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Engineering *Corynebacterium glutamicum* as a designer-bug for the bio-based production of chemical building blocks and biofuel

Dissertation

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Abstract

The restriction of fossil raw materials, as well as the impact of their use on the environment heads to a global social and economic crisis. Therefore, a strong political and technical interest arises for the replacement of petroleum-derived chemicals and fuels by such derived from biomass. For that purpose, microorganisms have been developed as biocatalysts to produce chemicals and fuels from renewable feedstocks. Also the well-known amino acid producer *Corynebacterium glutamicum* was used in the last decade for the bio-based production of building block chemicals, as well as for biofuels.

In this work, *C. glutamicum* was engineered for efficient aerobic production of pyruvate from glucose by modifying the acetate auxotrophic mutant *C. glutamicum* Δ*aceE* Δ*pqo* with subsequent deletions of the *ldhA*, *alaT* and *avtA* genes, to avoid L-lactate and L-alanine synthesis. Additional attenuation of the acetohydroxyacid synthase led to both significantly decreased L-valine formation, and extensively increased pyruvate accumulation. In shake flask experiments, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC-T *ilvN* Δ*alaT* Δ*avtA* (*C. glutamicum* ELB-P) produced up to 200 mM pyruvate in a growth-decoupled manner, with a maximum substrate specific yield ($Y_{P/S}$) of 1.49 mol per mol of glucose. In fed-batch fermentations at high cell densities more than 500 mM pyruvate could be produced with a $Y_{P/S}$ of 0.97 mol per mol of glucose, by implementation of microaerobic conditions from the middle until the end of growth phase.

Since pyruvate is a central precursor for the 1,4-dicarboxylic acids malate, fumarate and succinate, *C. glutamicum* ELB-P was further used as a basis for the production of these compounds. At first, a fermentation system was established leading to succinate formation as major product of *C. glutamicum* ELB-P under oxygen deprivation conditions. A $Y_{P/S}$ of 0.98 mol succinate per mol of glucose was obtained. To investigate long-term succinate production, a tri-phasic fed-batch fermentation system was established, including an aerobic growth phase on acetate for biomass formation. This was followed by a self-induced microaerobic phase at the end of growth, using minimal aeration. At last an anaerobic production phase on glucose was established in the same bioreactor by gassing with CO_2. Under these conditions *C. glutamicum* ELB-P formed more than 330 mM succinate from about 325 mM glucose, leading to a $Y_{P/S}$ of 1.02 mol succinate per mol of glucose. The availability of reduction equivalents was identified as a bottle-neck to raise final titre and $Y_{P/S}$. Therefore, several mutant strains were constructed in order to redirect the carbon flux into the pentose phosphate pathway, providing additional reduction equivalents. Combined deregulation of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in *C. glutamicum* ELB-P resulted in the most promising succinate titre with about 164 mM from 3% (*w*/*v*) glucose and a $Y_{P/S}$ of 1.07 mol per mol of glucose during batch experiments in sealed bottles. Subsequent deletion of the *pgi* gene, encoding phosphoglucoisomerase, led to inability of the mutant to consume glucose under the succinate production conditions. Neither

heterologous overproduction of a transhydrogenase (to provide NADH), nor homologous overproduction of the glyceraldehyde-3-phosphate dehydrogenase (to increase glycolytic flux) could abolish this effect.

Inactivation of succinate dehydrogenase (SDH) or fumarase (Fum) in *C. glutamicum* ELB-P should interrupt the reductive branch of tricarboxylic acid cycle for the anaerobic production of fumarate and malate, respectively. However, deletion of the corresponding genes did not lead to satisfying production of fumarate or malate. In both cases as well as in a SDH/Fum negative double mutant, unexpected succinate accumulation was observed, indicating an alternative succinate production route.

Last, based on an aerobic 2-ketoisovalerate producing strain, a *C. glutamicum* mutant was engineered for the production of isobutanol from glucose under oxygen deprivation conditions. By inactivation of L-lactate and malate dehydrogenase, implementation of ketoacid decarboxylase from *Lactococcus lactis*, and expression of the *pntAB* genes, encoding a transhydrogenase of *Escherichia coli*, as well as overexpression of the native *adhA* gene, isobutanol production with a $Y_{P/S}$ of 0.77 mol per mol of glucose was obtained. In fed-batch fermentations with an aerobic growth phase and an oxygen-deprived production phase, about 175 mM isobutanol were produced with a $Y_{P/S}$ of about 0.48 mol per mol of glucose.

Kurzdarstellung

Die Begrenzung fossiler Rohstoffe, sowie der Einfluss ihrer Nutzung auf die Umwelt steuern auf eine globale soziale und wirtschaftliche Krise hin. Daher entsteht großes politisches und technisches Interesse, erdölentstammende Chemikalien und Treibstoffe durch aus Biomasse gewonnene zu ersetzen. Hierfür wurden Mikroorganismen als Biokatalysatoren entwickelt, welche Chemikalien und Treibstoffe aus erneuerbaren Rohstoffen produzieren. Auch der weithin bekannte Aminosäureproduzent *Corynebacterium glutamicum* wurde im letzten Jahrzehnt für die biobasierte Produktion von chemischen Grundbausteinen und auch Biotreibstoffen eingesetzt.

In dieser Arbeit wurde *C. glutamicum* zur effizienten Produktion von Pyruvat aus Glukose weiterentwickelt, indem die Acetat-auxotrophe Mutante *C. glutamicum* Δ*aceE* Δ*pqo* durch nachträgliche Deletion der *ldhA*-, *alaT*- und *avtA*-Gene modifiziert wurde, um die Synthese von L-Laktat- und L-Alanin als Nebenprodukte zu verhindern. Die zusätzliche Abschwächung der Acetylhydroxysäure-Synthase führte sowohl zu erheblich verringerter L-Valinbildung, als auch erheblich gesteigerter Pyruvatanhäufung. *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC-T *ilvN* Δ*alaT* Δ*avtA* (*C. glutamicum* ELB-P) produzierte wachstumsentkoppelt bis zu 200 mM Pyruvat in Schüttelkolbenexperimenten, mit einem maximalen substratspezifischen Ertrag ($Y_{P/S}$) von 1,49 Mol pro Mol Glukose. In “Fed-Batch”-Fermentationen mit hohen Zelldichten konnten mehr als 500 mM Pyruvat mit einem $Y_{P/S}$ von 0,97 Mol pro Mol Glucose produziert werden, indem von Mitte bis Ende der Wachstumsphase mikroaerobe Bedingungen geschaffen wurden.

Da Pyruvat eine zentrale Vorstufe für die 1,4-Dicarbonsäuren Malat, Fumarat und Succinat darstellt, wurde *C. glutamicum* ELB-P als Basis für die Produktion dieser Substanzen weiter genutzt. Zunächst wurde ein Fermentationssystem etabliert, welches zur Bildung von Succinat, mit einem $Y_{P/S}$ von 0,98 Mol pro Mol Glukose, als Hauptprodukt von *C. glutamicum* ELB-P unter Sauerstoffentzug führt. Um längerfristige Succinatproduktion zu untersuchen, wurde ein triphasisches „Fed-Batch“-Fermentationssystem entwickelt, welches eine aerobe Wachstumsphase auf Acetat zur Biomassebildung beinhaltete. Gegen Ende der Wachstumsphase wurde durch minimale Begasung mit Luft eine selbst-induzierte mikroaerobe Phase ermöglicht. Dieser folgte im selben Biorektor durch das Einleiten von CO_2 eine anaerobe Produktionsphase auf Glukose. Unter diesen Bedingungen bildete *C. glutamicum* ELB-P mehr als 330 mM Succinat aus etwa 325 mM Glucose, was einem $Y_{P/S}$ von 1,02 Mol Succinat pro Mol Glukose gleichkommt. Die Verfügbarkeit von Reduktionsäquivalenten wurde als Flaschenhals identifiziert, um den finalen Titer und den finalen $Y_{P/S}$ zu erhöhen. Daher wurden verschiedene Mutantenstämme konstruiert, um den Kohlenstofffluss in den Pentosephosphatweg, unter Bildung zusätzlicher Reduktionsäquivalente, umzuleiten. Eine kombinierte Deregulierung von Glucose-6-phosphat-Dehydrogenase und 6-Phosphogluconat-Dehydrogenase in *C. glutamicum* ELB-P

resultierte in einem vielversprechenden Succinattiter von etwa 164 mM aus 3% (*w*/*v*) Glukose und einem $Y_{P/S}$ von 1,07 Mol pro Mol Glukose, bei „Batch“ Experimenten in geschlossenen Flaschen. Eine zusätzliche Deletion des *pgi* Gens, welches die Phosphoglucoisomerase kodiert, führte zum Unvermögen der Mutante, Glukose unter den Succinatproduktionsbedingungen zu verwerten. Weder die heterologe Überproduktion einer Transhydrogenase noch die homologe Überproduktion der Glycerinaldehyd-3-phosphat-Dehydrogenase konnten diesen Effekt aufheben.

Die Inaktivierung der Succinat-Dehydrogenase (SDH) bzw. Fumarase (Fum) in *C. glutamicum* ELB-P sollte den reduktiven Ast des Tricarbonsäurezykluses für die Produktion von Fumarat bzw. Malat als Endprodukt unterbrechen. Allerdings führte die Deletion der entsprechenden Gene zu keiner zufriedenstellenden Produktion von Fumarat bzw. Malat. In beiden Fällen, sowie bei einer SDH/Fum-negativen Doppelmutante, wurde die unerwartete Bildung von Succinat beobachtet, was auf einen alternativen Succinatproduktionsweg hindeutet.

Zuletzt wurde, basierend auf einem aerob 2-Ketoisovalerat produzierenden Stamm, eine *C. glutamicum* Mutante entwickelt, die zur Produktion von Isobutanol aus Glukose unter Sauerstoffausschluss diente. Durch Inaktivierung der L-Laktat- und Malat-Dehydrogenasen, Implementierung der Ketosäure-Decarboxylase aus *Lactococcus lactis* und die Expression der *pntAB* Transhydrogenasegene aus *Escherichia coli*, sowie die Überexpression des nativen *adhA* Gens, wurde Isobutanolproduktion mit einem $Y_{P/S}$ von 0,77 Mol pro Mol Glukose erreicht. In „Fed-Batch“-Fermentationen mit einer aeroben Wachstums- und einer sauerstofffreien Produktionsphase, wurden 175 mM Isobutanol mit einem $Y_{P/S}$ von ca. 0,48 Mol pro Mol Glucose produziert.

A) INTRODUCTION

1. *Corynebacterium glutamicum*

Corynebacterium glutamicum (Figure 1) was first isolated in 1957 and described as an immobile, non-spore forming, Gram-positive, rod-shaped soil microorganism (Kinoshita et al. 1957/2004). The bacterium is facultative anaerobic and grows well in presence of external electron acceptors such as oxygen or nitrate (Liebl, 2006; Nishimura et al. 2007; Takeno et al. 2007).

The genus *Corynebacterium* is assigned to the class *Actinobacteria*, which contains Gram-positive bacteria with a high (>50%) G+C content (Stackebrandt et al. 1997; Ventura et al. 2007). They are part of the subdivision eubacteria and were consolidated in the past together with the genera *Mycobacterium*, *Nocardia* and *Rhodococcus* to the order of the mycolic acid containing *Actinomycetes* (Stackebrandt et al. 1997). After more recent phylogenetic studies, these genera form now among others the closer related order of *Corynebacteriales* (Gao and Gupta, 2012). There are pathogenic (e.g., *C. diphtheria*) as well as non-pathogenic bacteria (e.g., *C. glutamicum*) in the genus *Corynebacterium* (Barksdale, 1970). The biotin-auxotrophic species *C. glutamicum* is the most investigated of the genus *Corynebacterium* (Abe et al. 1967).

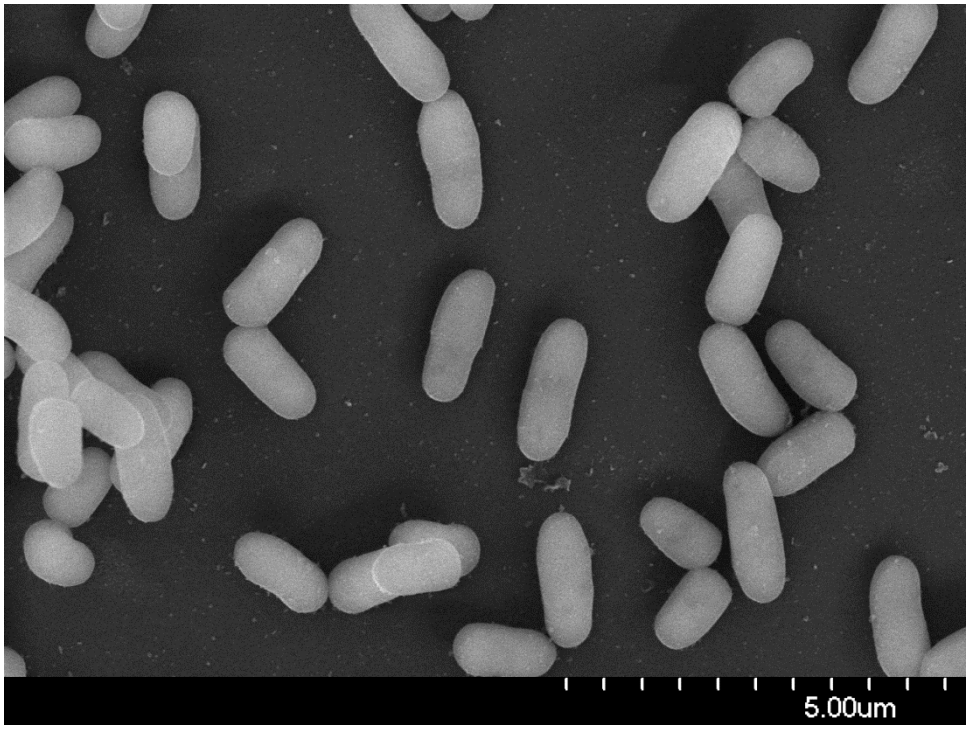

Figure 1: *Corynebacterium glutamicum* - field emission electron microscopical picture made with a Hitatchi S-52000 microscope; accelerating voltage: 10 kV, magnification: x10.0 k (Philipp von Zaluskowski, Institut für Mikrobiologie und Biotechnologie der Universität Ulm)

C. glutamicum has the so called GRAS-status, meaning that it is "generally regarded as safe". Therefore, it is industrially used to produce high amounts of the food additive L-glutamate ($2.2 \cdot 10^6$ t/a), but also for industrial production of other amino acids, such as L-lysine ($1.4 \cdot 10^6$ t/a), L-valine, L-threonine and L-tryptophane (Food-Oct2010, Ajinomoto; Feed-use-AA-Oct2011; Takors et al. 2007). All of them are important supplements for animal feed, cosmetics and pharmaceuticals (Feed-use-AA-Oct2011, Ajinomoto; Leuchtenberger et al. 2005). Furthermore, organic acids, such as 2-ketoisovaleric acid (KIV), pyruvic acid, L-lactic acid, D-lactic acid and succinic acid can be produced with *C. glutamicum* (Krause et al. 2010; Wieschalka et al. 2012a; Inui et al. 2004a; Okino et al. 2008b, 2008a). In recent years, the organism has been engineered to widen the biotechnological product spectrum. Alcohols which have potential as biofuels but also as chemical building blocks were produced successfully, e.g., ethanol, isobutanol, or xylitol (Inui et al. 2004b; Smith et al. 2010; Blombach et al. 2011; Sasaki et al. 2010). Furthermore, production of diamines came more and more to the forth. These compounds, e.g. putrescine and cadaverine, serve as precursors for industrial and economical important polyamides (Schneider and Wendisch, 2010; Kind et al. 2010a, 2010b; Kind and Wittmann, 2011; Mimitsuka et al. 2007; Schneider and Wendisch, 2011). The worldwide demand for the named products is still increasing. Better comprehension of the metabolic pathways of *C. glutamicum*, as well as further improvement of the production strains and the fermentation techniques is therefore economically interesting. For future improvement of the industrial exploitation of *C. glutamicum* it is necessary to deeply understand metabolism and its regulation in this organism. One preposition for this task is that its 3.28 Mbp sized genome (G+C content of 53.8%) has been completely sequenced by two independent groups, allowing e.g., genome-based metabolomic flux studies (Ikeda and Nakagawa, 2003; Kalinowski et al. 2003).

