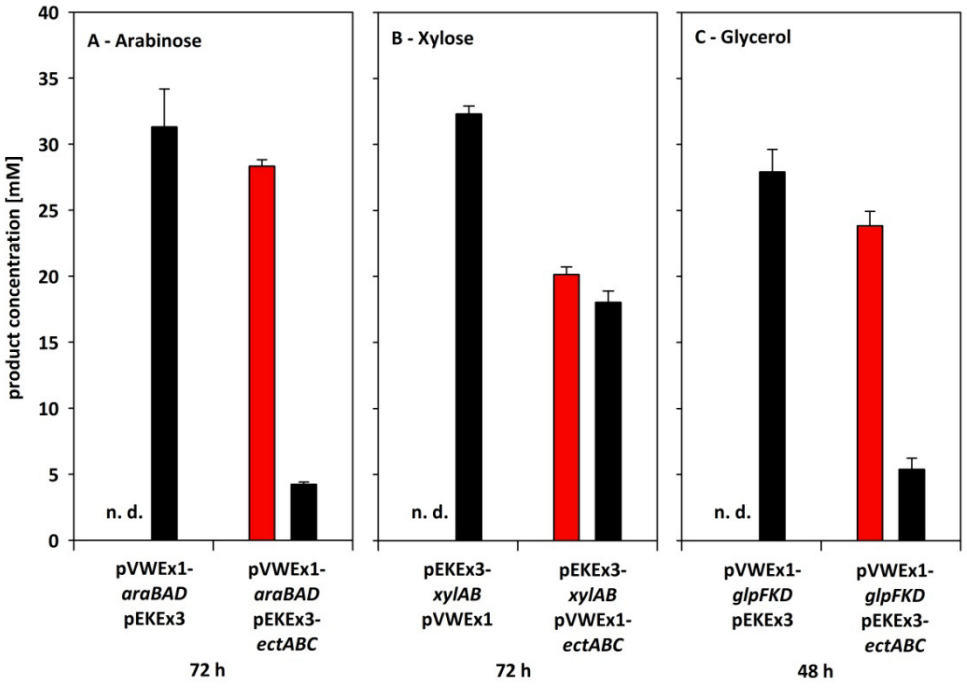

Figure 33: Production of ectoine from arabinose, xylose and glycerol.
Supernatants of arabinose and xylose grown cultures were analyzed after 72 h, while glycerol grown cultures were analyzed after 48 h. Strains carry vectors necessary for the utilization of the respective carbon source and the ectoine operon encoded on pEKEx3 (arabinose, glycerol) or pVWEx1 (xylose). Black bars: lysine, red bars: ectoine.

Ectoine was efficiently synthesized from all three carbon sources (Figure 33, red bars). After 72 hours, strains cultivated with the pentoses arabinose and xylose showed an formation of ectoine of 28.3 mM and 20.1 mM, respectively. In glycerol grown cultures, 23.8 mM ectoine were detected within 48 hours. Lysine was excreted during ectoine overproduction especially in xylose grown cultures, and therefore, it is important to minimize the excretion the by-product.

3.5.5 Yields of ectoine production

The yields for ectoine production were calculated after complete consumption of the carbon source. The production rates of the process were not determined, as the synthesis was only followed in 24 h steps.

Table 8: Yields for ectoine synthesis from all tested carbon sources.
Yields were calculated after complete consumption of carbon source. N. d.: not detected.

Strain	**WT**	**DM1729**			
Carbon source	Glucose	Glucose	Arabinose	Xylose	Glycerol
Yield lysine [g/g]	n. d.	0.145 ± 0.011	0.128 ± 0.012	0.133 ± 0.003	0.113 ± 0.023
Yield ectoine [g/g]	0.074 ± 0.006	0.146 ± 0.000	0.113 ± 0.002	0.080 ± 0.002	0.100 ± 0.007

The expression of the ectoine operon in the wild type led already to a significant formation of the compatible solute. This yield was further enhanced by the introduction of the biosynthetic genes into the strain DM1729, in which the utilization of the carbon source glucose led the excretion of 0.145 g/g. For the alternative carbon sources, higher yields are achieved for lysine than for ectoine synthesis, while there are no differences on glucose (Table 8). With arabinose and with glycerol the ectoine yields were 12 % lower than the lysine yields from these carbon sources. On xylose, an increased formation of the by-product lysine further reduced the ectoine yield. However, it was shown that efficient synthesis of ectoine is possible by heterologous expression of the ectoine biosynthetic genes, and furthermore, that this production is possible from sustainable carbon sources.

4 Discussion

4.1 Production of aspartate

The amino acid aspartate is a chemical building block with broad applications as food additive or as a biodegradable specialty polymer and holds potential to be synthesized independently from petrochemical resources. In cells, aspartate is not an end-product of a single pathway, but it is a central precursor for the synthesis of amino acids, for the cell wall component diaminopimelic acid, and for the B-vitamin pantothenate. Several subsequent pathways are dependent on its availability, and promoting the synthesis of aspartate does not necessarily mean that the amino acid is accumulating in the culture medium. Therefore, strategies were developed to enhance its synthesis and to minimalize the utilization of aspartate by the production host *Corynebacterium glutamicum*.

Aspartate excretion as a by-product in *C. glutamicum* was first described during glutamate production triggered by biotin limitation (Shiio *et al.*, 1962). Later on, aspartate excreting mutants were derived from *C. glutamicum* ATCC 14067 in several rounds of random mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine, which introduces point mutations by transition from GC→AT (Mori and Shiio, 1984; Shiio *et al.*, 1982). These mutants are characterized by reduced activities of citrate synthase and the pyruvate kinase and excreted aspartate under biotin limitation. However, aspartate excretion in these mutants was not stable and also restricted by other nutritional requirements not necessary related to the biosynthesis of the amino acid. Furthermore, the biochemical characterization of these strains was focused on measurements of citrate synthase and pyruvate kinase and other enzymes possibly influencing aspartate excretion, e.g. malate synthase, were not further investigated. Although these mutants overproduce aspartate, the obtained results were not integrated in this work, due to the undefined genetic background of these strains.

4.1.1 Synthesis of aspartate under aerobic conditions

Under aerobic conditions, the overproduction of aspartate was not enabled successfully with the implemented genetic modifications of *C. glutamicum*. The main focus was the expression of the *E. coli* aspartase (AspA) and the endogenous aspartate aminotransferase (AspB), but the expression of the genes alone did not promote aspartate synthesis. Therefore, deletions of the *fum* and *panD* gene were introduced into the genome of *C. glutamicum*, presumably improving the synthesis of fumarate or oxaloacetate, respectively. In both genetic backgrounds no aspartate was excreted, but the expression of *aspA* and *aspB* promoted growth of these strains, hinting on an indeed improved supply with the

respective precursor. With the deletion of the *panD* gene, cells are impaired in the synthesis of CoA, which is a cofactor of the pyruvate dehydrogenase complex (PDHC) marking the entry point of the oxidative branch of the TCA cycle. This inhibition was thought to promote the flux into the reductive branch, caused by the increased availability of pyruvate for anaplerotic reactions. Instead, the supernatants of the Δ*panD* strains showed a significant accumulation of pyruvate overflow metabolites (valine and alanine), but aspartate overproduction was not detected. In contrast to that, the excretion of 2 g/l aspartate as a by-product of glutamate overproduction (32 g/l from 100 g/l glucose) was detected in a defined pyruvate kinase (*pyk*, cg2291) mutant (Sawada *et al.*, 2010). In Δ*pyk*, a stoichiometric production of one molecule of PEP and one molecule pyruvate per molecule glucose is achieved by the PTS mediated sugar uptake and subsequent glycolysis. This was exploited during biotin-limited and thereby growth-arrested glutamate overproduction. Under these conditions, only PEP and not pyruvate serves as anaplerotic precursor, caused by the biotin dependence of pyruvate carboxylase. Thus, enhanced supply with PEP promoted aspartate excretion in growth arrested cells.