C. glutamicum grows aerobically on a variety of substrates, such as carbohydrates (e.g., glucose, fructose, and sucrose), organic acids (e.g., pyruvate, lactate, citrate and acetate) or alcohols (e.g., ethanol), as single or combined carbon and energy sources (Liebl, 2006; Netzer et al. 2004; Dominguez et al. 1997; Frunzke et al. 2008; Arndt et al. 2008). The pathways of carbon metabolism and important enzymes, playing a major role for this work, are shown in Figure 2. In general, the central metabolism of *C. glutamicum* is a tangled network, consisting of glycolysis (Embden-Meyerhof-Parnas pathway), pentose phosphate pathway (PPP), and tricarboxylic acid (TCA) cycle (Yokota and Lindley, 2005; Eikmanns, 2005). The corresponding enzymes, genes and reactions are displayed in Table 1, classified after their metabolic role in the main pathways.

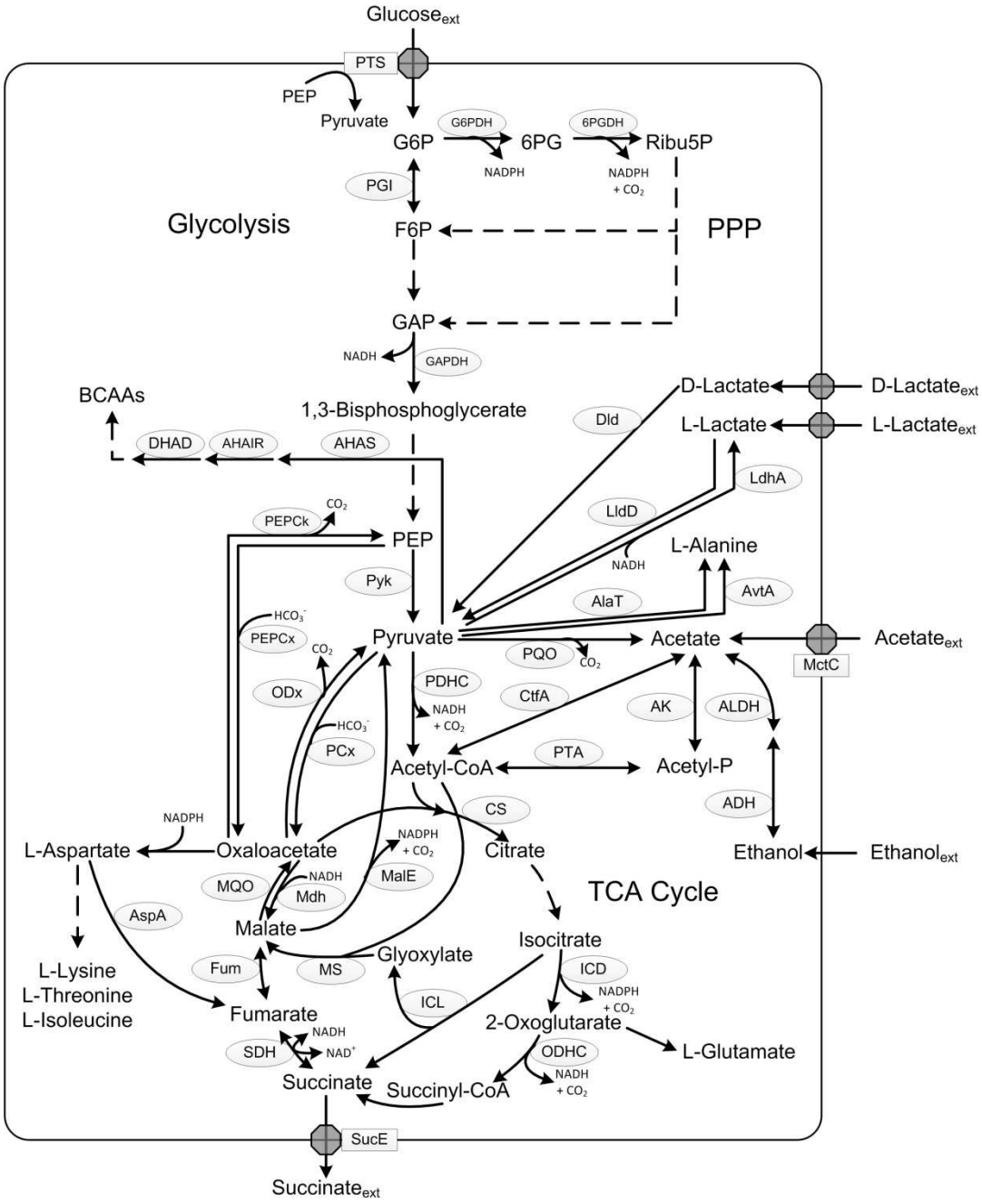

Figure 2: The central metabolism in *C. glutamicum* with the corresponding enzymes for major pathways of carbon metabolism. Abbreviations: 6PG, 6-phosphogluconate; 6PGDH, 6PG dehydrogenase; ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; AHAS, acetohydroxyacid synthase; AHAIR, acetohydroxyacid isomeroreductase; AK, acetate kinase; AlaT, alanine aminotransferase; AspA, aspartase; AvtA, valine-pyruvate aminotransferase; BCAAs, branched-chain amino acids; CoA, coenzyme A; CtfA, CoA transferase; CS, citrate synthase; DHAD, dihydroxyacid dehydratase; Dld, quinone-dependent D-lactate dehydrogenase; F6P, fructose-6-phosphate; Fum, fumarase; G6P, glucose-6-phosphate; G6PDH, G6P dehydrogenase; GAP, glyceraldehyde-3-phosphate; GAPDH, GAP dehydrogenase; ICD, isocitrate dehydrogenase; ICL, isocitrate lyase; LdhA, NAD^+-dependent L-lactate dehydrogenase; LldD, quinone-dependent L-lactate dehydrogenase; MalE, malic enzyme; MctC, monocarboxylic acid transporter; Mdh, malate dehydrogenase; MQO, malate:quinone oxidoreductase; MS, malate synthase; ODHC, oxoglutarate dehydrogenase complex; ODx, oxaloacetate decarboxylase; P, phosphate; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; PEPCk, PEP carboxykinase; PEPCx, PEP carboxylase; PGI, phosphoglucoisomerase; PPP, pentose phosphate pathway; PQO, pyruvate:quinone oxidoreductase; PTA, phosphotransacetylase; PTS, phosphotransferase system; Pyk, pyruvate kinase; Ribu5P, ribulose-5-phosphate; SDH, succinate dehydrogenase; SucE, succinate exporter; TCA, tricarboxylic acid

Table 1: Genes, enzymes and structures for major pathways of the central metabolism of *C. glutamicum* with their corresponding reactions or purposes

Gene(s)	Enzyme/structure name (Abbreviation)	Reaction or purpose	Reference
Glycolysis			
pgi	Phosphoglucoisomerase (PGI)	G6P ↔ F6P	Sugimoto and Shiio, 1989
gapA / ***gapB***	Glyceraldehyde-3-phosphate dehydrogenases A and B (GAPDH)	GAP + NAD^+ +ATP → 1,3-Bisphosphoglycerate + NADH + ADP	Eikmanns, 1992 Omumasaba et al. 2004
pyk	Pyruvate kinase (Pyk)	PEP + ADP → Pyruvate + ATP	Gubler et al. 1994
aceE ***sucB*** ***lpd***	E1p, E2 and E3 pyruvate dehydrogenase complex subunits (PDHC)	Pyruvate + NAD^+ + CoA→ Acetyl-CoA + CO_2 + NADH	Schreiner et al. 2006 Hoffelder et al. 2010 Schwinde et al. 2001
Pentose phosphate pathway			
zwf ***opcA***	Glucose-6-phosphate dehydrogenase (G6PDH)	G6P + $NADP^+$ → 6-Phosphoglucono-δ-Lactone + NADPH	Moritz et al. 2000 Sugimoto and Shiio, 1987
gnd	6-phosphogluconate dehydrogenase (6PGDH)	6PG + $NADP^+$ → Ribu5P + CO_2 + NADPH	Moritz et al. 2000
Transporters			
ptsI ***ptsH*** ***ptsG***	Phosphotransferase system (glucose) (PTS)	$Glucose_{ext}$ + PEP → $G6P_{int}$ + Pyruvate (glucose uptake)	Moon et al. 2007
mctC	Monocarboxylic acid transporter (MctC)	Uptake of pyruvate, propionate and acetate	Jolkver et al. 2009
sucE	Succinate exporter (SucE)	Export of succinate	Huhn et al. 2011 Fukui et al. 2011
Ethanol metabolism			
adhA	Alcohol dehydrogenase (ADH)	Ethanol ↔ Acetaldehyde	Arndt et al. 2008 Auchter et al. 2011
ald	Acetaldehyde dehydrogenase (ALDH)	Acetaldehyde ↔ Acetate	Arndt et al. 2008 Auchter et al. 2009

Acetate metabolism			
pqo	Pyruvate:quinone oxidoreductase (PQO)	Pyruvate → Acetate + CO_2	Schreiner and Eikmanns, 2005 Schreiner et al. 2006
ackA	Acetate kinase (AK)	Acetate + ATP ↔ Acetyl-P + ADP Propionate + ATP ↔ Propionyl-P + ADP	Shiio et al. 1969
pta	Phosphotransacetylase (PTA)	Acetyl-P + CoA ↔ Acetyl-CoA + P Propionyl-P + CoA ↔ Propionyl-CoA + P	Shiio et al. 1969
cat	CoA transferase (CtfA)	Acetate + CoA ↔ Acetyl-CoA	Veit et al. 2009
Lactate metabolism			
dld	Quinone-dependent D-lactate dehydrogenase (Dld)	D-Lactate + MQ→ Pyruvate + MQH_2	Kato et al. 2010
ldhA	NAD^+-dependent L-lactate dehydrogenase (LdhA)	Pyruvate + NADH ↔ L-Lactate + NAD^+	Inui et al. 2004a
lldD	Quinone-dependent L-lactate dehydrogenase (LldD)	L-Lactate + MQ → Pyruvate + MQH_2	Stansen et al. 2005
TCA cycle and glyoxylate shunt			
gltA	Citrate synthase (CS)	OAA + Acetyl-CoA + H_2O → Citrate + CoA	Eikmanns et al. 1994
icd	Isocitrate dehydrogenase (ICD)	Isocitrate + $NADP^+$ → 2-Oxoglutarate + CO_2 + NADPH	Eikmanns et al. 1995
odhA ***sucB***	E1o and E2o subunits oxoglutarate dehydrogenase complex (ODHC)	2-Oxoglutarate + NAD^+ + CoA → Succinyl-CoA + CO_2 + NADH	Usuda et al. 1996 Kataoka et al. 2006
sdhA ***sdhB*** ***sdhCD***	Succinate dehydrogenase (SDH)	Succinate + NAD^+/FAD ↔ NADH/$FADH_2$ + Fumarate	Kurokawa and Sakamoto 2005
fum	Fumarase (Fum)	Fumarate ↔ Malate	Genda et al. 2006
mqo	Malate:quinone oxidoreductase (MQO)	Malate + MQ → OAA + MQH_2	Molenaar et al. 1998, 2000 Genda et al. 2003
mdh	Malate dehydrogenase (Mdh)	OAA + NADH → Malate + NAD^+	Molenaar et al. 2000
aceA	Isocitrate lyase (ICL)	Isocitrate → Glyoxylate + Succinate	Reinscheid et al. 1994a
aceB	Malate synthase (MS)	Glyoxylate + Acetyl-CoA → Malate + CoA	Reinscheid et al. 1994b

Anaplerotic reactions (PEP-pyruvate-OAA-node)

ppc	Phosphoenolpyruvate carboxylase (PEPCx)	PEP + CO_2 → OAA + P	Eikmanns et al. 1989
pyc	Pyruvate carboxylase (PCx)	Pyruvate + CO_2 + ATP → OAA + ADP + P	Peters-Wendisch et al. 1997 Peters-Wendisch et al. 1998
pck	Phosphoenolpyruvate carboxykinase	OAA + ATP → PEP + ADP	Jetten and Sinskey, 1993 Riedel et al. 2001
odx	Oxaloacetate decarboxylase (ODx)	OAA → Pyruvate + CO_2	Klaffl and Eikmanns, 2010
malE	Malic enzyme (MalE)	Malate + $NADP^+$ → Pyruvate + NADPH + CO_2	Gourdon et al. 2000

Amino acid production

gdhA	Glutamate dehydrogenase (GDH)	2-Oxoglutarate + $NADP^+$ + NH_4^+ ↔ L-Glutamate + NADPH	Shiio and Ujigawa, 1978
glnA	Glutamate synthetase (GS)	L-Glutamate + NH_4^+ + ATP → L-Glutamine + ADP+P_i	Jakoby et al. 1997
gltB ***gltD***	Glutamate-2-oxoglutarate aminotransferase (GOGAT)	L-Glutamine + 2-Oxoglutarate + 2 NADH → 2 L-Glutamate + 2 NAD^+	Beckers et al. 2001
alaT	Alanine aminotransferase (AlaT)	L-Glutamate + Pyruvate ↔ L-Alanine + 2-Oxoglutarate	Marienhagen and Eggeling, 2008
avtA	Valine-pyruvate aminotransferase (AvtA)	L-Alanine + KIV ↔ L-Valine + Pyruvate	Marienhagen and Eggeling, 2008
aspA	putative Aspartase[1] (AspA)	L-aspartate → Fumarate + NH_4^+	Kalinowski et al. 2003[1]
ilvB ***ilvN***	Acetohydroxyacid synthase subunits (AHAS)	2 Pyruvate → α-Acetolactate + CO_2 Pyruvate + α-Ketobutyrate → α-Acetohydroxybutyrate + CO_2	Keilhauer et al. 1993
ilvC	Acetohydroxyacid isomeroreductase (AHAIR)	α-Acetolactate + $NADP^+$→ 2,3-Dihydroxyisovalerate + NADPH α-Acetohydroxybutyrate + $NADP^+$ → 2,3-Dihydroxy-3-methylvalerate + NADPH	Keilhauer et al. 1993
ilvD	Dihydroxyacid dehydratase (DHAD)	2,3-Dihydroxyisovalerate → KIV 2,3-Dihydroxy-3-methylvalerate → KMV	Radmacher et al. 2002

[1]AspA (*aspA*) is up to now just annotated as aspartase; Abbreviations: ATP, adenosine triphosphate; GAP, glyceraldehyde-3-phosphate; KIV, 2-ketoisovalerate; KMV, 2-keto-3-methylvalerate; MQ, $menaquinone_{ox}$; MQH_2, $menaquinone_{red}$; OAA, oxaloacetate; PEP, phosphoenolpyruvate; P_i, inorganic phosphate

The uptake of glucose (Figure 2, Table 1), fructose and sucrose by *C. glutamicum* is mediated by the phosphotransferase system (PTS). The system consists of the unspecific components EI (encoded by *ptsI*), and the histidine-containing protein HPr (encoded by *ptsH*) and one of the EII components (EII_{Glc}, EII_{Fru}, EII_{Suc}, encoded by *ptsG*, *ptsF* and *ptsS*, respectively), which are specific for the respective sugars. Uptake and phosphorylation by *C. glutamicum* are driven by hydrolysis of phosphoenolpyruvate (PEP) (Moon et al. 2007). During growth on glucose as sole carbon source, the sugar is mainly metabolized in glycolysis, gaining 2 mol of NADH and 2 mol of pyruvate for each mol of glucose. Besides, a part of the carbon at the level of glucose-6-phosphate (G6P) is not converted by phosphoglucoisomerase (PGI) to fructose-6-phosphate (F6P), but channelled into the PPP, generating NADPH for anabolic processes in the reactions mediated by glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) (Figure 2, Table 1). Since the reaction of 6PGDH generates CO_2, the oxidative route is irreversible under physiological conditions (Yokota and Lindley, 2005). The PPP refuels, by reactions catalysed by transaldolases and transketoloases, glycolysis at the levels of F6P and glyceraldehyde-3-phosphate (GAP) (Yokota and Lindley, 2005).

The pyruvate formed during glycolysis serves partially for the synthesis of amino acids of the pyruvate-family (L-alanine, L-leucine, and L-valine), initiated by the enzymes alanine aminotransferase (AlaT), valine-pyruvate aminotransferase (AvtA) and the key enzyme for the synthesis of branched-chain amino acids (BCAAs), the acetohydroxyacid synthase (AHAS). The major part of pyruvate is decarboxylated under NADH-formation and activated to acetyl-CoA. Responsible for the oxidative decarboxylation is the pyruvate dehydrogenase complex (PDHC), which is a multienzymatic complex in *C. glutamicum* (Table 1). It consists of the three subunits pyruvate decarboxylase (E1p), dihydrolipoamide transacetylase (E2) and dihydrolipoamide dehydrogenase (E3) (Eikmanns, 2005; Sauer and Eikmanns, 2005). Deletion of the *aceE* gene encoding E1p (Table 1) inactivates the PDHC completely, leading to a growth deficiency on carbohydrates, which can be bypassed with substrates entering the central metabolism at the level of acetyl-CoA, e.g., acetate (Schreiner et al. 2005; Blombach et al. 2009). As shown in Figure 2, the import of acetate is accomplished by the monocarboxylic acid transporter (MctC), while the activation to acetyl-CoA is carried out by acetate kinase (AK) and phosphotransacetylase (PTA) (Jolkver et al. 2009; Gerstmeir et al. 2003). Alternatively, acetate can be activated by CoA transferase (CtfA) (Veit et al. 2009). Besides, *C. glutamicum* possesses a pyruvate:quinone oxidoreductase (PQO), which can convert pyruvate to acetate and CO_2. However, the inability of a Δ*aceE* mutant to grow on glucose as sole carbon source excludes a functional bypass of the inactivated PDHC by a reaction-cycle of PQO, AK and PTA or CtfA, at least in a *C. glutamicum* Δ*aceE* mutant with no further modifications. Just by overexpression of the *pqo* gene (Table 1), acetate auxotrophy of a PDHC-negative *C glutamicum* mutant is partially abolished (Schreiner et al. 2006).

Ethanol can also serve as precursor for acetate formation and bypass growth defects of an *aceE* deletion (Blombach et al. 2009). The alcohol enters the cell probably by diffusion. It is degraded intracellular by alcohol dehydrogenase (ADH) activity and successive conversion by acetaldehyde dehydrogenase (ALDH) to acetate (Kotrbova-Kozak et al. 2007; Arndt and Eikmanns, 2007; Auchter et al. 2011; Arndt et al. 2008; Auchter et al. 2009). The formed acetate can then be activated via AK and PTA or CtfA (Jolkver et al. 2009; Gerstmeir et al. 2003).

Acetyl-CoA is commonly channelled into the TCA cycle by citrate synthase (CS) and to a major part degraded in energy metabolism to CO_2 (Figure 2). The formed NADH of both glycolysis and TCA cycle is usually converted by the NADH dehydrogenase to NAD^+, introducing electrons into the membrane-bound respiratory chain for adenosine triphosphate (ATP) generation (Bott and Niebisch, 2003). As several compounds of the TCA cycle, e.g., oxaloacetate (OAA) which is a precursor for amino acids of the aspartate-family (L-aspartate, L-asparagine, L-lysine, L-isoleucine, L-methionine, and L-threonine), as well as 2-oxoglutarate, which is a precursor for amino acids of the glutamate-family (L-glutamate, L-arginine, L-glutamine, and L-proline), are important for anabolic processes, the PEP-pyruvate-OAA-node (Table 1) plays a major role for carbon distribution within the central metabolism in order to replenish the intermediates withdrawn from the TCA cycle (Eikmanns, 2005; Sauer and Eikmanns 2005). As shown in detail in Figure 2, the metabolites at this node either (i) serve as precursors for anabolic purposes, (ii) enter the TCA cycle directly, or (iii) enter the TCA cycle via so-called anaplerotic reactions (Eikmanns, 2005; Sauer and Eikmanns 2005). Particularly, the bicarbonate-fixing reactions of PEP carboxylase (PEPCx) and pyruvate carboxylase (PCx), refilling the OAA-pool during growth on carbohydrates as carbon source, are of major importance for this work, as they enable reductive TCA cycle activity, which is mediated by soluble malate dehydrogenase (Mdh), and reversed reactivity of fumarase (Fum) as well as succinate dehydrogenase (SDH) (Figure 2, Table 1). Moreover, during growth on substrates entering the central metabolism at the level of acetyl-CoA, such as acetate and ethanol, the PEP-pyruvate-OAA-node (iv) fulfils initial reactions for gluconeogenesis, facilitated by the GTP-dependent PEP-carboxykinase (PEPCk), the malic enzyme (MalE) and probably by the OAA decarboxylase (ODx) (Riedel et al. 2001; Gourdon et al. 2000; Klaffl and Eikmanns, 2010). Growth on such substrates activates the glyoxylate shunt with its key enzymes isocitrate lyase (ICL) and malate synthase (MS), serving to provide malate and oxaloacetate from acetyl-CoA for anaplerosis (Kornberg, 1966).

Lactate is in *C. glutamicum* not only a product of straight pyruvate conversion to L-lactate by LdhA under oxygen deprivation conditions (Inui et al. 2004a). Lactic acid can also be taken up as D- or L-isomers and converted to pyruvate by quinone-dependent D-lactate dehydrogenase (Dld) and quinone-dependent L-lactate dehydrogenase (LldD), respectively (Kato et al. 2010; Stansen et al. 2005). Therefore, lactate can be utilized as

carbon source, entering the metabolism at the level of pyruvate (Figure 2, Table 1). In *C. glutamicum*, the reaction from lactate to pyruvate is bound to a direct electron transfer on menaquinone (Kato et al. 2010; Stansen et al. 2005).

Unlike other organisms, *C. glutamicum* is able to consume glucose and other sugars or organic acids, such as fructose, gluconate, lactate or acetate, simultaneously (Dominguez et al. 1997; Frunzke et al. 2008; Stansen et al. 2005; Wendisch et al. 2000). Up to now, diauxic growth was just reported for combinations of glucose plus glutamate, acetate plus ethanol and glucose plus ethanol (Krämer et al. 1990; Arndt and Eikmanns, 2007; Arndt et al. 2008). For this work, especially the co-utilization of glucose and acetate (Wendisch et al. 2000) is of great importance. These substrates are degraded as described above, but both with a lower consumption rate than being used as sole carbon source, resulting in similar total carbon consumption rates (Wendisch et al. 2000). The glucose consumption rate is controlled by the master regulator SugR (encoded by *sugR*) (Engels and Wendisch, 2007; Gaigalat et al. 2007; Tanaka et al. 2008). In presence of acetate, but also of pyruvate or lactate, SugR represses the genes of the PTS (*ptsG*, *ptsI* and *ptsH*; Table 1). The transcription of genes for the enzymes of the pathways involved in acetate metabolism (*pta-ack* operon, *aceA*, *aceB*; Table 1) is regulated by the global regulators RamA (activator) and RamB (repressor) (Cramer et al. 2006; Gerstmeir et al. 2004). GntR1 and GntR2 are involved in glucose uptake and gluconate degradation in *C. glutamicum* (Letek et al. 2006). These two functionally redundant regulators adjust carbon storage and uptake by repression of genes coding for gluconate degrading enzymes and activation of *ptsG* and *ptsS* transcription (Frunzke et al. 2008). Binding of GntR1 and GntR2 to their target sequences can be inhibited by the effectors gluconate and glucono-δ-lactone (Frunzke et al. 2008). Moreover, these regulators are responsible for repression of *gnd* and a weak repression of the *tkt-tal-zwf-opcA-devB* cluster, encoding the enzymes of the PPP (Frunzke et al. 2008). Taken together, the regulatory network in *C. glutamicum* comprises besides the regulators SugR, RamA and RamB, which are not only responsible for the regulations described above, a repertoire of several master and local transcription regulators, and the cAMP-sensing GlxR protein, which is as global regulator possibly involved in the transcription of 14% of all predicted genes in *C. glutamicum* (reviewed in Schröder and Tauch 2010).

2. Production of chemical building blocks and biofuels with *C. glutamicum*

In 2004, the U.S. Department of Energy (DOE) identified building block chemicals of interest, which can be produced from sugars via biological or chemical conversions (Werpy and Petersen, 2004; Table 2).

Table 2: Top chemical building blocks from carbohydrates according to a study of the DOE

Building blocks from carbohydrates	
C_3	3-hydroxy propionic acid
C_3	glycerol
C_4	3-hydroxybutyrolactone
C_4	aspartic acid
C_4	succinic, fumaric and malic acids
C_5	glutamic acid
C_5	itaconic acid
C_5	levulinic acid
C_5	xylitol/arabinitol
C_6	2,5-furan dicarboxylic acid
C_6	glucaric acid
C_6	sorbitol

Building block chemicals are defined as molecules with multiple functional groups that possess the potential to be transformed into new families of useful molecules (Werpy and Petersen, 2004). Therefore, chemical building blocks, such as certain organic acids, serve as precursors for a variety of bulk chemicals and commercial important polymers (Werpy and Petersen, 2004). These days, they are produced primarily from fossil fuels via chemical synthetic processes, which comes at a high environmental cost, particularly in form of higher CO_2 emissions (Okino et al. 2008a). The limitation of oil resources, accompanied by steadily rising oil prices and the strong impact on the environment, raises the requirement of safe and efficient bioprocesses for the production of hitherto crude oil-derived chemical building blocks from renewable resources. One general idea for the development of a safe, clean, efficient and sustainable petroleum-independent industry is the establishment of so called biorefineries (Sauer et al. 2008; Bozell and Petersen, 2010). These facilities combine biological, chemical and technical tools for the integration and conversion of biomass to produce fuels, power and chemicals (Kamm and Kamm, 2004, 2007; Sauer et al. 2008). Anyhow, up to now the development of biorefineries is challenged by the lack of conversion technology as well as the identification of potential targets for primary chemicals and secondary intermediates (Bozell and Petersen, 2010). Microbial production of organic acids is a promising approach for obtaining building-block chemicals by substitution of conventional synthesis step-by-step, under usage of renewable raw materials as substrate (Dodds and Gross, 2007; Sauer et al. 2008). An increase of the market for such substances is predicted, once competitive microbial production processes are established (Werpy and Petersen, 2004). Most of these organic acids are already a product, or at least an intermediate in the metabolism of microorganisms (Sauer et al. 2008). The compounds of interest to this work are

the 1,4-dicarboxylic acids malate, fumarate and succinate, which all are intermediates in the TCA cycle. These three acids have the potential to replace the important oil-derived commodity chemical maleic anhydride, as reactive precursor for polymer synthesis (Sauer et al. 2008).

Malate is used in pharmaceuticals, cosmetics, and food industry (acidulant E296) (Rosenberg et al. 1999; Bressler et al. 2002; Zhang et al. 2011). The annual production in 2008 mounted up to $10 \cdot 10^3$ t (Sauer et al. 2008). To date, it is industrially produced from petroleum-derived substrates, either (i) chemically by hydration of maleic or fumaric acid as racemate, or (ii) as pure L-malate by enzymatic hydration of fumarate (Bressler et al. 2002; Giorno et al. 2001; Zhang et al. 2011). Malate is also a product of a wide range of microorganisms, using microaerophilic or aerobic processes; e.g. *Saccharomyces cerevisiae* or *Aspergillus flavus* (overview given by Zhang et al. 2011). The production of malate with *Escherichia coli* has been obtained by approaches combining metabolic evolution with rational strain design (Jantama et al. 2008; Zhang et al. 2011).

Fumarate is produced in a scale of about $12 \cdot 10^3$ t per year via chemical conversion of maleic anhydride from petrochemical raw materials (Sauer et al. 2008; Roa Engel et al. 2008). It is used for different food applications and as starting material for polymerisation and esterification reactions (Roa Engel et al. 2008). Psoriasis can be caused by deficiency of patients to produce fumaric acid in their skin. Therefore, fumarate is also used for the treatment of this disease (Altmeyer et al. 1994; Mrowietz et al. 1998). At last, fumarate can be used in cattle-feed, reducing the emission of methane by the animals (McGinn et al. 2004). Besides fumarate production via petrochemical routes, attempts have been made for enzymatic conversion of maleic acid to fumaric acid (Goto et al. 1997, 1998). Microbial production by genetic modification of organisms is hardly explored. However, natural fumarate producing genera of fungi were identified: *Rhizopus*, *Mucor*, *Cunninghamella*, and *Circinella* (Roa Engel et al. 2008). Production of fumarate with these fungi was promoted in the past and optimized especially with *Rhizopus oryzae* at low pH fermentations (overview given by Roa Engel et al. 2008, 2011).

Succinate is a versatile base material for a multiplicity of industrial applications. Sauer et al. 2008 denoted succinate humorously as "the LEGO® of chemical industry". It was estimated in 2010 that it is a precursor or part for more than $25 \cdot 10^6$ t industrial products per year, including 1,4-butanediol, tetrahydrofuran, γ-butyrolactone, adipic acid, n-methylpyrrolidone and linear aliphatic esters (Bozell and Petersen, 2010; Zeikus et al. 1999). Succinate is broadly used for coating, for plasticizers, as surfactant, detergent or foaming agent, as ion chelator, for green solvents, and biodegradable plastics, but also as acidulant, flavouring agent or anti-microbial agent in the food industry (Zeikus et al. 1999; McKinlay et al. 2007; Bozell and Petersen, 2010). Furthermore, it has health-related functions in pharmaceutical and antibiotic products (Zeikus et al. 1999). As component of bio-based

polymers, succinate is a base material for nylons and polyesters (Zeikus et al. 1999). The annual production reached approximately $16 \cdot 10^3$ t in 2008 (Sauer et al. 2008), being mostly produced chemically from maleic anhydride (Lee et al. 2005). Since succinate is also an important intermediate of the TCA cycle and a product of mixed-acid fermentation, e.g. in *Mannheimia succiniproducens* (Lee et al. 2006), microbial production is basically possible, and found slowly its way towards production in industrial relevant scale. Nowadays, the chemical companies BASF and PURAC produce about $10 \cdot 10^3$ t succinate per year with *Basfia succiniproducens* (Zelder, 2012). But also other regular succinate producing microorganisms, such as *Anaerobiospirillum succiniciproducens* (Davis et al. 1976), and *M. succiniciproducens* (Lee et al. 2002) are of great interest for succinic acid production in industrial scale. The current state of art in succinate production with metabolically engineered *C. glutamicum* is reviewed in Wieschalka et al. 2012b.

Succinate, aside from L-lactate and trace amounts of acetate, is a major fermentation product of *C. glutamicum*, when it consumes glucose under oxygen deprivation conditions (Dominguez et al. 1993; Inui et al. 2004a; Okino et al. 2005). L-lactate is formed by the LdhA from pyruvate (Figure 2, Table 1). The reactions mediated by PEPCx and PCx, followed by reductive activity of the TCA cycle, lead to the formation of succinate from PEP or pyruvate. For this purpose, the Mdh reduces OAA to malate, in contrast to oxidative TCA cycle flux, where malate is naturally oxidised to OAA by the malate:quinone oxidoreductase (MQO), transferring electrons to menaquinone (Molenaar et al. 2000; Figure 2, Table 1). The reductive Mdh reaction in contrary is NADH-dependent. Combined with the last reaction step from fumarate to succinate, which also needs reduction equivalents, the NADH-demand is very high for anaerobic succinate production. Likewise, malate and fumarate are intermediates in the reductive branch of the TCA cycle. The production of these compounds was planned to be realised in this work by interruption of the reductive TCA cycle, either (i) at the reaction of SDH, leaving fumarate as end-product, or (ii) at the reaction of Fum, leaving malate as end-product. A high substrate specific yield ($Y_{P/S}$) was expected from this approach for all of the three dicarboxylic acids, since in the OAA forming reactions, catalysed by PEPCx and PCx, additional carbon from CO_2 is fixed (Figure 2, Table 1). Looking at the metabolism of *C. glutamicum*, aside from the importance of the reductive TCA cycle reactions, pyruvate was identified as central precursor for the desired products (Figure 2). Accordingly, the first step of this work was the development of a strong pyruvate producer, serving as a platform for the pyruvate-derived products of the TCA cycle.

Pyruvate itself is an interesting compound, as it is widely used in polymer and chemical synthesis, as well as for food, and pharmaceuticals (Li et al. 2001; Zhu et al. 2008). It also serves in several health-related applications, including weight loss diets (Stanko et al. 1992b, 1992a; Roufs, 1996), exercise endurance (Stanko et al. 1990), and acne treatment (Cotellessa et al. 2004; Ghersetich et al. 2004). It can be used as antioxidant (DeBoer et al. 1993) and acts

positively on graft tolerance (Cicalese et al. 1997, 1999). For these operation fields, pyruvate is chemically produced by decarboxylation of tartaric acid (Howard and Fraser, 1932), however, in a very cost-inefficient way (Li et al. 2001). Therefore, fermentative production of pyruvate is of strong interest and was added to the goals of this work. As described in detail in Wieschalka et al. 2012a, efficient pyruvate production can be achieved by down-regulation or complete shut-down of pyruvate consuming reactions. Basis for this work was a *C. glutamicum* strain with inactivated PDHC and a deletion of *pqo* (Blombach et al. 2007, 2008). This mutant produces about 19 mM L-valine, 28 mM L-alanine and already about 55 mM pyruvate from 150 mM glucose.