The growth of the *panD* mutants constructed in this work differed to recent findings for *panD* mutants in *E. coli* and *C. glutamicum* (Dusch *et al.*, 1999a; Kennedy and Kealey, 2004). The defined *panD* mutant generated here grew without the addition of β-alanine (Figure 7, p. 52), while the *C. glutamicum* insertion mutant ND2, and the *E. coli* mutant carrying a point mutation inactivating the decarboxylase, grew only with supplementation of the β-amino acid. The latter two mutants were supplemented in minimal medium with 5 µM or 1 mM β-alanine, respectively, without determining the minimal amount necessary to complement the deletion. However, only small amounts of β-alanine enabled growth of the mutants. Prior to growth experiments in minimal medium performed here, cells were precultivated in complex medium and it is not known, if cells are fully depleted of β-alanine. A residual intracellular pool accumulated during precultivation eventually causes the observed growth of the *panD* mutant. Nonetheless, after a passage on CgXII minimal medium plates supplemented with glucose for 24 h, the *panD* mutant generated here resumed growth on fresh minimal medium plates. In the above mentioned Δ*pyk* strains, the excretion of aspartate was linked to a growth arrest, and therefore, the *panD* gene was also deleted in the genetic background of Δ*sdhCAB* Δ*ldhA*. But also under anaerobic and thereby growth arrested conditions, the deletion of *panD* did not further promote aspartate excretion (3.2.5, p. 65). Another pathway for the synthesis of β-alanine is not known for *C. glutamicum*. In eukaryotes, β-alanine is synthesized in three steps from uracil with the dihydropyrimidine dehydrogenase, the dihydropyrimidinase and the β-alanine synthase. This pathway is present only in some eukaryotes, and enables the use of pyrimidines as sole nitrogen source (Campbell, 1957a, b). Recently, a dihydropyrimidine dehydrogenase was discovered in *E. coli*, but, like in *C. glutamicum*, the complete pyrimidine catabolic pathway is not present (Hidese *et al.*, 2011).

The deletion of the gene for the aspartokinase gene (*lysC*) was another strategy to diminish aspartate drain off and enable accumulation of the amino acid. The aspartokinase LysC catalyzes the phosphorylation of aspartate and is the entry-point for the synthesis of threonine, lysine and diaminopimelic acid. The *lysC* gene contains an additional promoter mediating the transcription of the β-subunit of LysC and forms an operon with the aspartate semialdehyde dehydrogenase gene *asd* (Kalinowski *et al.*, 1990). However, a deletion of the aspartokinase gene by homologous recombination was not possible (3.1.6.1, p. 52). Apparently, the viability of cells is strictly dependent on the availability of aspartylphosphate. All steps of the recombination were performed on complex medium, supplying the cells with all amino acids. Not provided, however, is the cell wall precursor diaminopimelic acid, which can only be synthesized in the course of lysine synthesis (Yeh *et al.*, 1988). Also the supply with threonine does not enable the synthesis of the cell wall component. Threonine is synthesized in four enzymatic steps from aspartylphosphate and might serve as substrate for diaminopimelate, however, the dephosphorylation of homoserine phosphate to threonine by threonine synthase ThrC is irreversible (Malumbres *et al.*, 1994).

A disruption mutant of *lysC* was reported in the lysine producing strain *C. glutamicum* ATCC 21799, formerly known as *Corynebacterium lactofermentum* or *Brevibacterium lactofermentum* (Jetten *et al.*, 1995). This strain is devoid of homoserine dehydrogenase, which causes threonine auxotrophy, and carries a feedback-resistant aspartokinase with a three times higher activity compared to *C. glutamicum* ATCC 13032, used in this work. The disruption of *lysC* was performed by introducing a chloramphenicol acetyltransferase into the gene. However, this strain grows in minimal medium with a four times higher doubling time compared to the parental strain, and the addition of lysine or diaminopimelate has no influence on the growth behavior. The synthesis of lysine showed a strong reduction but was not diminished completely. The authors proposed that lysine is synthesized either by an unknown path from threonine or by isoenzymes of the aspartokinase, similar to *E. coli* and *B. subtilis*. These organisms possess three aspartokinases (ask) with different functions and regulations (Cohen *et al.*, 1969; Patte *et al.*, 1967; Zhang *et al.*, 1990). In *E. coli*, Ask I and Ask II, encoded by *thrA* and *metL*, respectively, are bifunctional enzymes with aspartokinase and homoserine dehydrogenase activity. ThrA is allosterically inhibited by threonine, and its synthesis is repressed on the transcriptional level by threonine and isoleucine. MetL is involved in the synthesis of methionine and is repressed by this amino acid. In contrast, Ask III (LysC) is a monofunctional enzyme, whose activity and transcription is regulated by lysine. In *B. subtilis*, Ask I (DapG) is involved in the synthesis of the cell wall precursor diaminopimelate, while Ask II (LysC) is involved in lysine synthesis and Ask III (YclM) in threonine and methionine synthesis. Deletion of Ask II and Ask III alone does not affect growth in minimal medium, but a combined deletion leads to lysine, threonine and methionine auxotrophy (Zhang *et al.*, 1990). The deletion of Ask I in *B. subtilis* can be compensated by Ask II and III, if no

inhibition by accumulation of amino acids (lysine or threonine and lysine, respectively) occurs (Roten *et al.*, 1991). For *C. glutamicum* ATCC 13032, only the monofunctional aspartokinase LysC is known, which is allosterically inhibited by a combination of threonine and lysine. This might explain the disability to disrupt the gene and the essentiality of LysC for *C. glutamicum*.

4.1.2 Overproduction of aspartate under anaerobic conditions

In this work, a fermentative process for the overproduction of aspartate with *C. glutamicum* was developed. The strain *C. glutamicum* Δ*sdhCAB* Δ*ldhA* (SL) with deleted succinate dehydrogenase and lactate dehydrogenase genes was used as basis for aspartate production. Further adaptations of the strain are depicted in Figure 34 and targeted improvements in product formation (e.g. expression of *aspA* and *aspB*), of the precursor supply (e.g. expression of *ppc* and *pyc*), the reduction of by-products (deletion of *cat*, *alaT* and *avtA*), or cofactor recycling (e.g. expression of *mdh* and *gapA*), and are discussed in the following sections.

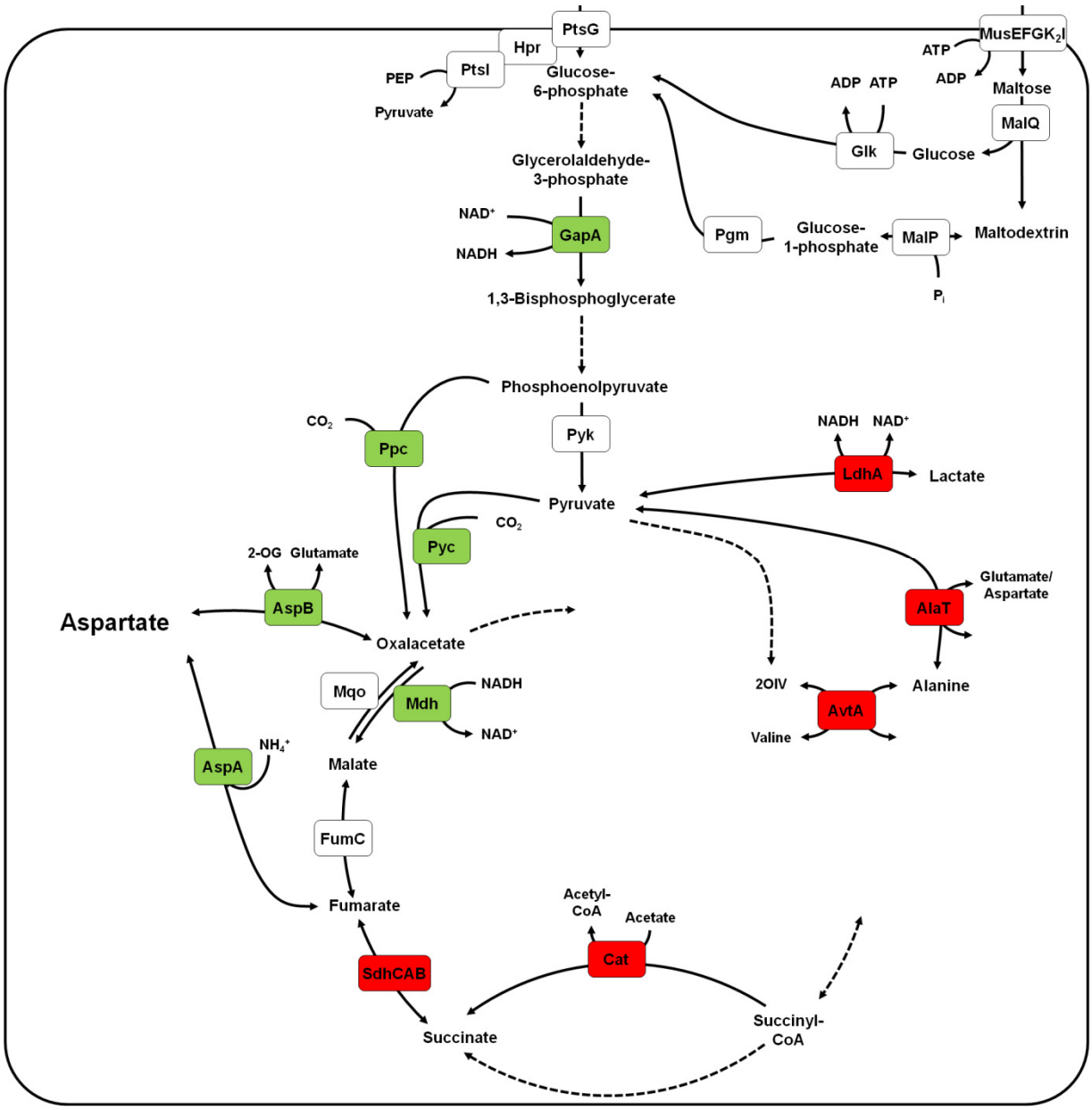

Figure 34: Modifications in the central carbon metabolism of *C. glutamicum* to enable efficient aspartate overproduction.

Red boxes: deletions; green boxes: overexpressions of respective genes. Abbreviations: PtsG: PTS transporter glucose; Hpr, PtsI: unspecific PTS components; MusEFGK$_2$I: ABC-transporter for maltose uptake; MalQ: 4-α-glucanotransferase; MalP: maltodextrin phosphorylase; Pgm: α-phosphoglucomutase; Glk: glucokinase; GapA: glyceraldehyde-3-phosphate dehydrogenase; Pyk: pyruvate kinase; LdhA: lactate dehydrogenase (NAD-dependent); IlvBN: acetohydroxyacid synthase subunits; IlvE: branched chain amino acid aminotransferase; AlaT: alanine transaminase; AvtA: alanine valine transaminase; Ppc: PEP-carboxylase; Pyc: pyruvate carboxylase; Cat: Succinyl-CoA:Acetate CoA transferase; SucCD: succinyl-CoA synthetase; SdhCAB: succinate dehydrogenase; AspA: aspartase; Fum: fumarase; Mqo: malate quinone oxidoreductase; Mdh: malate dehydrogenase; AspB: aspartate aminotransferase.

4.1.2.1 Anaerobic fermentation of aspartate in the strain SL

One basic requirement for aspartate production is to prevent the metabolization of the amino acid. This task was accomplished under anaerobic conditions. In *C. glutamicum*, the change from oxic to anoxic conditions induces a growth arrest and the switch to a fermentative metabolism that is characterized by the excretion of succinate, lactate, and acetate (Dominguez and et al., 1993; Inui *et al.*, 2004b). The overproduction of aspartate under anaerobic conditions without growth requires elimination of the main fermentation products by the deletion of the genes for succinate dehydrogenase and lactate dehydrogenase. The strain *C. glutamicum* Δ*sdhCAB* Δ*ldhA* pEKEx3 excretes aspartate anaerobically with a yield of 0.55 mol/mol glucose and forms alanine as main by-product (Figure 10, p. 55).

In general, anaerobic consumption of carbon sources is coupled to the reduction of a terminal electron acceptor other than oxygen or the excretion of reduced fermentation products. Based on this, anaerobic fermentations for the production of organic acids like succinate, lactate and ethanol were developed (Inui *et al.*, 2004a; Litsanov *et al.*, 2012a; Okino *et al.*, 2008a). Anaerobic processes for amino acid synthesis with *C. glutamicum* were developed recently for alanine and valine (Hasegawa *et al.*, 2013; Hasegawa *et al.*, 2012; Jojima *et al.*, 2010). The processes are based on sufficient supply with the precursor pyruvate and a decreased flux into the reductive branch of the TCA cycle. However, the main difference to the aspartate production described here is that both amino acids are produced with amino acid dehydrogenases and that alanine and valine are excreted as reduced fermentation products. This strategy was not applicable for aspartate, as the aspartate dehydrogenases analyzed here were not active under the tested conditions (3.2.3.5, p. 64). Therefore, aspartate is not excreted as a reduced product in the process described here. Other fermentation products besides succinate, lactate, and acetate were not detected in supernatants of the strain SL (Figure 18, p. 64; Figure 20, p. 67). Possible candidates in this strain reside from the glycolysis and reductive branch of the TCA cycle, while the production of organic acids from the oxidative branch seems unlikely, as the *gltA* gene for citrate synthase and *aceA*, coding for the E1p subunit of the pyruvate dehydrogenase complex, are downregulated (Inui *et al.*, 2007).