The limitation of oil resources, and the strong impact on the environment by the use of oil, not only affects the allocation of bulk chemicals for different industrial and medical processes, but also has a great influence on the fuel market. Therefore, it is necessary to develop safe and efficient bio-production processes for biofuels from renewable biomass. Besides ethanol, higher alcohols, such as butanol or isobutanol have the potential to serve as valuable biofuels. Higher alcohols have significant advantages in comparison to ethanol: e.g., isobutanol can be used pure or blended in any concentration with gasoline, is compatible to all existing engines or pipelines, but also is safer to handle due to a lower vapour pressure (Dürre, 2007). Furthermore, it is not corrosive or hygroscopic (Dürre, 2007). These abilities turn isobutanol into an interesting target for microbial biofuel-production. In addition, it is also a valuable compound for chemical industry as precursor for the production of isobutene, butyl rubber and specialty chemicals (Gogerty and Bobik, 2010; Macho et al. 2001).

As described in detail in Blombach et al. (2011), for the production of isobutanol with *C. glutamicum* a new pathway had to be introduced in an already existing *C. glutamicum* mutant. Such a plan for isobutanol production was already realised with *E. coli* (Atsumi et al. 2008). In the case of *C. glutamicum*, isobutanol production was described for the first time. Anyhow in parallel Smith et al. (2010) followed the same idea with *C. glutamicum*, during the time we developed our isobutanol producer, however yielding a less efficient mutant strain.

3. Defining the targets

The goals of this work were defined by the interest to exploit *C. glutamicum* as a platform for the bio-based production of pyruvate and the 1,4-dicarboxylic acids malate, fumarate and succinate. The first step was the allocation of the central precursor pyruvate by development of a strong pyruvate producing mutant strain, and finding the best fermentation conditions for this mutant. These steps already included the goals to reduce the concentration of amino acids as by-products in culture supernatants, as well as blocking the production of L-lactate as

major anaerobic product of *C. glutamicum*. By development of an anaerobic production system, the reductive branch of the TCA cycle should be activated and reductive flux should be enhanced by optimizing the incubation conditions, and by further genetical modification of the mutants. Since succinate is already an end-product of fermentations with *C. glutamicum* under oxygen deprivation, demanding a high availability of reduction equivalents, the supply of reduction equivalents should be improved for succinate production. In the case of malate and fumarate, the aim was to stop the reaction chain of the reductive TCA cycle at the reactions from fumarate to succinate and from malate to fumarate, to initiate production and secretion of the particular product. By introduction of a new pathway into *C. glutamicum* and under usage of the established oxygen deprivation fermentation systems, additionally the production of the biofuel isobutanol was targeted. In the entity of this work, the necessity, as well as possibilities and prospects of bio-based building block production with microorganisms, especially concerning *C. glutamicum*, should be worked out and summarized.

B) RESULTS

The results of this work have partially been published in Blombach et al. (2011) and Wieschalka et al. (2012a). A third manuscript, Wieschalka et al. (2012b), summarizing my own and all current achievements in bio-based production of organic acids with *C. glutamicum*, has been submitted at the date of this thesis submission. For better understanding, some of the already published results will be picked up.

First, a brief introduction on the procedure of gene and partial gene exchange within the genome of *C. glutamicum* is given. Second, the published achievements for the production of pyruvate with *C. glutamicum* (Wieschalka et al. 2012a) are summarized and complemented with the results of my latest work on this field. The third section describes the design of succinic acid-producing *C. glutamicum* mutants by metabolic engineering, as well as the optimization of organic acid production conditions. Furthermore, the third section includes detailed descriptions of my experiments outlined in the publication on organic acid production with *C. glutamicum* (Wieschalka et al. 2012b). In the fourth section the procedures and results of the first attempts ever made to develop malate and fumarate producing *C. glutamicum* mutants, are presented. Finally, the fifth section summarizes the published achievements on isobutanol production with *C. glutamicum* (Blombach et al. 2011).

1. Construction of *C. glutamicum* mutant strains

For the construction of organic acid- and isobutanol-producing *C. glutamicum* mutants, the genome of *C. glutamicum* had to be modified by deletions and single base exchanges. All gene deletions, partial gene deletions or single-base exchanges described in the following sections, were obtained by means of transformation with derivatives of the suicide vectors pK18*mobsacB* and pK19*mobsacB* (Schäfer et al. 1994). Both plasmid types contain a multiple cloning site (MCS) within the *lacZα* complement, a resistance cassette Kan^R against the antibiotic kanamycin, the *sacB* gene, encoding the levansucrase from *Bacillus subtilis*, and an origin of replication for *E. coli* (Figure 3). For each modification in *C. glutamicum*, the modified DNA fragment that should replace the original part in the genome was cloned into the MCS of the respective plasmid. After transformation into *C. glutamicum*, selection followed on agar-plates containing kanamycin. Due to the lack of an origin of replication for *C. glutamicum*, just bacteria were able to form colonies on the selective medium, which had integrated the whole plasmid into their genome by homologous recombination (Figure 3). These integrants were then plated out on complex medium plates containing sucrose. The enzyme levansucrase encoded by the *sacB* gene, catalyses the formation of levan (a branched fructose polymer) from sucrose (Jäger et al. 1992). Levan is an exopolysaccharide, usually used for cell wall assembly of some bacteria. However, in *C. glutamicum*, levan evolves

toxicity (Jäger et al. 1992). Probably due to the inability of *C. glutamicum* to export levan, the polymer accumulates within the cell, causing a toxic disturbance of transportation processes across the membrane (Jäger et al. 1992). Therefore, the selection pressure mediated by sucrose, forces the bacteria to cut the plasmid out again, by a second homologous recombination (Figure 3). Afterwards, in 50% of all cases the wildtype is restored, in the other half the mutated DNA fragment remains in the genome, replacing the original sequence (Schäfer et al. 1994). False positives were sorted out, checking for kanamycin resistance again. In the further progress, the kanamycin sensitive clones were discriminated in wildtype and mutant either by colony PCR (in the case of complete or partial gene deletion), or by sequencing (in the case of single-base exchange).

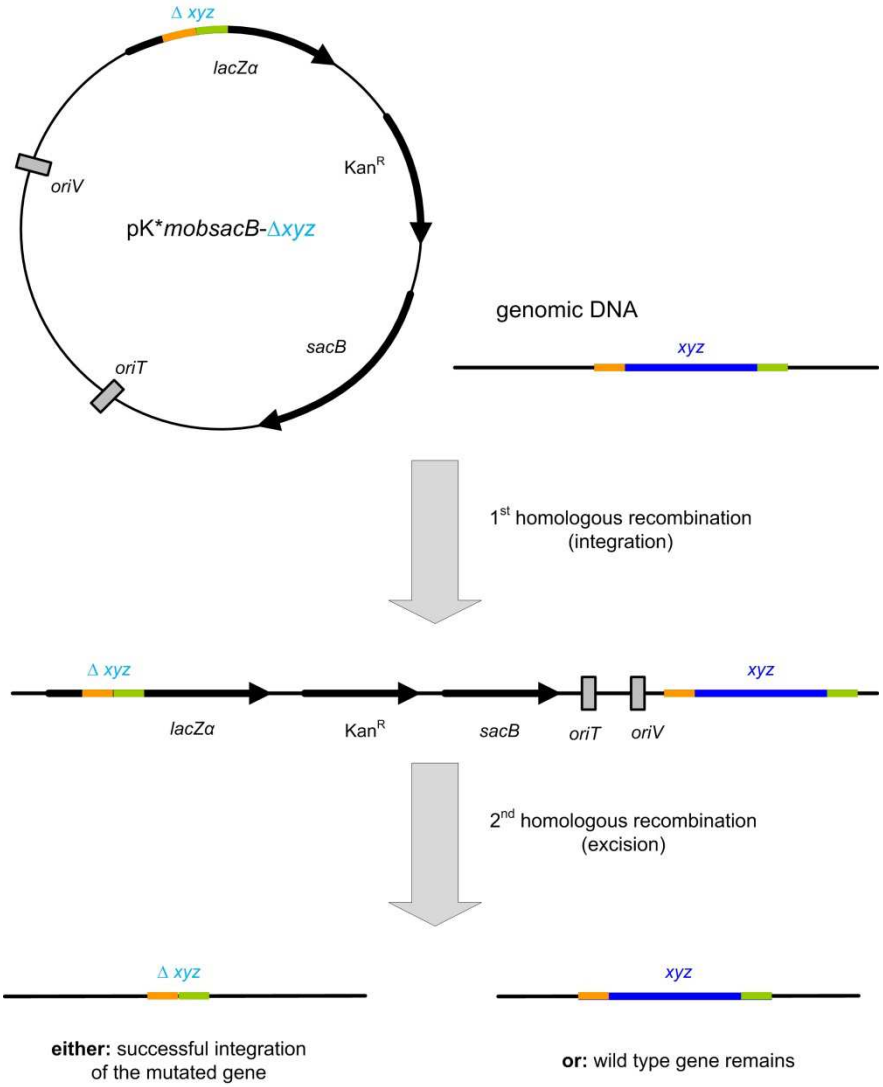

Figure 3: Schematic depiction of the principle of gene replacement in the chromosomal DNA of *C. glutamicum* via homologous recombination (after Schäfer et al. 1994); the asterisk within the plasmid name symbolizes the plasmid label 18 or 19; *xyz*: target sequence; Δ*xyz*: modified target sequence; *lacZα*: complement of the β-galactosidase gene *lacZ* (in this picture interrupted by the target sequence); Kan^R: kanamycin resistance cassette; *sacB* encodes the levansucrase from *B. subtilis*; *oriT*/*oriV*: origin of replication for *E. coli*; sizes of lines and arrows are just schematic and do not reflect the actual sizes of any sequence

2. Engineering *C. glutamicum* for the production of pyruvate

The construction of pyruvate producing *C. glutamicum* mutants, and the experiments performed with these strains for aerobic pyruvate production are published in Wieschalka et al. 2012a. This chapter gives a summary of the achievements made and shows the latest further results on pyruvate production.

Looking at the central metabolism of *C. glutamicum*, pyruvate can be identified as a central precursor for a variety of pathways and substances in the PEP-pyruvate-OAA node (Figure 2). To create a strong pyruvate-producing strain of *C. glutamicum*, pyruvate drawing reactions had to be down-regulated or even to be eliminated in the course of strain development. Recently, the PDHC of *C. glutamicum* was inactivated by deletion of the *aceE* gene (Schreiner et al. 2005). The resulting strain *C. glutamicum* Δ*aceE* was auxothrophic for acetate or ethanol (Schreiner et al. 2005; Blombach et al. 2009), and produced significant amounts of L-alanine (30 mM), L-valine (30 mM) and pyruvate (30 mM) from glucose when acetate was depleted and growth stopped (Blombach et al. 2007). Using this strain as a basis, the genes *pqo* and *ldhA* coding for PQO and LdhA were stepwise deleted, as described in Wieschalka et al. (2012a). The resulting strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* accumulated more than 50 mM pyruvate in a growth-decoupled manner, with a substrate specific product yield ($Y_{P/S}$) of 0.48 mol pyruvate per mol of glucose, besides L-alanine and L-valine as by-products (Wieschalka et al. 2012a). In a further step, performing a chromosomal exchange of the *ilvN* gene with a truncated substitute (C-T *ilvN*), the native AHAS was substituted by a leaky variant (ΔC-T IlvN). This led to an almost threefold increased $Y_{P/S}$ (1.36 mol pyruvate per mol of glucose) and a strong increase of pyruvate production (193 mM), while L-valine and L-alanine formation was reduced to 1 mM and 9 mM, respectively (Wieschalka et al. 2012a). Additional deletion of the genes encoding AlaT and AvtA resulted in cumulative reduction of L-alanine as unwanted side-product by 50% (~4 mM). The final strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC-T *ilvN* Δ*alaT* Δ*avtA* (further denoted as *C. glutamicum* ELB-P) produced in shake flask experiments up to 200 mM pyruvate, with a $Y_{P/S}$ of 1.49 mol per mol of glucose and a very low by-product yield of 0.03 mol L-alanine and 0.01 mol L-valine per mol of glucose (Wieschalka et al. 2012a). To study its relevance for industrial applications, subsequent fed-batch fermentations were performed with *C. glutamicum* ELB-P. Under oxygen surplus, adjusting dissolved oxygen (DO) at a level of 30%, significant lower glucose consumption rates and $Y_{P/S}$ were observed in comparison to shake flask experiments. Therefore, low oxygen tension from the middle until the end of growth phase was implemented in the bioreactor. Using fixed agitation and aeration, a self-induced microaerobic phase was established due to cell respiration. These conditions were identified as a premise for efficient pyruvate production in the bioreactor (Wieschalka et al. 2012a). In such fed-batch fermentations at high cell densities 500 mM pyruvate were obtained, with a maximum $Y_{P/S}$ of 0.97 mol per mol of glucose and a

productivity of 0.92 mmol·$g_{(CDW)}^{-1}$·h^{-1} (i.e., 0.08 g·$g_{(CDW)}^{-1}$·h^{-1}) during production phase (Wieschalka et al. 2012a). These results show that the final mutant *C. glutamicum* ELB-P is a strong and useful pyruvate producer.

During the course of strain development for pyruvate production, the concentration of the by-product L-alanine was diminished by stepwise deletion of the genes *alaT* and *avtA*, encoding enzymes for L-alanine forming pathways, resulting finally in *C. glutamicum* ELB-P (Wieschalka et al. 2012a). By deletion of *alaT* or both *alaT* and *avtA* the growth rate decreased dramatically from 0.40 h^{-1} to 0.12 h^{-1} in comparison to the parental strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC-T *ilvN* (Figure 4A-C). Therefore, the minimal medium (modified CGXII minimal medium; pH 6.8, adjusted with 5 M KOH (Eikmanns et al. 1991), with $(NH_4)_2SO_4$ (5 g/l) and $MnSO_4$ x H_2O (0.1 mg/l)) had to be adjusted to ensure proper growth of the mutants. Addition of 2 mM L-alanine to the medium enhanced the growth rate up to 0.35 h^{-1} and also increased the maximal OD_{600} of both strains in shake-flask experiments with 1% (*w*/*v*) acetate and 3% (*w*/*v*) glucose as carbon source to about 15.8, even though the maximal OD_{600} of about 19.5 of the parental strain could not be restored completely (Figure 4A-C). Nevertheless, the addition of L-alanine was sufficient to establish suitable aerobic growth conditions for the pyruvate producing mutants.

In addition to the modifications described above, further efforts have been made to improve pyruvate production with *C. glutamicum*. The $Y_{P/S}$ of 1.49 mol pyruvate per mol of glucose of *C. glutamicum* ELB-P (Wieschalka et al. 2012a) is below the maximal theoretical $Y_{P/S}$ of 2 mol pyruvate per mol of glucose. Suggesting that there is a drain of carbon towards the reductive branch of the TCA cycle in the pyruvate producing strain, it was investigated, whether an inactivation of the Mdh leads to higher pyruvate amounts. This enzyme reduces oxaloacetate to malate (Figure 2) and therefore probably is responsible for reductive TCA cycle flux. The inactivation of the Mdh was performed by knockout of the *mdh* gene as described in chapter "1. Construction of *C. glutamicum* mutant strains". The successful deletion was proven by colony PCR. *C. glutamicum* ELB-P Δ*mdh* was characterized in 500 ml shake-flask fermentations with 50 ml minimal medium containing 3% (*w*/*v*) glucose, 1% (*w*/*v*) potassium acetate and 2 mM L-alanine. The results are shown in Figure 5. *C. glutamicum* ELB-P Δ*mdh* grew until acetate was exhausted and produced pyruvate as major product from glucose in a growth-decoupled manner until after approximately 72 h the whole batch of glucose was consumed. The maximal concentration of pyruvate in the supernatants averaged 167 mM, and thereby the $Y_{P/S}$ was lowered by 28% in comparison to *C. glutamicum* ELB-P. Pyruvate uptake started when the glucose concentration approached zero. Interestingly, *C. glutamicum* ELB-P Δ*mdh* produced up to 10 mM L-glutamate as side-product, while the concentrations of both L-alanine and L-valine remained with about 4 mM and 1 mM, respectively, at the level of the parental strain.

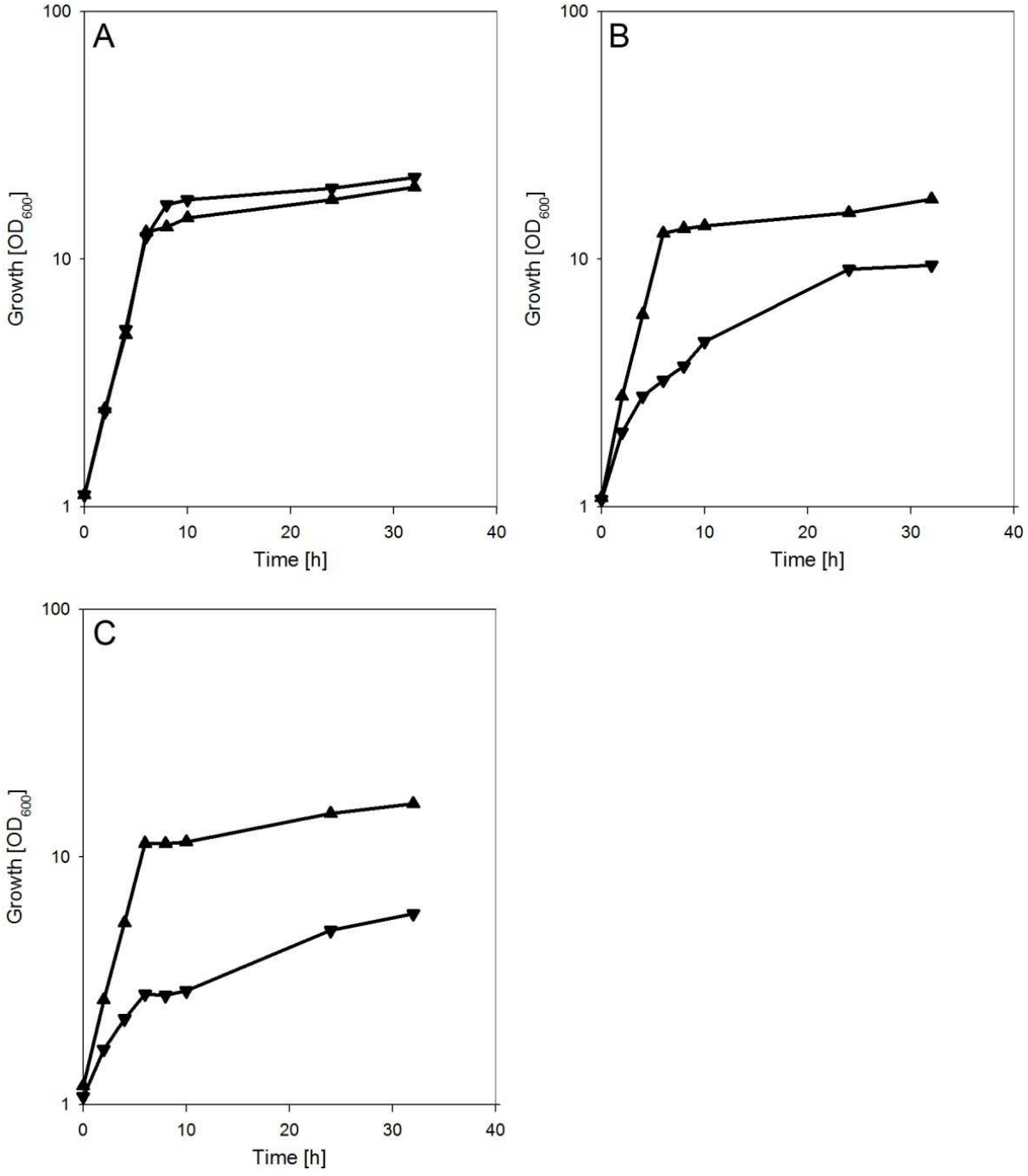

Figure 4: Growth of (A) *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC-T *ilvN*, (B) *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC-T *ilvN* Δ*alaT* and (C) *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC-T *ilvN* Δ*alaT* Δ*avtA* (*C. glutamicum* ELB-P) in modified CGXII medium containing 3% (*w*/*v*) glucose and 1% (*w*/*v*) potassium acetate, either without ▼ or with ▲ addition of 2 mM L-alanine. At least four independent fermentations were performed, all of them showing comparable results.

In summary, these results show the successful development of the pyruvate producing mutant *C. glutamicum* ELB-P. Additional deletion of the *mdh* had no beneficial effect on pyruvate production, indicating that there is no drain of carbon towards the reductive branch of the TCA cycle under aerobic conditions. Furthermore, efficient experimental setups for pyruvate production could be developed, concerning medium adjustment with L-alanine, as well as optimization of fed-batch fermentation strategies by implementation of low oxygen tension in mid-growth-phase.

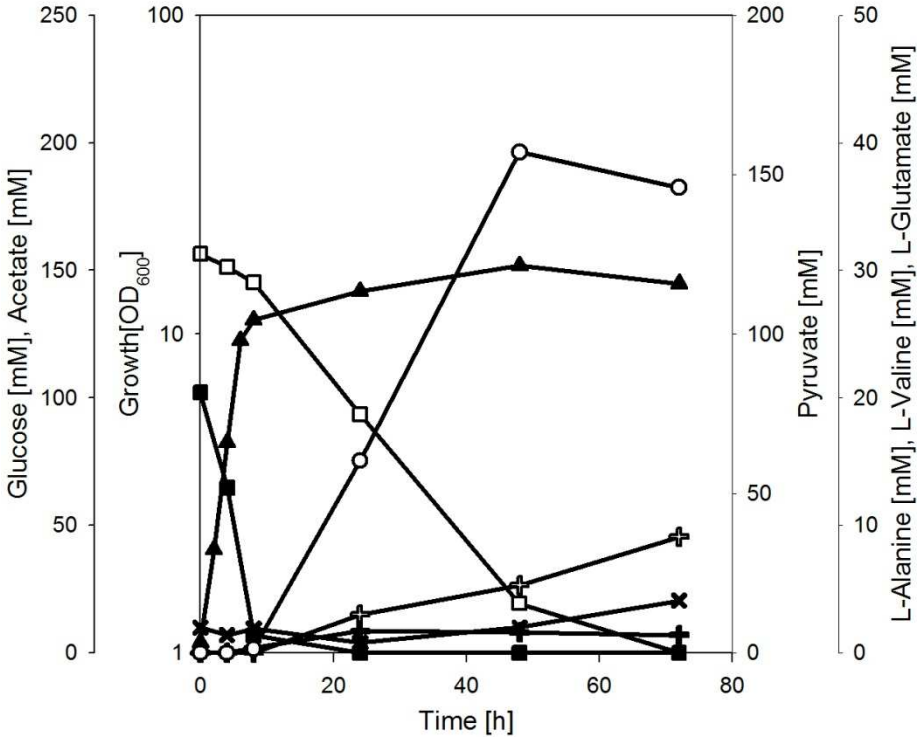

Figure 5: Growth, substrate consumption, and product formation during a representative shake-flask batch cultivation of *C. glutamicum* ELB-P Δ*mdh* in modified CGXII medium containing 3% (*w*/*v*) glucose, 1% (*w*/*v*) potassium acetate and 2 mM L-alanine. ▲ growth; □ glucose; ■ potassium acetate; ✖ L-alanine; ✚ L-valine; ✜ L-glutamate; ○ pyruvate. At least six independent fermentations were performed, all of them showing comparable results.

3. Tailoring *C. glutamicum* as a designer bug for the production of succinate

A major aim of this work was the development of an efficient succinate-producing *C. glutamicum* mutant by metabolic engineering. This chapter describes all efforts and achievements made during the course of designing such a mutant and concomitant establishment of fermentation processes for optimized production conditions.

For the formation of succinate, the pyruvate producing strain *C. glutamicum* ELB-P was used as a basis. In a first attempt, succinate production under aerobic conditions was investigated with this mutant. With such a strain background, succinate production ought to be realised by reductive TCA cycle activity. However, the results obtained with *C. glutamicum* ELB-P Δ*mdh* (see above), had already revealed that there is no drain of carbon towards the reductive branch of the TCA cycle under aerobic conditions. Therefore, the carbon flux should be directed towards the reductive branch by overproduction of PCx and PEPCx in *C. glutamicum* ELB-P, enhancing the OAA-forming carboxylation reactions (Figure 2). This was initiated by transforming *C. glutamicum* ELB-P with the plasmid pAN6*pyc*^{P458S}*ppc* from Litsanov et al. (2012a), containing a Kan^{R} and expressing *ppc*, encoding PEPCx, and *pyc*^{P458S}, encoding a deregulated PCx variant, under control of the *tac* promoter (P_{tac}) and the *lac* operator (*lacI*). Successful uptake of the plasmid was proven by clone selection on kanamycin, re-isolation of the plasmid and subsequent PCR, using an oligodeoxynucleotide (primer) pair, which amplified a fragment including parts of the plasmid as well as of the *pyc*^{P458S} gene. *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc* was cultivated at 30 °C in 500 ml baffled Erlenmeyer-flasks with 50 ml modified CGXII medium containing 3% (*w*/*v*) glucose, 1% (*w*/*v*) potassium acetate and 2 mM L-alanine. Additionally, in parallel performed experiments, a tenfold excess (2 mg/l) of the for carboxylation reactions essential cofactor biotin was tested. Expression of *pyc*^{P458S} and *ppc* was induced by addition of 0.5 mM IPTG to the medium after 4 h of growth. The mutant did not produce succinate under aerobic conditions, even in presence of a ten-fold excess of biotin (data not shown).

Under oxygen deprivation conditions *C. glutamicum* wild-type (WT) already produces succinate, besides lactate and trace amounts of acetate (Inui et al. 2004a). Therefore, in a further approach *C. glutamicum* ELB-P was used for the production of succinate under anaerobic conditions. For this purpose, the bacteria were precultured in 500 ml baffled Erlenmeyer flasks with 50 ml complex medium (2xTY medium; bacto-tryptone (16 g/l), yeast extract (10 g/l), NaCl (5 g/l)), containing 0.5% (*w*/*v*) acetate. From these, 125 ml Müller-Krempel bottles with 50 ml modified CGXII medium, supplemented with 2% (*w*/*v*) glucose were inoculated to an OD_{600} of about 15. While shaking with sealed lid at 30 °C on a rotary shaker (120 rpm), *C. glutamicum* ELB-P produced up to 1 mM fumarate, 7 mM malate and 20 mM pyruvate. The main product was succinate. In average, almost 100 mM could be detected, with a $Y_{P/S}$ of 0.78 mol succinate per mol of glucose (Figure 6, Table 3). When glucose was exhausted, the bacteria always started to consume the produced pyruvate.

Table 3: $Y_{P/S}$ and productivity of *C. glutamicum* ELB-P and *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc* after anaerobic incubation with 2% (*w*/*v*) of glucose in 125 ml Müller-Krempel bottles

***C. glutamicum* strain**	**Product $Y_{P/S}$ [mol succinate /mol glucose]**	**Production rate [mmol·$g_{(DCW)}^{-1}$·h^{-1}]**
ELB-P	0.78 ± 0.08	0.81 ± 0.06
ELB-P pAN6_*pyc*^{P458S}*ppc*	0.86 ± 0.05	0.89 ± 0.28

Incubating *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc* under the same conditions, led to a slightly beneficial effect on the $Y_{P/S}$ (0.86 mol succinate per mol of glucose) and on the production rate (0.89 ± 0.28 mmol·$g_{(DCW)}^{-1}$·h^{-1} in comparison to 0.81 ± 0.06 mmol·$g_{(DCW)}^{-1}$·h^{-1}) with 2% glucose as carbon source (Table 3).

During the time these strains were developed, the succinate exporter SucE was identified (Huhn et al. 2011; Fukui et al. 2011; Figure 2, Table 1). When *C. glutamicum* ELB-P was transformed with plasmid pXMJ19-cgl2211 overproducing SucE (Huhn et al. 2011), no beneficial effect was observed on succinate production rate or $Y_{P/S}$ (data not shown). Therefore, it was assumed that the natural transport force of *C. glutamicum* was sufficient to export all succinate produced by the so far constructed mutant strains.

Altogether, *C. glutamicum* ELB-P and its derivative *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc* already produced succinate well under anaerobic conditions, and therefore represented a promising platform for further improvement.

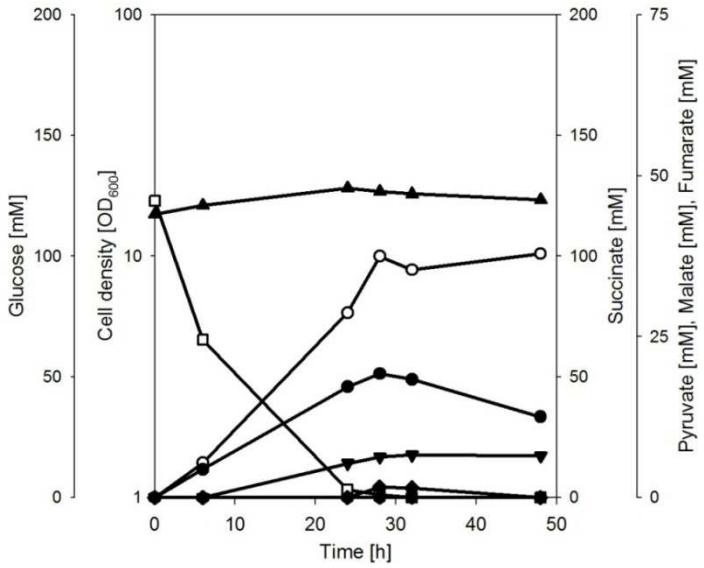

Figure 6: Cell density, substrate consumption and product formation during a representative anaerobic batch experiment of *C. glutamicum* ELB-P in minimal medium containing 2% (*w*/*v*) glucose. ▲ cell density; □ glucose; ● pyruvate; ○ succinate; ▼ malate; ◆ fumarate. At least six independent fermentations were performed, all of them showing comparable results.

Addition of $NaHCO_3$ leads to continuous consumption of higher glucose levels, resulting in a higher $Y_{P/S}$ and final succinate titres during batch fermentations in Müller-Krempel bottles

As a next step the final succinate titres, achieved under oxygen deprivation conditions at 30 °C with *C. glutamicum* ELB-P and *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc*, should be enhanced by just increasing the initial glucose concentration under the experimental conditions described above.

However, with 3% (*w/v*) glucose in the medium, glucose consumption and succinate production with *C. glutamicum* ELB-P stopped when about 2% (*w/v*) glucose were consumed (Figure 7A). The $Y_{P/S}$ and productivity obtained until production stopped were similar to those in the experiments with 2% (*w/v*) glucose. It was presumed that the limiting factor for succinate production was the availability of CO_2 for the carboxylation of PEP or pyruvate to OAA. To compensate the sudden break in glucose consumption, the supply of CO_2 was improved by addition of bicarbonate (200 mM $NaHCO_3$) to the Müller-Krempel bottles. The result shown in Figure 7B indicates that addition of $NaHCO_3$ led to complete glucose consumption as well as improved succinate formation. More than 150 mM succinate were formed under these conditions, with a higher $Y_{P/S}$ of 0.91 ± 0.02 mol succinate per mol of glucose in comparison to experiments without bicarbonate (Table 4, Table 3). However, the bacteria produced succinate with a lower production rate of just 0.70 ± 0.06 $mmol \cdot g_{(DCW)}^{-1} \cdot h^{-1}$ in comparison to fermentations without $NaHCO_3$ (0.81 ± 0.06 $mmol \cdot g_{(DCW)}^{-1} \cdot h^{-1}$). Pyruvate appeared still as major by-product (about 20 mM), followed by malate and lactate (both <10 mM) (Figure 7B). Fumarate levels were less than 1 mM (data not shown).

When $NaHCO_3$ was supplied to the medium, the succinate productivity of *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc* remained with 0.75 ± 0.02 $mmol \cdot g_{(DCW)}^{-1} \cdot h^{-1}$on a similar level as in the experiments with this mutant strain in absence of bicarbonate (Table 4, Table 3). Also the $Y_{P/S}$ did not increase with addition of $NaHCO_3$ (Table 4), suggesting that the slightly beneficial effect of pAN6*pyc*^{P458S}*ppc* in the experiments with 2% (*w/v*) glucose was due to improved CO_2 fixation under "non-CO_2-surplus" conditions. Moreover, *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc* did not show improved succinate production in the tested experimental setups in presence of a ten-fold excess of the essential cofactor biotin (data not shown).

The results obtained with *C. glutamicum* ELB-P and *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc*, feeding 3% (*w/v*) glucose in presence or absence of 200 mM $NaHCO_3$ clearly showed that the supply of CO_2 for the reactions towards OAA is crucial for anaerobic succinate production. Anyhow, the question remained, why the bacteria were able to produce succinate from up to 2% (*w/v*) glucose, without external CO_2 supply to the medium. This finding was investigated in further experiments to identify the source of CO_2 in absence of $NaHCO_3$, which are described in the following paragraph.