The addition of nitrate as terminal electron acceptor to anaerobic cultures of the strain SL improved the slow glucose utilization caused by the deletion of *sdhCAB* and *ldhA*, but came along with a high excretion of alanine and a concomitant reduction of aspartate in the supernatants (Figure 13, p. 58 and Figure 14, p. 59). Nitrate promotes biomass formation in *C. glutamicum* which is tenfold lower than under aerobic conditions (Nishimura *et al.*, 2007; Takeno *et al.*, 2007). The organism possesses the *narKGHJI* gene cluster encoding orthologous proteins of *E. coli* for nitrate/nitrite antiport, the nitrate reductase and an assembly subunit (Bott and Niebisch, 2003). *C. glutamicum* lacks pathways for

ammonification and denitrification and the reduction of nitrate leads to an equimolar formation of toxic nitrite. Nitrate respiration induces many genes of the SOS response which is crucial for long term survival, to protect cells from reactive nitrogen species (Nishimura *et al.*, 2011). This toxic effect might cause the concentration-dependent, bi-phasic metabolization of glucose that was observed for cultures supplemented with nitrate (Figure 13, p. 58), consisting of an initial phase of accelerated, and a second phase of decelerated glucose consumption. On the other hand, two mol NADH per mol glucose are generated during glycolysis, which need to be regenerated by two molecules of nitrate. The bi-phasic glucose consumption might therefore be caused by a depletion of nitrate, as the second phase with decelerated glucose consumption started, when strains supplemented with 30 mM nitrate had consumed around 10-15 mM glucose. However, neither nitrate nor nitrite was measured in this experiment. During nitrate respiration alanine was detected as the main fermentation product (Figure 14, p. 59). The amino acid is synthesized in *C. glutamicum* from pyruvate by the aminotransferases AlaT and AvtA, which use glutamate, aspartate and valine as amino group donors (Marienhagen and Eggeling, 2008; Marienhagen *et al.*, 2005). During the aerobic production of lysine, valine and pyruvate, an increased accumulation of alanine was detected in the production phase, caused by the insufficient conversion of pyruvate into desired products (Blombach *et al.*, 2007; Krömer *et al.*, 2004; Wieschalka *et al.*, 2012). Alanine is therefore considered as a product of overflow metabolism, which might also occur in the strain SL under anaerobic nitrate respiration. This overflow is supported by transcriptomic data for the anaplerotic node (Nishimura *et al.*, 2011). The analysis revealed besides a minor increase in the expression of the anaplerotic PEP carboxylase gene *ppc*, a reduced expression of the citrate synthase gene *gltA* and increased expression of the gluconeogenetic phosphoenolpyruvate carboxykinase gene *pck* and malic enzyme gene *malE*. This indicates only a minor role of the TCA cycle under nitrate respiration. Nonetheless, a reduced expression of TCA cycle genes does not necessarily imply a reduced flux through the cycle, which also depends on the activity of the respective enzymes.

4.1.2.2 Enhancing the aspartate overproduction by the overexpression of the aspartate aminotransferase gene *aspB*

The overproduction of aspartate in *C. glutamicum* was enhanced by 18 % by the expression of the aspartate aminotransferase AspB (Figure 18, p. 64). Neither the expression of aspartases (AspA) nor of aspartate dehydrogenases (AspDH) improved aspartate overproduction. Moreover, the expression of the aspartase of *E. coli* $AspA_{Ec}$ reduced the aspartate yield to 26 % compared to the control strains (Figure 16, p. 62). This result is in line with the results obtained for the enzymatic assays that showed a preference of the aspartases for the degradation of aspartate (Table 6, p. 60). In a previous study, it was shown that the expression of the $AspA_{Ec}$ in a lysine producing strain of *C. glutamicum* and the

feeding of a glucose/fumarate mixture improves lysine yields and leads to a concomitant excretion of aspartate (Menkel *et al.*, 1989). Aspartate overproduction with aspartases under aerobic conditions is therefore enabled in *C. glutamicum*, when sufficient amounts of fumarate are provided. Apparently, the deletion of the succinate dehydrogenase genes does not enable an accumulation of fumarate in the strain SL. Another possibility is a futile cycle between fumarate, aspartate and oxaloacetate mediated by the reactions of AspA and AspB. Aspartate formed from fumarate by the aspartase would then be converted to oxaloacetate by aspartate aminotransferase (compare to scheme shown in Figure 34, p. 90). To test this hypothesis, a strain with a deleted *aspB* gene (Δ*sdhCAB* Δ*ldhA* Δ*aspB*, SLB) was constructed. In this strain, aspartate synthesized by aspartases cannot be transaminated to oxaloacetate. But neither the expression of the endogenous aspartase nor the expression of homologous enzymes from *B. subtilis* or *E. coli* was able to compensate the deletion (Figure 17, p. 63), indicating that no fumarate is accumulating by the deletion of the *sdhCAB* genes.

In another study, a similar strategy was used to establish fumarate production under oxygen-deprived conditions. In a strain optimized for pyruvate production, the additional deletion of the *sdhCAB* gene did not enable fumarate accumulation intracellulary or in culture supernatants (Wieschalka, 2012). In a succinate-producing derivative of *E. coli*, the deletion of the fumarate reductase, that catalyzes the reduction of fumarate to succinate, does not lead to an excretion of fumarate but malate in anaerobic fermentations (Zhang *et al.*, 2011). Explanations for these results are scarce, but are related to a possible toxicity of fumarate to the cells. Another possible explanation that prevents the accumulation of fumarate, might be a cycle between the malate dehydrogenase (Mdh) and the malate:quinone oxidoreductase (Mqo). The enzymes catalyze the opposite reactions from oxaloacetate to malate (Mdh) and back to oxaloacetate (Mqo) leading to a net transfer of electrons from NAD to quinone (Molenaar *et al.*, 2000; Molenaar *et al.*, 1998). This cycle would prevent aspartate formation with AspA as fumarate synthesis is blocked, but does not interfere with the transamination of oxaloacetate by AspB.

Under aerobic conditions, it was shown that AspB is essential, as the growth of mutants was inhibited in minimal medium (Figure 6, p. 50). In a previous study, a disruption mutant of the *aspB* gene was created in a restriction-deficient mutant of *C. glutamicum* ATCC13032 (McHardy *et al.*, 2003). This mutant showed no growth defect, which is in contrast to the defined mutants (Δ*aspB* and Δ*sdhCAB* Δ*ldhA* Δ*aspB*) analyzed here. However, the *aspB* mutants obtained here indicate a highly specific reaction of AspB, as no other transaminase was apparently able to take over its function. This is in contrast to other aminotransferases like IlvE, which has a broad substrate spectrum and is involved in the synthesis of leucine, isoleucine and valine. The function of IlvE can at least partially be replaced by AvtA (Marienhagen *et al.*, 2005). In *E. coli*, the aspartate aminotransferase AspC is involved in the synthesis not only of aspartate, but also of tyrosine and of phenylalanine. These functions can

be taken over by either IlvE, which synthesizes phenylalanine, or TyrB that can replace AspC in tyrosine, phenylalanine and aspartate synthesis (Gelfand and Steinberg, 1977; Jensen and Calhoun, 1981; McHardy *et al.*, 2003; Powell and Morrison, 1978).

The overexpression of the *aspB* gene proved to be beneficial for the anaerobic aspartate production process and increased the aspartate overproduction to 0.83 mol/mol glucose (Figure 18, p. 64). In contrast to AspA, AspB depends on glutamate as amino donor. Therefore, the synthesis of one mol of aspartate consumes one mol of NADPH, which is needed by glutamate dehydrogenase for the amination of 2-oxoglutarate. In *C. glutamicum*, the $NADP^+$ is reduced in the pentose phosphate pathway by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase or in the TCA cycle by isocitrate dehydrogenase. Although information about the activities of these enzymes under anaerobic conditions is scarce, a limitation of NADPH-, and therefore, of glutamate-supply is unlikely, as the overexpression of the *aspB* gene improved aspartate production, indicating a high intracellular amount of glutamate. Moreover, the expression of the transhydrogenase PntAB, reducing $NADP^+$ to NADPH, had a negative effect on aspartate overproduction (3.2.7.5, p. 72). Taken together, the production of aspartate is presumably not limited by the synthesis or the availability of glutamate.