Table 4: $Y_{P/S}$ and productivities of *C. glutamicum* ELB-P and *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc* after anaerobic incubation with 3% (*w*/*v*) of glucose and 200 mM $NaHCO_3$ in 125 ml Müller-Krempel bottles

***C. glutamicum* strain**	**Product $Y_{P/S}$ [mol succinate /mol glucose]**	**Production rate [mmol·$g_{(DCW)}^{-1}$·h^{-1}]**
ELB-P	0.91 ± 0.02	0.70 ± 0.06
ELB-P pAN6_*pyc*^{P458S}*ppc*	0.87 ± 0.01	0.75 ± 0.02

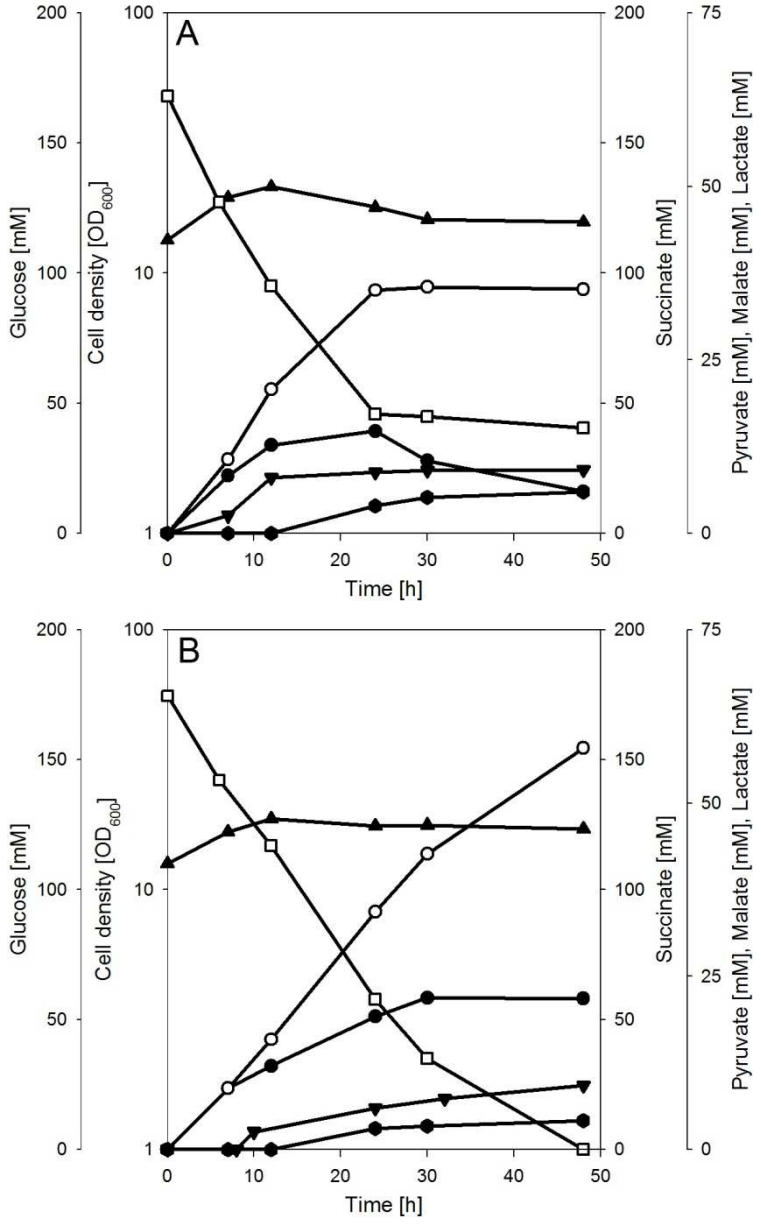

Figure 7: Cell density, substrate consumption and product formation during a representative anaerobic batch experiment of *C. glutamicum* ELB-P in modified CGXII medium containing 3% (*w*/*v*) glucose, in (A) absence or (B) presence of 200 mM $NaHCO_3$. ▲ cell density; □ glucose; ● pyruvate; ○ succinate; ▼ malate; ⬢ lactate. At least three independent fermentations were performed, all of them showing comparable results.

C. glutamicum ELB-P is able to use urea as CO_2-donor for succinate production under oxygen deprivation conditions

The addition of $NaHCO_3$ to the medium of *C. glutamicum* ELB-P and *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc* enabled complete consumption of 3% (*w*/*v*) glucose and concomitant production of about 150 mM succinate. However, the capability of the mutants to produce succinate from 2% (*w*/*v*) of glucose raised the question, why this was possible without addition of $NaHCO_3$. The results obtained with addition of bicarbonate to the medium clearly showed that CO_2 supply is a limiting factor for the conversion of glucose to succinate (Figure 7A+B). It was concluded that there must be already a source for CO_2 in the medium, which ensures succinate production from 2% (*w*/*v*) of glucose. Minimal medium for *C. glutamicum* contains 5 g urea per litre as nitrogen source (Eikmanns et al. 1991). Urea can be converted intracellular by urease to ammonia and CO_2 (Siewe et al. 1998). To test, whether the conversion of urea by urease is the origin of CO_2 in our anaerobic experiments, *C. glutamicum* ELB-P was incubated at an initial OD_{600} of about 15 in Müller-Krempel bottles, containing modified CGXII medium supplied with 2% (*w*/*v*) glucose, either in presence, or absence of 5 g urea per litre. The results shown in Figure 8A indicate that *C. glutamicum* ELB-P consumes 2% (*w*/*v*) glucose in minimal medium, which contains urea, and produces succinate as major product (~90 mM). The results shown in Figure 8B indicate evidently that without addition of urea to the medium, glucose is hardly consumed (depletion of ~55 mM), leading to minor accumulation of succinate (~30 mM) or any other product.

Taken together with the results from the experiments with $NaHCO_3$ addition, urea seemed to be a promising CO_2 storage for the production of succinate since the productivity was higher with urea as sole CO_2 source (Table 3, Table 4), probably due to direct intracellular availability. Therefore, urea was tested as substitute for bicarbonate, repeating the experiments in Müller-Krempel bottles with 3% (*w*/*v*) glucose under usage of double urea concentrations (10 g/l) and in absence of $NaHCO_3$. Under these experimental conditions *C. glutamicum* ELB-P consumed the initially added glucose completely and produced up to 150 mM succinate with pyruvate as major by-product (about 25 mM), followed by malate and lactate (both <10 mM) (Figure 8C). Fumarate levels were less than 1 mM (data not shown). These results show that urea in principle can be used as substitute for $NaHCO_3$. However, urea consumption is bound to a strong ammonia release into the medium. A high ammonia concentration might enhance amino acid side-product formation and complicate product purification.

It can be concluded that urea represents an additional source to generate CO_2 for the carboxylation of PEP and pyruvate to OAA. However, the use of $NaHCO_3$ was judged as more economical, since ammonia enrichment within the medium should be avoided to improve product purification. Therefore, the concentration of urea was kept at 5 g/l for all experiments that followed and $NaHCO_3$ was supplied to enhance CO_2 availability.

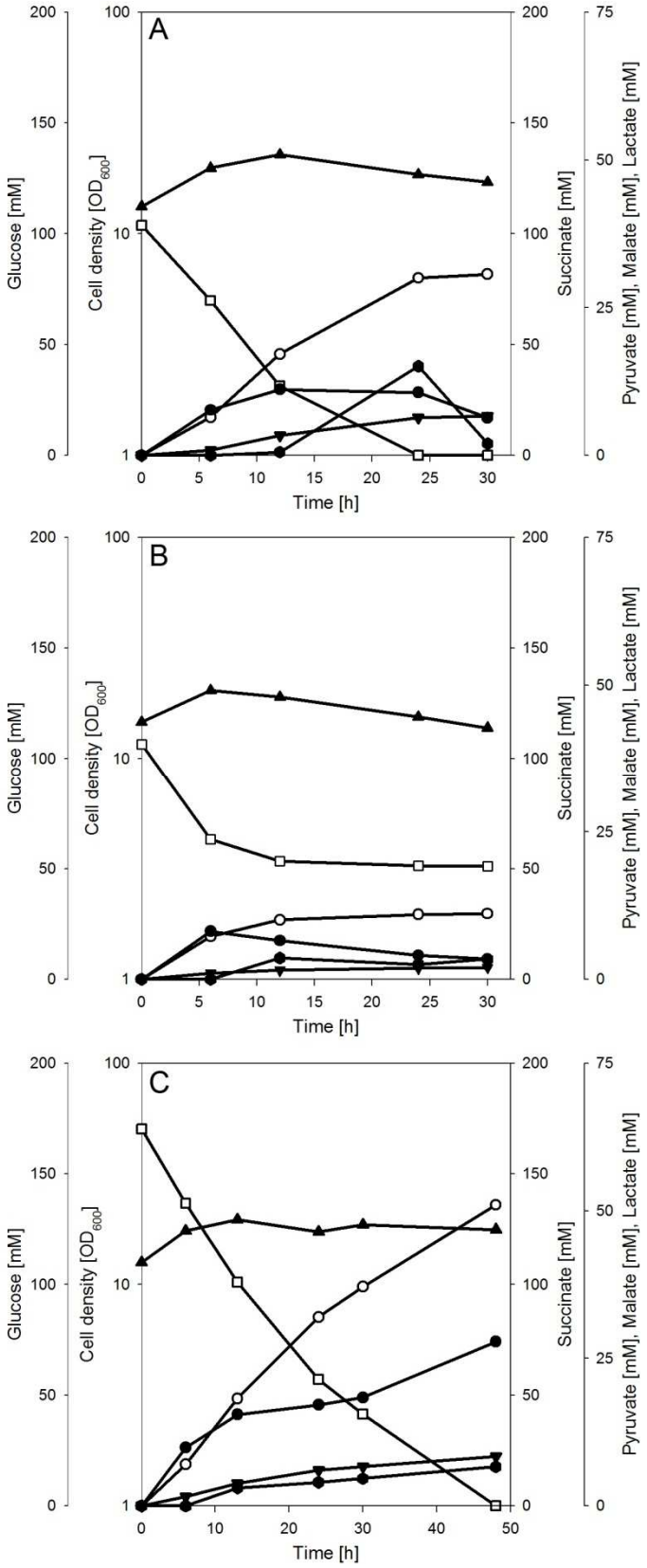

Figure 8: Cell density, substrate consumption and product formation during a representative anaerobic batch experiment of *C. glutamicum* ELB-P in modified CGXII medium containing (A) 2% (*w*/*v*) glucose and 5 g/l urea, (B) 2% (*w*/*v*) glucose and no urea, or (C) 3% (*w*/*v*) glucose and 10 g/l urea. ▲ cell density; □ glucose; ● pyruvate; ○ succinate; ▼ malate; ⬢ L-lactate. At least three independent fermentations were performed, all of them showing comparable results.

Culture conditions affect succinate production behaviour of *C. glutamicum* ELB-P

Culture conditions were further optimized for anaerobic succinate production at 30 °C, concerning the bottle type and size, as well as the composition of the gas phase in the headspace. It could be shown that these parameters have a tremendous impact on production behaviour of *C. glutamicum* ELB-P under oxygen deprivation conditions (in collaboration with Maria-Elisabeth Böhm, 2012). The most interesting conditions used for batch fermentations and the resulting effects on yield and production rate are summarized in Table 5. All experiments were performed at an initial OD_{600} of about 15, either in 125 ml Müller-Krempel bottles, or in 100 ml screw cap bottles, containing 50 ml modified CGXII medium, supplied with 2% (*w*/*v*) glucose or 3% (*w*/*v*) glucose plus 200 mM $NaHCO_3$. In one part of the experiments, the air gas phase was replaced by nitrogen. For both bottle types, the replacement of the air gas phase with nitrogen resulted in an increased succinate yield (0.92 versus 0.78 mol succinate per mol of glucose in Müller-Krempel bottles; 0.76 versus 0.70 mol succinate per mol of glucose in screw cap bottles). The reason is that the nitrogen flooded bottles are completely oxygen free. With air gas phase the bottles become anaerobic during the course of the experiment by oxygen consumption of the bacteria. The addition of $NaHCO_3$ led to a decrease in the production rate from 0.90 to 0.69 $mmol \cdot g_{(CDW)}^{-1} \cdot h^{-1}$ in Müller-Krempel bottles, and from 0.75 to 0.68 $mmol \cdot g_{(CDW)}^{-1} \cdot h^{-1}$ in screw cap bottles (Table 5).

In an additional step, reductive flow towards the TCA cycle should be enhanced, by modifying the metabolism of *C. glutamicum* ELB-P at the level of a CO_2-independent reaction, using the different experimental setups described above. For that purpose, the Mdh (Figure 2, Table 1) should be overproduced in *C. glutamicum* ELB-P. The Mdh coding gene *mdh* (annotated as *cg2613*) from *C. glutamicum* ATCC13032 was amplified via PCR and cloned behind a constitutive P_{tac} into the vector pBB1 (pMM36 derivative: Cm^R, P_{tac}, tryptophane terminator (T_{trp}), *lacI*^{q}; Krause et al. 2009). The resulting plasmid pBB1-*cg2613* was checked by sequencing, amplified in *E. coli* DH5α, and after purification transformed into *C. glutamicum* ELB-P. Successful transformation of the plasmid was proven by selection on chloramphenicol and colony PCR, using a primer pair, amplifying a fragment, including parts of the plasmid, and of the *mdh* gene. However, *C. glutamicum* ELB-P pBB1-*cg2613*, showed no improved succinate production in any of the mentioned experimental setups (data not shown; performed in collaboration with Stephanie Färber, 2011).

The results shown in Table 5, revealed the usage of 100 ml screw cap bottles, and supplying the medium with 3% (*w*/*v*) glucose plus 200 mM $NaHCO_3$, and substituting the air in the gas phase by nitrogen as the most promising experimental setup. The production rate was a bit lower in this setting in comparison to the other experiments (0.68 $mmol \cdot g_{(CDW)}^{-1} \cdot h^{-1}$; Table 5), but most impressive is that the highest $Y_{P/S}$ was obtained with 0.98 mol succinate per mol of glucose. The additional overproduction of Mdh, by constitutively expressing *mdh* on the plasmid pBB1-*cg2613* had no further effect on succinate production.

Table 5: $Y_{P/S}$ and production rate of *C. glutamicum* ELB-P under oxygen deprivation conditions using different production settings

Bottle volume	Bottle type	N_2 atmosphere	Glucose[1]		200 mM $NaHCO_3$	Product $Y_{P/S}$ [mol succinate /mol glucose]	Production rate [mmol·$g_{(CDW)}^{-1}$·h^{-1}]
			2%	3%			
125 ml	M-Krempel		X			0.78 ± 0.08	0.81 ± 0.06
125 ml	M-Krempel	X	X			0.92 ± 0.18	0.90 ± 0.26
125 ml	M-Krempel	X		X	X	0.91 ± 0.05	0.69 ± 0.03
100 ml	screw cap		X			0.70 ± 0.00	0.74 ± 0.06
100 ml	screw cap	X	X			0.76 ± 0.15	0.75 ± 0.05
100 ml	screw cap	X		X	X	0.98 ± 0.12	0.68 ± 0.11

[1] in % (*w*/*v*); Abbreviation: M-Krempel, Müller-Krempel

Efficient succinate production with *C. glutamicum* ELB-P in one-stage fed-batch fermentations, using a tri-phasic setup and CO_2 gassing during stationary phase

To study the relevance of long-term succinate production at high cell densities with *C. glutamicum* ELB-P, fed-batch fermentations with controlled pH were established in a "Fed-Batch Pro Fermentation System" from DASGIP (Jülich, Germany) at 30 °C. In contrast to sealed-bottle experiments where the bottles were inoculated with resting cells (two-stage process), the bacteria were cultivated in the 400 ml bioreactors and directly used in a one-stage process to produce succinate, when the bacterial growth phase had ended. The reactors were filled with 250 ml modified CGXII medium, initially containing 4% (*w*/*v*) glucose, 1% (*w*/*v*) acetate, and 6 mM L-alanine. The pH was maintained at 7.0 by online measurement using a standard pH electrode (Mettler Toledo, Giessen, Germany) and addition of 4 M H_2SO_4 and 4 M KOH, respectively. 1:20 diluted Struktol 674 antifoam (Schill und Seilacher, Hamburg, Germany) was added manually drop wise (~20 µl) when necessary. DO was measured online, using an oxygen electrode (Mettler Toledo, Giessen, Germany). When the bacteria were cultivated at 30 °C in a biphasic system with an aerobic growth phase under oxygen surplus (i.e., with a constant pO_2 of 30% DO), directly followed by an anaerobic production phase, bubbling CO_2, *C. glutamicum* ELB-P grew with a µ of 0.28 h^{-1} to an OD_{600} of about 55, but showed only weak succinate production of up to 80 mM with a $Y_{P/S}$ of 0.46 mol succinate per mol of glucose (data not shown). Such an effect was already observed in pyruvate fed-batch fermentations with this strain (Wieschalka et al. 2012a). To facilitate a fermentation system including both proper growth and proper production conditions a tri-

phasic fed-batch fermentation system was established, which enabled the bacteria to adapt to the particular conditions. The arrangement included an aerobic growth phase (I) on acetate plus glucose, passing into a self-induced microaerobic phase (II) at the end of growth by minimal aeration and fixed agitation. Immediately after reaching stationary phase, aeration was stopped and replaced by CO_2 sparging to initiate (III) oxygen deprivation conditions and ensure CO_2 supply for succinate production (Figure 9AB; Wieschalka et al. 2012b).