Besides the endogenous AspB, also the enzymes of *E. coli* ($AspC_{Ec}$) and *B. subtilis* ($AspB_{Bs}$) were tested. $AspC_{Ec}$ is one of the best characterized PLP-dependent enzymes that belongs to the aminotransferase subgroup Iα, in which the binding of the substrate induces a conformational change (Okamoto *et al.*, 1994). The alignment of the $AspC_{Ec}$ sequence and the $AspB_{Cg}$ sequence shows only weak sequence identity between the enzymes. Five structural amino acids were determined in the crystal structure of $AspC_{Ec}$, mediating the binding of the PLP cofactor and the substrate (Jäger *et al.*, 1994; Jeffery *et al.*, 2000). $AspB_{Cg}$ does not share Lys258, necessary for PLP binding in AspC. However, $AspB_{Bs}$ also differs from the $AspC_{Ec}$ sequence and it is devoid of Arg280, necessary in *E. coli* for the binding and the recognition of aspartate. Nevertheless, the aminotransferases $AspB_{Bs}$ and $AspC_{Ec}$ mediated the overproduction of aspartate, but did not exceed aspartate formation of the endogenous enzyme.

Under anaerobic conditions, processes were developed by Jojima *et al.* and Hasegawa *et al.* that enable efficient overproduction of alanine and valine with amino acid dehydrogenases (Hasegawa *et al.*, 2012; Jojima *et al.*, 2010). Recently, the aspartate dehydrogenases of the thermophilic organisms *Archaeoglobus fulgidus* ($AspDH_{Af}$, AF1838) and *Thermotoga maritima* ($AspDH_{Tm}$, TM1643) were crystallized and analyzed in detail (Yang *et al.*, 2003; Yoneda *et al.*, 2006). Their structures were used as basis for alignments with the mesophilic enzymes of *Pseudomonas aeruginosa* PAO1 ($AspDH_{Pa}$) and *Ralstonia eutropha* ($AspDH_{Re}$) used here. All amino acids necessary for substrate binding are highly conserved, except $AspDH_{Af}$ L161, which is altered to isoleucine in *T. maritima* and to alanine in all mesophilic enzymes.

For the tested AspDHs, no activity was found in enzymatic assays and the expression of these enzymes had no influence on aspartate excretion in *C. glutamicum* (3.2.3.5, p. 64). In *E. coli*, a production scheme for aspartate was developed with $AspDH_{Pa}$. Efficient overproduction of aspartate was achieved in an enzymatic process with permeabilized cells that convert fumarate via malate and oxaloacetate to aspartate. This three step fed-batch process led to overproduction of 625 mM aspartate in six hours. However, an effective fermentative production scheme with living cells was not developed. The synthesis of aspartate with $AspDH_{Pa}$ leads to an accumulation of around 10 mM aspartate from 400 mM glucose. A higher titer was achieved from glucose plus succinate, where cells are inoculated with 80 mM glucose and succinate was added to the broth every two hours. Within 50 hours, 33 mM aspartate were produced from the glucose plus 500 mM succinate (Li *et al.*, 2011). However, this scheme was not applicable in *C. glutamicum*, as the tested AspDHs were inactive and additionally, the overall yield obtained in the fermentative process is rather low.

4.1.2.3 The deletion of the alanine valine aminotransferase increases aspartate overproduction

Alanine was detected as the main by-product during the fermentative production of aspartate. The general information about alanine transaminases is rather scarce, but for *C. glutamicum*, the two alanine synthesizing aminotransferases AlaT and AvtA were identified (Marienhagen *et al.*, 2005). AlaT uses glutamate or aspartate as amino donor while AvtA is part of the branched-chain amino acid (BCAA) pathway and accepts valine or alanine as amino donor, but not glutamate. Under aerobic conditions, AlaT was identified as the principal alanine-supplying aminotransferase in *C. glutamicum*, as a deletion of *alaT* decreased the intracellular content of alanine to a greater extent than did a deletion of the *avtA* gene (Marienhagen and Eggeling, 2008; Marienhagen *et al.*, 2005). A deletion of both genes prevented growth on minimal medium plates, which could be restored by the addition of alanine. The results obtained with the SL strains harboring the single and the double deletions of *alaT* and *avtA*, the strains SLT (Δ*sdhCAB* Δ*ldhA* Δ*alaT*), SLV (Δ*sdhCAB* Δ*ldhA* Δ*avtA*), and SLTV (Δ*sdhCAB* Δ*ldhA* Δ*alaT* Δ*avtA*), respectively, are different from these observations. Neither the deletion of *alaT* nor the deletion of *avtA* alone reduced the alanine content in the supernatants of anaerobically incubated cultures. Instead, the strains SLT and SLV excreted higher amounts of alanine than the control strain SL and only the deletion of both transaminases decreased alanine in the supernatants from 16.2 mM to 5.2 mM (Figure 21 A, p. 68). This residual amount of alanine in SLTV is presumably synthesized by unspecific side reactions of other aminotransferases. A similar effect was observed in a pyruvate-excreting strain of *C. glutamicum* that is devoid of *alaT* and *avtA*, but still excretes alanine as a by-product during the production phase (Wieschalka *et al.*, 2012). Furthermore,

the deletion of the alanine transaminases affected aspartate formation. The strains SLT and SLV formed less aspartate without overexpression of *aspB*, while the amount in SLTV stayed at the level of the control strain. With the additional expression of *aspB*, the aspartate content was increased in all strains. The highest aspartate excretion of 1.02 mol/mol glucose was reached with SLV.

The benefit of the *avtA* deletion might be found in the synthesis of the branched chain amino acids (BCAA). Pyruvate is the precursor for the BCAAs valine and leucine, and is also involved in the synthesis of isoleucine. The first step in the BCAA pathway is catalyzed by the acetohydroxy acid synthase IlvBN, which is feedback inhibited by valine, leucine and isoleucine. IlvN contains a regulatory C-terminal domain that is sensitive to all three BCAAs and binding of the amino acids negatively regulates the activity of IlvBN. Valine can be synthesized in two ways in *C. glutamicum*, either by IlvE or by AvtA. A preference for the synthesis or the degradation of valine by IlvE and AvtA is not known, but it was recently shown in a production process for valine, that the deletion of the *avtA* gene diminished alanine as a by-product (Hasegawa *et al.*, 2013). This hints on a catabolic function of AvtA and might lead to an accumulation of valine in the strain SLV, which in turn regulates the flux in the BCAA pathway by inhibiting the activity of IlvBN (Figure 35).

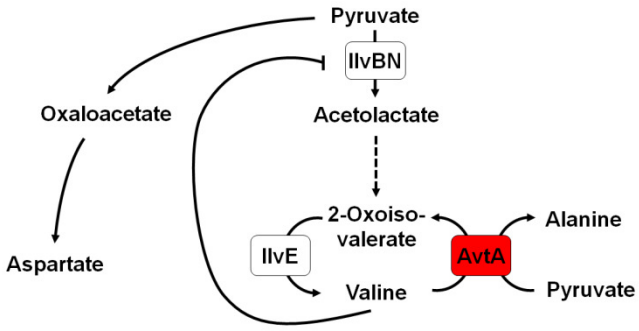

Figure 35: Inhibition of IlvBN by valine by deletion of alanine-valine aminotransferase.
Abbreviations: IlvBN: acetohydroxy acid synthase; IlvE: branched-chain amino acid aminotransferase; AvtA: Alanine-valine aminotransferase.

In *C. glutamicum* DM1729 and DM1933, the deletion of the *ilvB* gene promoted lysine overproduction (Blombach *et al.*, 2009). Overall, the deletion affected the transcription of around fifty genes, but none of the lysine biosynthetic genes. However, the increased lysine content is at least partially caused by a reduced flux into the BCAA pathway, which leads to an accumulation of pyruvate that serves as precursor for lysine synthesis. Therefore, the deletion of *avtA* might mimic the *ilvB* deletion in the proposed scheme and potentially improve the supply with pyruvate. In the first step, this led to an increased alanine content, but promoted in combination with the overexpression of *aspB* the

formation of aspartate. However, valine or other BCAA were not detected as by-product during aspartate overproduction.

The role of AlaT during anaerobic aspartate production is elusive. The enzyme is proposed as the principal alanine supplying enzyme under aerobic growth conditions, but a deletion of *alaT* during aspartate overproduction led to the highest alanine excretion. This underlines the proposed model for AvtA and the flux through the BCAA pathway, but does not match the observations for AlaT under aerobic conditions. Furthermore, the reduction of the by-product alanine is not completely transferred into the product aspartate, as it can be seen from the results obtained for the strain SLTV. Although the strain excretes less alanine than the other tested strains, the aspartate content does not exceed the content in SLV.

Taken together, with the deletion of the alanine aminotransferases a reduction of the alanine content can be achieved. Additionally, the valine-alanine aminotransferase AvtA occupies a special role, as it positively influences aspartate overproduction by a yet not completely understood mechanism.

4.1.2.4 Regeneration of reduction equivalents

The regeneration of reduction equivalents is of major concern, as it influences glucose consumption and therefore the velocity of the process. A maximum of two mol NADH per mol glucose is formed in the strain SL by the glyceraldehyde-3-phosphate dehydrogenase (GapA), which can be regenerated to NAD^+ by the malate dehydrogenase (Mdh). Limiting factors in this scheme are the phosphorylation of glycerol-3-phosphate by GapA and the carboxylation at the anaplerotic node. GapA is inhibited by high NADH levels and is therefore the rate-limiting enzyme in glycolysis controlling sugar consumption (Dominguez *et al.*, 1998; Omumasaba *et al.*, 2004). The Mdh synthesizes malate from oxaloacetate and fuels the reductive branch of the TCA cycle. Furthermore, the Mdh can contribute to the transfer of electrons to the quinone pool under aerobic conditions, when malate is reoxidized to oxaloacetate by the malate:quninone oxidoreductase (Mqo) (Molenaar *et al.*, 2000; Molenaar *et al.*, 1998).

To improve the velocity of aspartate formation, the overexpression of the glycolytic *gapA* and the reductive *mdh* was tested separately and in combination (3.2.7.2, p. 70). Both, GapA and Mdh had positive effects on aspartate overproduction (Figure 23, p. 70). For GapA, the effect was further evaluated and was shown to enhance the aspartate production rate 1.5-fold to 0.154 mM/h*gCDW and the glucose consumption rate 1.4-fold to 0.763 molC/h*gCDW, but not the total yield (Table 7, p. 74). The combined expression of *gapA* with *mdh* negatively influenced the velocity of the production compared to the single *gapA* overexpression. This might contribute to the reduced activity of GapA during combined expression with *mdh*, which was at the level of the control strain (Figure 22, p. 69.)

Besides its function in NAD^+-regeneration, Mdh is competitive to AspB for the substrate oxaloacetate. The initial strategy was to replace the Mdh with aspartate dehydrogenases (AspDH), which would serve both, NAD^+-regeneration and product formation. But this proved to be futile, as no activity was detected for the tested AspDHs, when they are expressed in *C. glutamicum*. The expression of the NAD-dependent glutamate dehydrogenase Gdh_{Pa} of *Peptostreptococcus asaccharolyticus* was another strategy to regenerate NAD^+ in an *mdh* deficient strain. The enzyme catalyzes the reductive amination of 2-oxoglutarate to glutamate, the precursor for AspB, and would serve precursor supply and NAD^+ regeneration (Marx *et al.*, 1999). The expression of the gdh_{Pa} gene could restore the impaired glucose consumption of the *mdh* mutant, but not the aspartate production that was achieved in a strain with intact Mdh (Figure 24, p. 72). The strategy of combining an increased expression of glycolytic genes with NAD-regenerating, product-forming enzymes was followed for the anaerobic production of alanine and valine, too (Hasegawa *et al.*, 2013; Hasegawa *et al.*, 2012; Jojima *et al.*, 2010). For alanine synthesis, the expression of *gapA* was combined with the expression of an alanine dehydrogenase, which increased the productivity 2.7-fold. In an anaerobic production scheme for valine, several genes of the glycolytic pathway (phosphofructokinase, phosphoglucose isomerase, triosephosphate isomerase, pyruvate kinase, and *gapA*) were overexpressed and combined with the heterologous expression of a leucine dehydrogenase, which improved glucose consumption 7.6-fold and the valine production 9.0-fold. The velocity of aspartate overproduction might therefore be further enhanced, if NAD^+-regeneration can be coupled to product formation.

4.1.2.5 Anaplerosis and carbon sources

The synthesis of aspartate is draining off oxaloacetate from the TCA cycle, which is replenished at the anaplerotic node by the carbon-fixing reactions of the PEP carboxylase (Ppc) and the pyruvate carboxylase (Pyc). The formation of the initial aspartate concentration of 0.55 mol/mol glucose obtained in standard medium is presumably promoted by the hydrolysis of urea. Under conditions of surplus nitrogen, urea enters the cell by passive diffusion, while an active uptake is induced under nitrogen starvation (Nolden *et al.*, 2000; Siewe *et al.*, 1998). The urease is a multisubunit complex encoded by the *ureABCEFGD* gene cluster that hydrolyzes urea into two molecules of ammonium and one molecule of CO_2, which can be fixed by Pyc and Ppc. Furthermore, aspartate overproduction was greatly enhanced by the addition of $NaHCO_3$ to the medium, which delivers bicarbonate ions (HCO_3^-) for these carboxylations (Figure 12, p. 57). Nevertheless, the overexpression of *ppc* and pyc^{P458S} did not further enhance aspartate excretion, when $NaHCO_3$ was added to the medium (Figure 19, p. 65). Similar effects were observed during anaerobic succinate synthesis with bicarbonate, where the expression of pyc^{P458S} led to decreased production rates or yields (Okino *et al.*, 2008a; Wieschalka,

2012). In contrast, the expression of *pyc*^{P458S} from the strong constitutive *tuf* promoter (P_{tuf}) greatly enhanced succinate production (Litsanov *et al.*, 2012a). In the latter production scheme, formate instead of bicarbonate was added to the medium, which was reduced to two molecules of CO_2 by the NADH-forming formate dehydrogenase (Fdh) of *Mycobacterium vaccae*. A side effect of this reduction is the import of CO_2 into the cells.