During the early growth phase, the DO was kept constant at about 30% for 6 h to accelerate growth. Afterwards the pO_2 dropped to 0-5% DO, due to cell respiration, stirring at a maximum of 400 to 750 rpm and gassing with 1 vvm (volume of gas per volume of medium per minute) air. By addition of adequate amounts from a 50% (*w*/*v*) acetate stock solution after 8 h, *C. glutamicum* ELB-P continued to grow with a µ of 0.27 h^{-1} (Table 6), to a final OD_{600} of about 50 (Figure 9A), reaching a biomass yield of 10.51 ± 1.13 $g_{(DCW)}^{-1} \cdot mol_C^{-1}$ (Table 6). In this phase the pO_2 dropped to 0-5% DO, stirring at 400 to 1000 rpm, while gassing with 1.25 vvm air. The high cell density led to self-induced microaerobic conditions (Figure 9B). The pO_2 peak at the end of microaerobic phase (marked with the asterisk in Figure 9B) indicated end of aerobic growth, as O_2 usage stopped, leading to increasing DO in the medium. Immediately, aeration was replaced by CO_2 sparging and production phase started (Figure 9AB). A batch of glucose at the beginning of stage III should ensure carbon availability for succinate production.

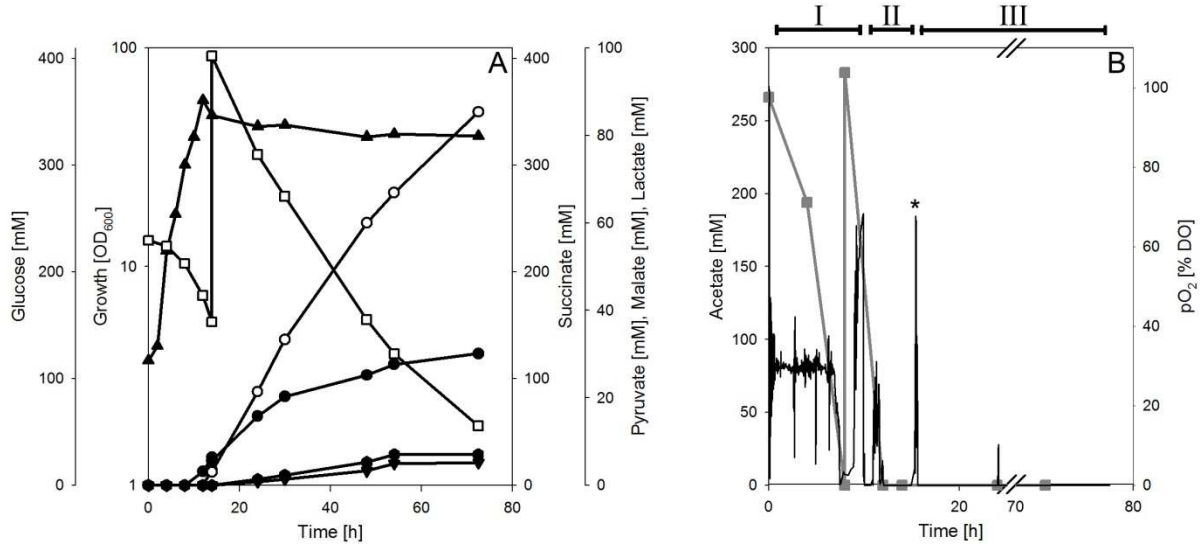

Figure 9: (A) Growth, glucose consumption, and product formation, as well as (B) acetate consumption and the course of the pO2 of *C. glutamicum* ELB-P during a representative pH-controlled tri-phasic fed-batch cultivation in a 400 ml bioreactor with minimal medium, initially containing 4% (*w*/*v*) glucose, 1% (*w*/*v*) acetate and 6 mM L-alanine. ▲ growth; □ glucose; ● pyruvate; ○ succinate; ▼ malate; ⬢ lactate; roman numerals indicate the three fermentation stages (I) aerobic growth phase, (II) self-induced microaerobic phase, (III) oxygen deprivation by CO_2 gassing; the asterisk within figure (B) marks the rising pO_2 when aerobic growth ended. At least five independent fermentations were performed, showing comparable results.

Table 6: Relevant process parameters of tri-phasic fed-batch fermentations with *C. glutamicum* ELB-P for one-stage succinate production under oxygen deprivation conditions

Process parameters	Growth condition 400 ml bioreactor (fed-batch)
growth rate μ [h^{-1}]	0.27 ± 0.03
biomass yield [$g_{(CDW)} \cdot mol_C^{-1}$]	10.51 ± 1.13
glucose consumption rate during production phase [$mmol \cdot g_{(CDW)}^{-1} \cdot h^{-1}$]	0.65 ± 0.1
production rate [$mmol \cdot g_{(CDW)}^{-1} \cdot h^{-1}$]	0.58 ± 0.11
product $Y_{P/S}$ [mol succinate /mol glucose]	1.02 ± 0.03

The glucose consumption rate during production was 0.65 ± 0.1 $mmol \cdot g_{(DCW)}^{-1} \cdot h^{-1}$ (Table 6). In the anaerobic phase the mutant formed up to 350 mM succinate within 60 h from glucose (Figure 9A). The $Y_{P/S}$ was 1.02 ± 0.03 mol succinate per mol of glucose and the production rate reached 0.58 ± 0.11 $mmol \cdot g_{(DCW)}^{-1} \cdot l^{-1} \cdot h^{-1}$ (Table 6).

By optimization of one-stage fed-batch fermentation conditions in the bioreactor a tri-phasic fermentation protocol was successfully established, which enabled the bacteria to adapt from growth conditions to production conditions. In this new experimental setup, *C. glutamicum* ELB-P produced up to 350 mM succinate with a $Y_{P/S}$ of 1.02 mol succinate per mol of glucose, which is higher than the best yields obtained in sealed bottle experiments (Table 6, Table 5).

Modification of the flux distribution between glycolysis and pentose phosphate pathway in *C. glutamicum* ELB-P had a slightly positive impact on succinate production

To further improve *C. glutamicum* ELB-P, bottlenecks for succinate production had to be identified. As it can be read off Figure 2 and as calculated in Figure 10, the conversion of 1 mol glucose leads to 1 mol succinate, assuming that the PPP activity is zero, while one mol of pyruvate is left at the end of glycolysis. The remaining pyruvate cannot be converted to succinate as all reduction equivalents, which are formed during glycolysis, are consumed during the course of succinate formation from the first mol of pyruvate. This calculation indicates that more reduction equivalents are needed to keep the reductive branch of the TCA cycle running. Reduction equivalents can be generated by redirection of the carbon flux through the PPP (Figure 2 and Figure 10). It was shown in several publications on Lysine production strains, that enhanced PPP flux can be achieved by deregulation of G6PDH

(encoded by *zwf* and *opcA*; Table 1) and 6PGDH (encoded by *gnd*; Table 1) (Becker et al. 2007; Ohnishi et al. 2005).

Thus, sequential chromosomal exchanges of gene sections of *zwf* and *gnd* were performed in *C. glutamicum* ELB-P (in collaboration with Maria-Elisabeth Böhm, 2012). A part of the genomic *zwf* gene was replaced by homologous recombination with a mutated part, containing a single-base-exchange from G to A at position 727, and leading to the deregulated version of G6PDH: ZwfA243T. The chromosomal exchange was performed after the method described in "1. Construction of *C. glutamicum* mutant strains" (p. 19f), transforming the suicide vector pK18*mobsacB_zwf*XLA243T (provided by Evonik Industries AG), into *C. glutamicum* ELB-P and subsequent accomplishment of the necessary selection steps. The successful single-base-exchange was proven by sequencing the target gene. Afterwards, a region of the genomic *gnd* gene additionally was replaced by homologous recombination with a mutated part containing a single-base-exchange from C to T at position 1082, leading to the deregulated version of 6PGDH: GndS361F. For that purpose the plasmid pK19*mobsacB_gnd*S361F had to be constructed. Therefore, the respective region of the *gnd* gene was amplified in two parts with overlapping primers in the middle. The primers used in the overlapping region were created to carry already the desired base-exchange at the respective position. Via crossover PCR with both fragments a *gnd* fragment was constructed, carrying the exchanged nucleotide. The sequence of this fragment was verified by sequencing and then cloned into pK19*mobsacB*. The chromosomal exchange in *C. glutamicum* was performed after the method described in "1. Construction of *C. glutamicum* mutant strains" (p. 19f), transforming the suicide vector pK19*mobsacB_gnd*S361F, into *C. glutamicum* ELB-P and subsequent accomplishment of the necessary selection steps. The successful single-base exchange was proven by sequencing the target gene.

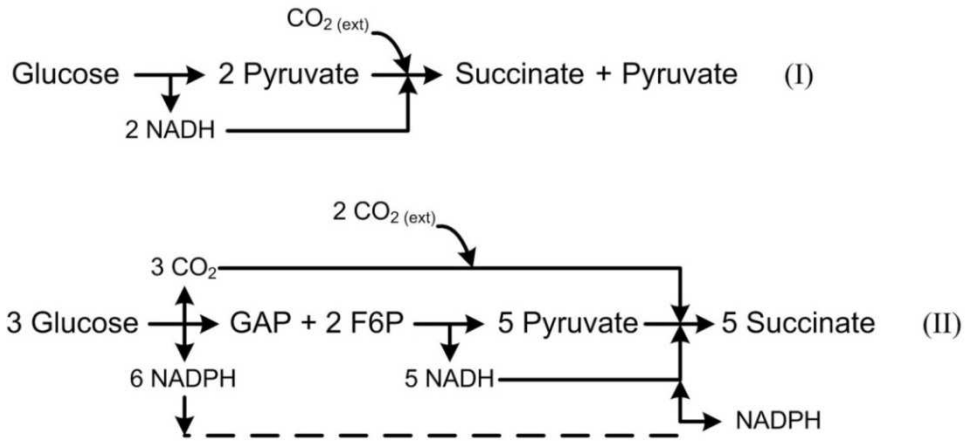

Figure 10: Schematic course of succinate formation, using either (I) glycolysis or (II) PPP for glucose metabolism

The deregulation of the G6PDH alone (data not shown; Böhm, 2012), as well as the double deregulation mutant *C. glutamicum* ELB-P ZwfA243T GndS361F had a sequential, slightly beneficial effect on succinate production. Figure 11 shows a representative diagram of an experiment with *C. glutamicum* ELB-P ZwfA243T GndS361F incubated at 30 °C in 100 ml screw cap bottles under oxygen deprivation conditions, with nitrogen replacing the air gas phase in the bottle headspace. The bacteria were inoculated at an initial OD_{600} of about 15 to modified CGXII medium, containing 3% (*w*/*v*) glucose and 200 mM $NaHCO_3$. With a production rate of 0.79 ± 0.06 $mmol \cdot g_{(CDW)}^{-1} \cdot h^{-1}$ about 150 mM succinate were produced, resulting in a $Y_{P/S}$ of 1.07 mol succinate per mol of glucose. Both $Y_{P/S}$ and productivity were increased in comparison to *C. glutamicum* ELB-P, which showed under the same conditions a $Y_{P/S}$ of 0.98 mol succinate per mol glucose and a production rate of 0.68 ± 0.06 $mmol \cdot g_{(CDW)}^{-1} \cdot h^{-1}$ (Table 5). Major side-product of *C. glutamicum* ELB-P ZwfA243T GndS361F was pyruvate, with about 25 mM (Figure 11). Lactate was not detectable as by-product in the supernatants.

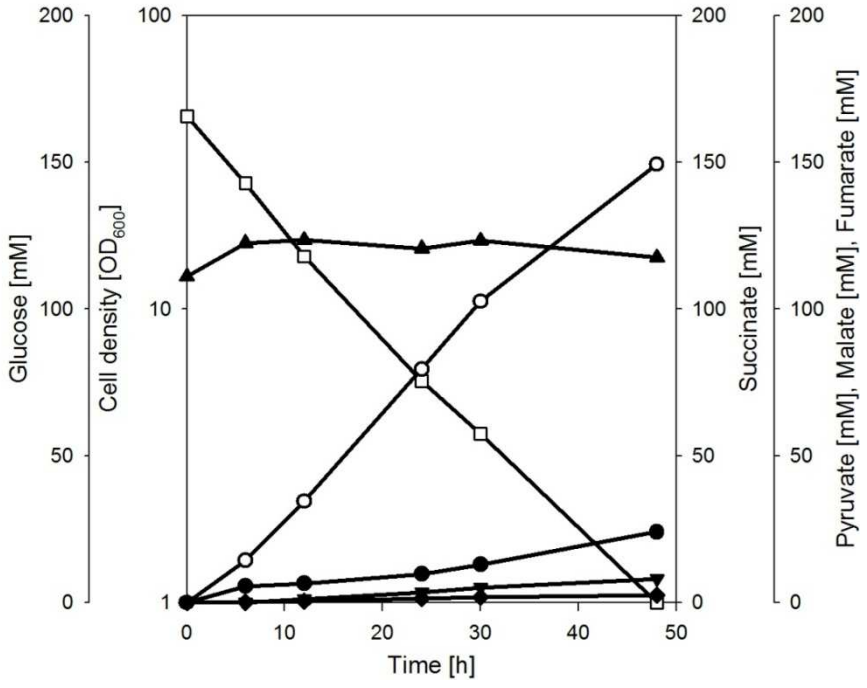

Figure 11: Cell density, substrate consumption and product formation during a representative anaerobic batch experiment of *C. glutamicum* ELB-P ZwfA243T GndS361F in modified CGXII medium, containing 3% (*w*/*v*) glucose and 200 mM $NaHCO_3$. ▲ cell density; □ glucose; ● pyruvate; ○ succinate; ▼ malate; ◆ fumarate. At least six independent fermentations were performed, all of them showing comparable results.

Taken together, the new strain *C. glutamicum* ELB-P ZwfA243T GndS361F was constructed successfully. Productivity and $Y_{P/S}$ of this new strain were slightly increased in comparison to the parental strain *C. glutamicum* ELB-P. Looking at the theoretical $Y_{P/S}$ of 1.67 mol succinate per mol of glucose, given by the scheme in Figure 10, the deregulation of the initial enzymes of the PPP was not sufficient to push succinate production up near the maximum.

As a next step, carbon was directed completely into the PPP, blocking glycolysis at the level of the enzyme PGI. This was realised by deletion of the PGI coding gene *pgi* (Table 1). The deletion of *pgi* was performed after the method described in "1. Construction of *C. glutamicum* mutant strains" (p. 19f), transforming the suicide vector pK18*mobsacB* pgi del (Bartek et al. 2010) into *C. glutamicum* ELB-P ZwfA243T GndS361F. The successful deletion was proven by colony PCR. In a *pgi* negative mutant, glucose metabolism has to be driven by the PPP alone. However, as shown in Figure 12, *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F did hardly consume glucose, leading to minimal accumulation of succinate or any other product. The surprising inability of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F to produce succinate was hence further investigated as described in the following paragraph.

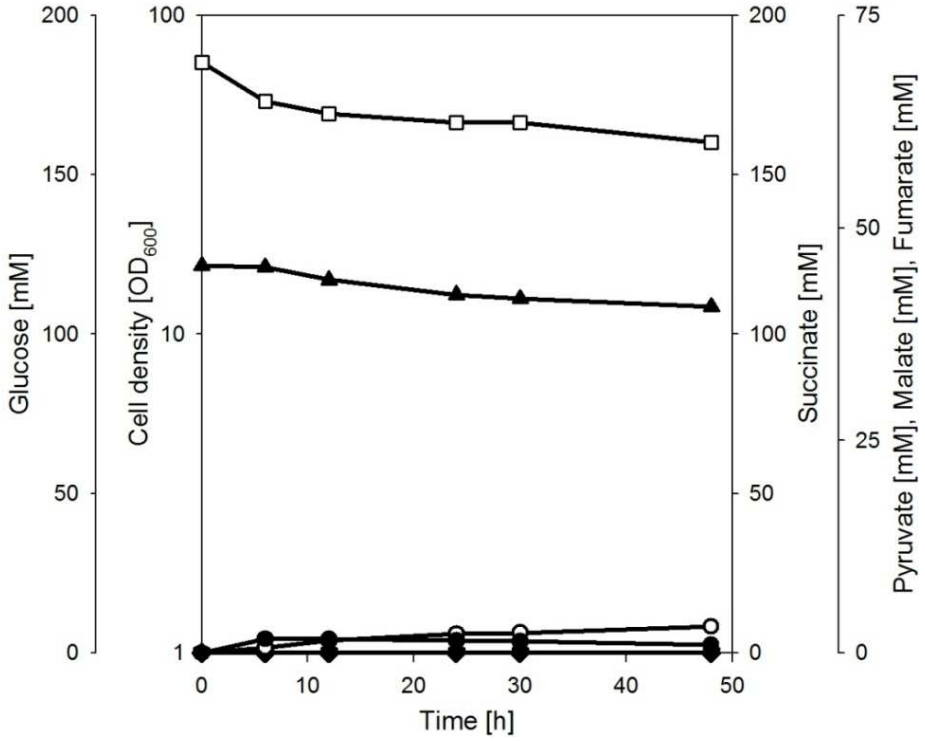

Figure 12: Cell density, substrate consumption and product formation during a representative anaerobic batch experiment of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F in modified CGXII medium containing 3% (*w*/*v*) glucose and 200 mM $NaHCO_3$. ▲ cell density; □ glucose; ● pyruvate; ○ succinate; ▼ malate; ◆ fumarate. At least three independent fermentations were performed, all of them showing comparable results.