These results hint on a limitation in the interconversion of HCO_3^- and CO_2, if $NaHCO_3$ is supplemented. $NaHCO_3$ dissociates into Na^+ and HCO_3^- ions, and a proportion of the bicarbonate ions are dehydrated to CO_2 in the medium. Only the uncharged CO_2 can enter the cells by diffusion, but must be hydrated to HCO_3^- by the anhydrase CynT (*cynT*, cg2954) to serve as a substrate for the carboxylation reaction. Recently, CynT proved to be indispensable for growth, but overexpression of the gene did not result in improved growth or yields during lysine synthesis (Mitsuhashi *et al.*, 2004). Taken together, rather the diffusion of CO_2 in the cells than the hydration is the limiting factor in this scheme, and therefore, the selection of the carbon source for anaplerosis might be a valuable target to improve the aspartate production process.

Inui *et al*. postulated that Ppc is the main anaplerotic enzyme under anaerobic conditions (Inui *et al.*, 2004b). It was found that a deletion of the *ppc* gene inhibited glucose consumption and succinate formation, while a strain with a deleted *pyc* gene consumed and produced as the wild type. The biochemical analysis of Ppc revealed a synergistic inhibition of the enzyme by aspartate and 2-oxoglutarate and non-synergistic inhibition by succinate and malate (Mori and Shiio, 1985b). The inhibition of Ppc by aspartate is competitive to PEP and bicarbonate, and can additionally be overcome by acetyl-CoA or fructose-1,6-bisphosphate (Eikmanns *et al.*, 1989; Mori and Shiio, 1985a). The intracellular amounts of the respective compounds were not determined, but a high concentration of bicarbonate is expected, as it is a medium component. Furthermore, aspartate is accumulating in the medium which might circumvent an inhibition of Ppc by the amino acid. Nevertheless, an inhibition of Ppc by aspartate during the production process cannot be excluded.

The importance of Ppc under anaerobic conditions finds support within the observation made for the utilization of maltose. Maltose as carbon source led to the highest aspartate production rate (0.176 mM/h*gCDW) and yield (0.80 g/g maltose) (Table 7, p. 74). One main difference between the carbon sources glucose and maltose is the uptake. The disaccharide is taken up by the recently identified ATP-binding cassette transporter (ABC) $MusEFGK_2I$ and converted to either glucose-6-phosphate or added to the storage polymer maltodextrin (Henrich *et al.*, 2013). In total, the uptake of one mol maltose and the conversion to two mol glucose-6-phosphate consumes two mol of ATP and one mol inorganic phosphate (Seibold *et al.*, 2009). On glucose, two mol of PEP are consumed for the import and the phosphorylation to obtain the same amount of glucose-6-phosphate (Yokota and Lindley, 2005). Taken together, the utilization of maltose consumes ATP, while glucose uptake is

associated to PEP consumption. The connection between anaplerosis and carbon source is, in this case, the role of Ppc. Ppc competes with the PTS for the substrate PEP, and if all synthesized succinate is formed by the anaplerotic reaction of Ppc, as postulated by Inui (2004), only fifty percent of the available carbon can be channeled into the reductive branch of the TCA. The positive effect of maltose on aspartate formation might therefore be the increased availability of PEP for the carboxylation at the anaplerotic node.

4.1.2.6 Comparison to other aspartate production processes

In this work, an anaerobic process with resting cells was developed that led to aspartate overproduction with yields of 0.59 g/g glucose or 0.80 g/g maltose. Other fermentative productions are described for *C. glutamicum* by Shiio *et al.*, Mori *et al.*, Sawada *et al.*, and Menkel *et al.*, as well as the already discussed production of aspartate in *E. coli* with the aspartate dehydrogenases of *P. aeruginosa*. The use of the dehydrogenase is effective during enzymatic conversion of fumarate with permeabilized cells similar to existing aspartase-based productions on the industrial scale, while the expression of the $aspDH_{Pa}$ gene in growing cells leads to a low substrate specific yield of 0.02 g/g glucose. The excretion of aspartate in *C. glutamicum* was first observed as a by-product during glutamate overproduction. Based on this, Shiio *et al.* and Mori *et al.* developed aspartate producing strains by random mutagenesis that overproduce aspartate with yields of 0.29 g/g glucose or 0.23 g/g glucose, respectively (Mori and Shiio, 1984; Shiio *et al.*, 1982). These mutants are cultivated aerobically under biotin-limiting conditions and aspartate excretion is detected in the transition to the stationary phase. This demonstrates that arresting cells in growth is a prerequisite to enable aspartate overproduction from glucose, similar to the results obtained in this work. Besides this, aspartate occurs as a by-product (2 g/l) of biotin-limited glutamate production (32 g/l) in a defined pyruvate kinase deficient mutant (Sawada *et al.*, 2010). However, several biotransformations are described with immobilized cells that overproduce aspartate in bioreactors (Eggeling and Sahm, 2009; Zajkoska *et al.*, 2013). In these bioreactors, cells that express aspartases are cross-linked to a polymer and fed with a fumarate/ammonium solution. For recombinant *E. coli*, a process is described where cells are embedded in a chitosan matrix and are able to convert 99.8 % of the fed fumarate and produce 6 g/g_{CDW}/h aspartate (Szymanska *et al.*, 2011). The fermentative process described here, cannot compete with these biotransformations in terms of productivity and yield, but it is not dependent on petrochemical resources and shows that alternative processes exist if the availability of the substrate fumarate is limited.

4.2 Anaerobic overproduction of β-alanine

Next to its function as vitamin precursor, the non-proteinogenic amino acid β-alanine has large scale application in the synthesis of hydroxypropionic acid and as supplement in the diet of athletes. In this work, overproduction of β-alanine was achieved with aspartate-producing strains of *C. glutamicum*. However, significant amounts of the available substrate aspartate were not converted into the product, but excreted to the culture supernatant (Figure 27, p. 78). In advance, the enzymatic activity of three different aspartate-α-decarboxylases (PanD) was tested. Compared to the homologous enzymes of *E. coli* and *B. subtilis*, the endogenous enzyme ($PanD_{Cg}$) showed the highest specific activity in crude extracts of *C. glutamicum,* which is with 55.4 mU/mg in the range of the previously reported specific activity of 34.5 mU/mg by Dusch, *et al*. (Dusch *et al.*, 1999b).

The aspartate-α-decarboxylase is a pyruvate-dependent multimer consisting of four PanD peptide chains, of which three undergo a self-induced auto-proteolysis between the residues Gly24 and Ser25. In *C. glutamicum* it was recently found, that one additional arginine residue in the N-terminal region of the pro-protein is needed to initiate the maturation of the protein (Cui *et al.*, 2013). However, the proteolysis generates a pyruvoyl-residue representing the prosthetic group of the enzyme (Schmitzberger *et al.*, 2003). A major disadvantage of the aspartate-α-decarboxylase is the inactivation of this prosthetic group by the substrate aspartate. During the decarboxylation, the pyruvoyl-residue is transaminated to alanine depending on time and concentration of aspartate. The effect was detected with *E. coli* PanD, which was tested for enzymatic preparation of β-alanine in matrices similar to the enzymatic preparation of aspartate with immobilized *E. coli* cells (Konst *et al.*, 2009; Webb *et al.*, 2004). The embedded proteins were mixed with fresh substrate every thirty minutes, but lost 30 % of the initial activity within two hours.

Besides the inactivation, also self-proteolysis of the enzyme might be rate limiting for β-alanine overproduction with aspartate-producing strains expressing the *panD* gene. For *Mycobacterium tuberculosis*, a complete self-proteolysis of purified aspartate-α-decarboxylase was observed after 24 hours at 37 °C (Chopra *et al.*, 2002). A similar pattern was observed *in vitro* with *E. coli* PanD, were self-proteolysis was incomplete after 24 hours at 37 °C but promoted by elevated temperatures (Ramjee *et al.*, 1997). How these conditions reflect the self-proteolysis under physiological conditions is not known, but it gives an indication of a possible limitation.

However, the utilization of an aspartate-producing derivative of *C. glutamicum* expressing the *panD* gene (Δ*sdhCAB* Δ*ldhA* pVWEx1-*aspB* pEKEx3-$panD_{Cg}$) leads to the overproduction of β-alanine with a yield of 0.24 g/g glucose. As the supply with the precursor molecule aspartate seems unlikely to limit the decarboxylation, further improvements need to address stability and activity of the aspartate-α-decarboxylase.

4.3 Aerobic overproduction of ectoine

The compatible solute ectoine has gained attention due to its chemical properties and the broad range of applications as protecting agent of DNA, proteins and the human skin. In this work, salt-independent overproduction of ectoine was established by the expression of the ectoine biosynthetic genes of *Chromohalobacter salexigens* in *C. glutamicum*. The *ectABC* gene cluster was transferred into derivatives of *C. glutamicum* and allowed efficient production of ectoine from glucose and the non-food carbon sources glycerol, arabinose, and xylose (Figure 33, p. 84). Ectoine biosynthesis is catalyzed by five enzymatic reactions and shares the initial steps of the synthesis of amino acids of the aspartate family that lead to aspartate semialdehyde. The major pathway controlling enzyme is the aspartokinase LysC due to its feedback inhibition by lysine and threonine. Ectoine is not a natural product of *C. glutamicum*, and the high amount that was obtained by the expression of *ectABC* in the wild type (0.074 g/g glucose) indicates that LysC is not, or only at a low level, influenced by the compatible solute (Figure 30, p. 81). Feedback inhibition of the aspartokinase of *C. salexigens* is only poorly studied, but as the organism possesses only one enzyme, an inhibition by ectoine seems unlikely (Pastor *et al.*, 2010). However, expression of the ectoine biosynthetic genes in the lysine-producing strain with deregulated aspartokinase DM1729 (pyc^{P458S}, hom^{V59A}, $lysC^{T311I}$) doubled the ectoine yield to 0.145 g/g glucose (Figure 31, p. 82).

Ectoine is synthesized on an industrial scale with the natural, Gram-negative producers *Halomonas elongata* or *C. salexigens* in a cell-recycling process called bacterial milking. Thereby, the biosynthesis and excretion of the compatible solute are separated in two phases. The biosynthesis is performed under high salt conditions with 2.57 M NaCl, and the excretion is induced by an osmotic downshock. This scheme has some drawbacks concerning the medium conditions, and is limited in the application for Gram-positive organisms. For instance, the halophilic *Marinococcus* sp. 52 synthesizes hydroxyectoine naturally, but does not excrete compatible solutes upon osmotic downshock (Frings *et al.*, 1995). However, a high yield of 540 mg/gCDW and a productivity of 32.5 $g/l*d^{-1}$ are obtained in this process with *C. salexigens* (Fallet *et al.*, 2010). For *H. elongata*, yields of 155 mg/gCDW per cycle are obtained, conferring to a productivity of 5.3 $g/l*d^{-1}$ (Sauer and Galinski, 1998). In the batch process performed in this work with *C. glutamicum* DM1729, 36.9 mM ectoine were produced within 24 h, corresponding 514 mg/gCDW and a productivity of 5.2 $g/l*d^{-1}$, which is well in the range of the natural producers. The higher productivity obtained with *C. salexigens* can be explained by the six times higher biomass (61 g/l compared to 10.2 g/l) used in the process. Additionally, controlled culture conditions in a fermenter might further improve ectoine production of *C. glutamicum*. One major advantage is the salt-independent production of the compatible solute, as, besides the cost, the high salt concentration negatively influences the work life of fermenters and other equipment.

Heterologous production of ectoine was recently shown for *C. glutamicum* and for *E. coli*, too. The ectoine biosynthetic genes of *C. salexigens* were used for production in a fed-batch culture of *E. coli* (Schubert *et al.*, 2007). Continuous excretion of ectoine was enabled and led to a final titer of 6 g/l ectoine after 160 hours. The final titer was rather low, caused by a high carbon loss of more than eighty percent during the production phase. Additionally, the specific activity of EctA in crude extracts of *E. coli* was reduced to one third compared to *C. glutamicum*, contributing to the obtained results.

In *C. glutamicum*, the *ectABCD* genes of *Pseudomonas stutzeri* A1501 were integrated in the genome under the control of the strong constitutive promoter P_{tuf} (Becker *et al.*, 2013). Further improvements of the strain were a deregulated aspartokinase and the deletion of the gene for the lysine exporter LysE. This strain excreted ectoine and small amounts hydroxyectoine simultaneously with an ectoine yield of 32 mmol/mol glucose in batch fermentation, which is 18 % of the yield obtained with *C. glutamicum* DM1729 under similar conditions. In fed-batch fermentation at high optical density (around OD_{660}=100), a yield of 6.7 g/l*d^{-1} was obtained (Becker *et al.*, 2013). However, a direct comparison between this production and the process shown in this work is difficult, as different genetic backgrounds and different ectoine biosynthetic genes were used.

C. glutamicum is a well investigated amino acid-producing strain, and several improvements for the overproduction of lysine can be transferred to improve ectoine synthesis. Nevertheless, as lysine is a by-product of ectoine synthesis, one important target is to prevent its excretion, similar to the strategy in the ectoine-producing *C. glutamicum* strain of Becker *et al*. Further improvements are useful at the level of aspartate semialdehyde, which is the last common precursor of lysine, threonine, isoleucine, and ectoine synthesis. Studies on the homoserine dehydrogenase Hom and the dihydropicolinate synthase DapA showed that the regulation of these enzymes influence lysine, threonine, and branched-chain amino acid production, respectively (Cremer *et al.*, 1988; Morbach *et al.*, 1996; Sahm *et al.*, 1996). Therefore, the additional downregulation of *dapA* in the strain DM1729 might further enhance ectoine overproduction.

Taken together, a constant, salt-independent production of ectoine competitive to existing productions with native producers can be achieved not only from glucose, but also from the non-food carbon sources arabinose and xylose. Moreover, efficient production is possible from glycerol, which is a stoichiometric waste product of biodiesel synthesis, and therefore, available in large amounts.

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Ehrenwörtliche Erklärung

Hiermit erkläre ich ehrenwörtlich, dass ich die vorgelegte Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel genutzt habe. Alle aus der Literatur entnommenen Zitate sind als solche kenntlich gemacht.

Die Anfertigung dieser Dissertation erfolgte unter der Betreuung von Prof. Dr. Volker F. Wendisch im Fachbereich Genetik der Prokaryoten der Fakultät Biologie an der Universität Bielefeld.

Ich versichere außerdem, dass ich die vorliegende Dissertation nur in diesem und keinem anderen Promotionsverfahren eingereicht habe, und dass diesem Promotionsverfahren keine endgültig gescheiterten Promotionsverfahren vorausgegangen sind. Ich bewerbe mich hiermit erstmalig um den Doktorgrad der Naturwissenschaften der Universität Bielefeld.

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Ort, Datum Unterschrift

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