C. glutamicum ELB-P Δ*pgi* ZwfA243T GndS361F consumes glucose in presence of an external electron acceptor

The surprising effect that *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F hardly consumed glucose under the established succinate production conditions was further investigated. Since it was possible to grow aerobic precultures of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F in complex medium for the inoculum of the sealed bottles, it was already proven that the mutation is not lethal in liquid medium. The question occurred, why resting *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F cells are not able to survive in minimal medium or to consume glucose at all. It was postulated that glucose metabolism is arrested, when carbon flux is driven alone by the PPP under oxygen deprivation conditions, because the NAD(P)H/NAD(P)$^+$ ratio is too high. To demonstrate that *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F is able to use glucose as carbon source when NAD(P)H can be oxidised, two experimental setups were followed: In the first, the strain was inoculated to an OD_{600} of about 15 to a baffled Erlenmeyer flask, containing modified CGXII medium plus 3% (*w*/*v*) glucose, and was shaken (rotary shaker, 120 rpm) aerobically at 30 °C. Under these circumstances, glucose was consumed, and pyruvate was formed as major product (~160 mM; Figure 13A). In the second, the bacteria were incubated on a rotary shaker (120 rpm) at 30 °C to an OD_{600} of about 15 in a 100 ml screw cap bottle, containing modified CGXII medium plus 2% (*w*/*v*) glucose and 50 mM nitrate as external electron acceptor (Figure 13B). Under these conditions, glucose was also consumed and pyruvate (~13 mM), as well as succinate were formed (~12 mM with a $Y_{P/S}$ of 0.86 mol succinate per mol of glucose).

The results obtained with oxygen or nitrogen as external electron acceptor show that *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F just did not consume glucose, when the experiments were performed in absence of an external electron acceptor. In presence of oxygen or nitrate this effect was abolished, as probably the reduction equivalents were regenerated due to NADPH oxidation by NADPH dehydrogenase activity or due to a side activity of the NADH dehydrogenase (Matsushita et al. 2001; Molenaar et al. 2000). The results of these two experiments thereby verified the ability of *C. glutamicum* to metabolize glucose, using the PPP alone. Afterwards explanations had to be found, why the influence of the NAD(P)H/NAD(P)$^+$ ratio was that high from the beginning of the cultivation under oxygen deprivation conditions. One explanation might be that the NADPH, which is formed during the PPP cannot be regenerated in the anaerobic reactions for succinate production, leading to intracellular NADPH accumulation. Another explanation might be that by the strong NADPH entry of the PPP the GAPDH activity is inhibited. Such an observation was already described by Jojima et al. (2010) and Litsanov et al. (2012b) for NADH overflow. To test both possibilities, *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F was further modified and investigated as described in the following sections.

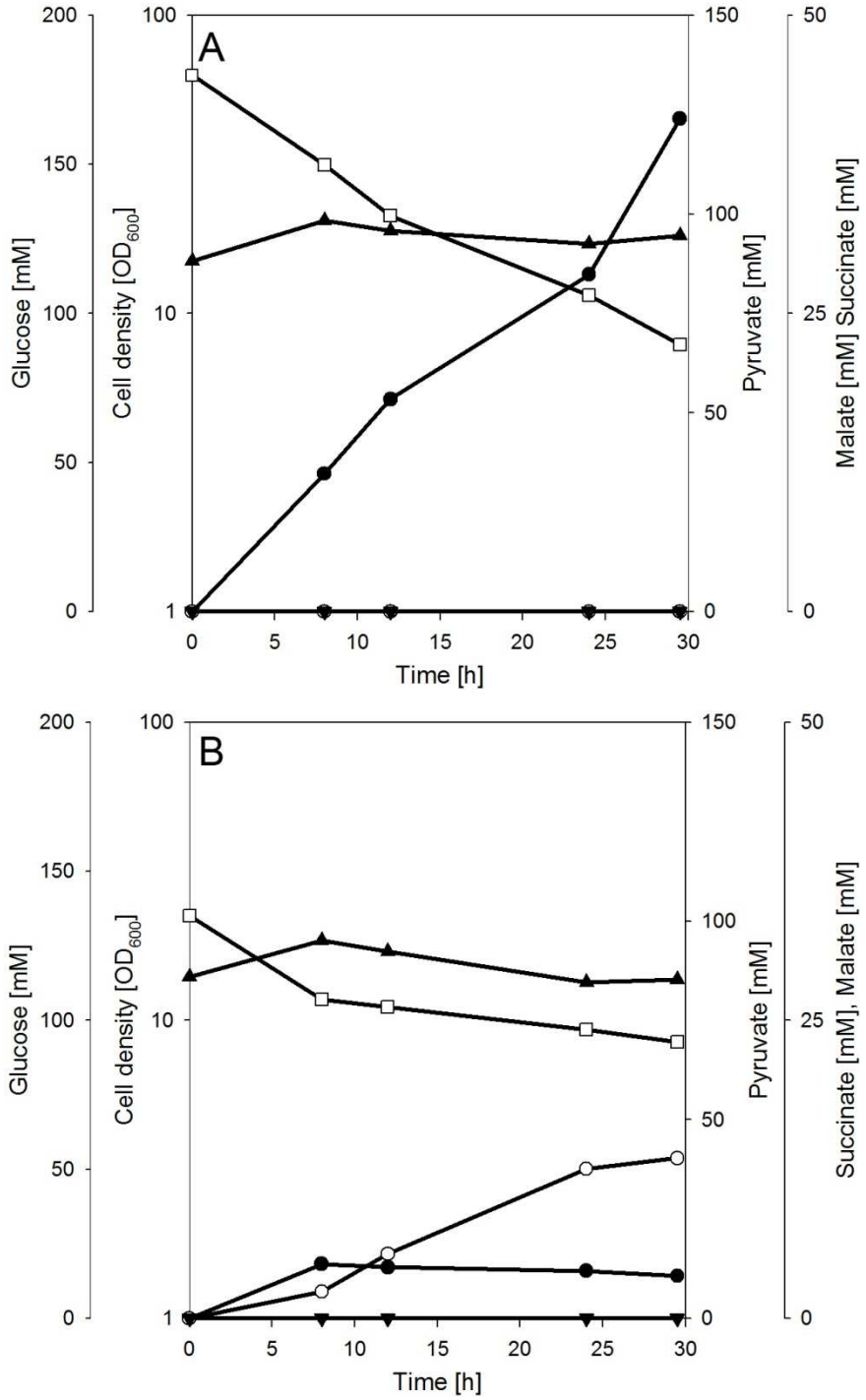

Figure 13: Cell density, substrate consumption and product formation during a representative batch experiment of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F in modified CGXII medium, containing 3% (*w*/*v*) glucose. The experiments were performed in (A) 500 ml baffled Erlenmeyer-flaks (aerobic) or in (B) 100 ml locked screw cap flasks, containing 50 mM nitrate. ▲ cell density; □ glucose; ● pyruvate; ○ succinate; ▼ malate. At least two independent fermentations were performed, showing comparable results.

Glucose consumption arrest of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F cannot be compensated by heterologous overproduction of a transhydrogenase from *E. coli*

Mdh and SDH catalyse the NADH-dependent reactions in the reductive branch of the citric acid cycle (Figure 2). However, it is known that at least the Mdh is capable to use also NADPH *in vitro* (Molenaar et al. 2000), leading to the assumption that the modification of the flux distribution between glycolysis and PPP should have no negative effect on succinate production. It was postulated that Mdh consumes the NADPH formed in the PPP, while SDH consumes the NADH formed during glycolysis, since NADPH consumption by SDH is not reported so far. But *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F was not able to consume glucose under oxygen deprivation (Figure 12). Hence, it was postulated that the Mdh might not be able to use NADPH as electron donor *in vivo*. To keep though glucose consumption active under oxygen deprivation conditions with *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F, conversion of NADPH to NADH should be realised within this mutant by heterologous overproduction of different transhydrogenases (TH). Figure 14 shows the theoretical production of 5 mol pyruvate from 3 mol of glucose, in presence of a TH and an external CO_2 source. This calculation implies a maximal theoretical $Y_{P/S}$ of 1.67 mol succinate per mol of glucose, when NADPH can be converted to NADH by a heterologous expressed TH, with an excess of still 1 mol of NADPH.

First, the membrane-bound ATP-dependent TH from *E. coli* PntAB (encoded by *pntAB*) was overproduced in *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F. For this purpose, the plasmid pBB1*pntAB* (pBB1, carrying the *pntAB* genes from *E. coli* under control of P_{tac}; Blombach et al. 2011), was transformed into *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F. Successful transformation of the plasmid was proven by selection of clones on chloramphenicol, re-isolation of the plasmid and subsequent PCR, using a primer pair, which amplified a fragment, including parts of the plasmid as well as of the *pntAB* operon.

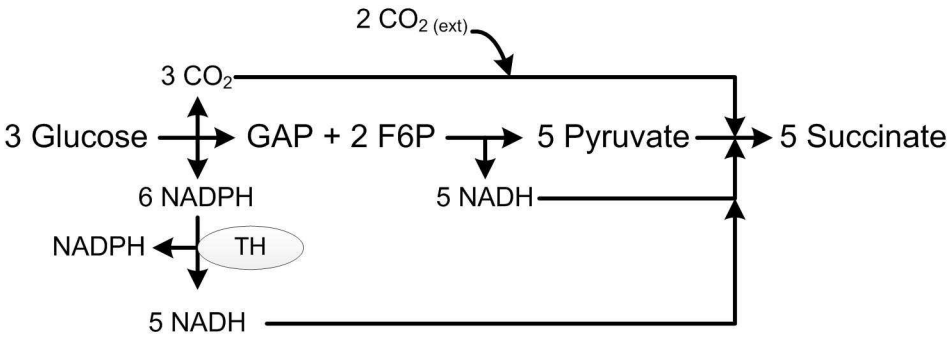

Figure 14: Schematic course of succinate formation, using the PPP for glucose metabolism and a heterologous overproduced NADPH converting transhydrogenase. Abbreviation: TH, transhydrogenase

However, introduction of the *pntAB* genes did neither improve glucose consumption, nor succinate production of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F (data not shown; Böhm, 2012). It could not be excluded whether this effect occurred because of the preference of PntAB for the reverse reaction: NADH + $NADP^+$ → NADPH + NAD^+ (Sauer et al. 2004), or because of the limitation of ATP under oxygen deprivation conditions. These restrictions should be circumvented by expressing the gene *udhA*, coding for the soluble TH UdhA from *E. coli*, in *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F, instead of those encoding PntAB. The UdhA catalyses the non-ATP-consuming reaction from NADPH plus NAD^+ to NADH plus $NADP^+$ (Sauer et al. 2004). For heterologous expression of *udhA*, the gene from *E coli* K12 was amplified via PCR and cloned behind the constitutive P_{tac} into the above described vector pBB1 (Krause et al. 2009). The resulting plasmid pBB1-*udhA* was checked by sequencing, amplified in *E. coli* DH5α, and after re-isolation transformed into *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F. Successful transformation of pBB1-*udhA* was proven by selection of clones on chloramphenicol, re-isolation of the plasmid and subsequent PCR, using a primer pair, which amplified a fragment, including parts of the plasmid as well as of the *udhA* gene. Activity of UdhA was tested after the method described by Kabus et al. (2007), revealing a low, but measureable specific UdhA activity of 0.019 ± 0.002 $U \cdot mg^{-1}_{(protein)}$ (one unit (U) corresponds to 1 $\mu mol \cdot min^{-1}$) in *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F pBB1-*udhA*. This new strain was then tested under the conditions established for the parental strains. However, also heterologous overexpression of *udhA* from *E. coli* did not result in an improvement of glucose consumption with the PGI negative strain background, leading to minimal accumulation of succinate or any other product (data not shown).

In summary, the THs UdhA and PntAB had no beneficial effect on glucose consumption or succinate production by *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F. Therefore, THs of *Bifidobacterium bifidum* S17, *B. longum* E18 and *B. breve* S18 should be additionally tested with *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F. The corresponding genes were amplified from genomic DNA of the different bacteria and cloned into pBB1 (Krause et al. 2009), as described for the construction of pBB1-*udhA*. However, all attempts to design one of these constructs ended with a mutated TH gene, for which reason this idea was not followed further.

Glucose consumption arrest of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F cannot be compensated by homologous overproduction of the glyceraldehyde-3-phosphate dehydrogenase

Since all tested THs could not abolish the impaired glucose consumption of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F, other bottlenecks were investigated. Another explanation for the inability of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F to consume glucose is

probably the strong NADPH entry of the PPP, which already at the beginning of the experiment inhibits GAPDH activity. Such an effect was already described by Jojima et al. (2010) and Litsanov et al. (2012b) for an overflow of NADH, and was successfully bypassed by GAPDH overproduction. Consequently, a potential inhibition of GAPDH by NADPH overflow should be compensated, overexpressing its gene *gapA*, like reported in the mentioned publications.

For homologous overexpression of *gapA* in *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F, the plasmid pAN6-*gap* (Kan^R, expressing *gapA* under control of P_{tac} and *lacI*; Litsanov et al. 2012b) was used. Successful transformation of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F was proven by re-isolation of the plasmid and subsequent PCR, using a primer pair, which amplified a fragment, including parts of the plasmid as well as of the *gapA* gene. Expression of *gapA* was induced with 0.5 mM IPTG. Although this approach had a beneficial effect on the aerobic growth during the preculture in complex medium (data not shown), *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F pAN6-*gap* showed neither enhanced glucose consumption, nor production of any product under oxygen deprivation conditions (data not shown).

Homologous overproduction of GAPDH could therefore not overcome the glucose consumption arrest of the PGI-negative mutant under oxygen deprivation conditions.

4. Production of malate and fumarate with *C. glutamicum*

C. glutamicum ELB-P should be further exploited for the production of the 1,4-dicarboxylic acids malate and fumarate. The strain was modified for this purpose and resulting mutants were analysed.

4.1 Engineering *C. glutamicum* for anaerobic fumarate production

For anaerobic fumarate production, the reductive branch of the TCA cycle in *C. glutamicum* ELB-P should be used. To prevent conversion of fumarate to succinate and consequently produce fumarate as end-product of anaerobic metabolism, the SDH was inactivated in *C. glutamicum* ELB-P.

By deletion of the corresponding genes *sdhA*, *sdhB* and *sdhCD* (Table 1) the reaction between succinate and fumarate (Figure 2) was interrupted, using the method described in chapter "1. Construction of *C. glutamicum* mutant strains". For that purpose, *C. glutamicum* ELB-P was transformed with the vector pK19*mobsacB*-Δ*sdhCAB* (pK19*mobsacB*, carrying a 1.1 kb overlap-extension PCR product, which covers the flanking regions of the *C. glutamicum sdhCAB* genes; Litsanov et al. 2012a). The successful deletion was proven by colony PCR. In a SDH-negative mutant the oxidative circuit of the TCA cycle is blocked. During growth under aerobic conditions on acetate, this interruption was expected to be bypassed by glyoxylate shunt activity. Production should take place during incubation under oxygen deprivation conditions, using the reductive branch of the citric acid cycle, as described for the succinate producers (see above).

C. glutamicum ELB-P Δ*sdhCAB* was cultivated in complex medium with 1% (*w*/*v*) acetate. Then, the bacteria were inoculated to an OD_{600} of about 15 to Müller-Krempel bottles, containing modified CGXII medium with 2.5% (*w*/*v*) glucose, and incubated on a rotary shaker (120 rpm) at 30 °C. As shown in Figure 15, less than 1 mM fumarate, about 20 mM pyruvate, 4 mM lactate and 8.5 mM malate were produced. Furthermore, still 60 mM succinate accumulated in the supernatants. Especially the latter finding was surprising, since without acetate in the medium, it should be impossible to form succinate within this strain background. Additionally, counting the products together and confronting them with the total glucose consumed, a loss of carbon was detected. More than 70 mM of glucose were converted into unknown by-products, as from about 825 mM of the initial carbon (in form of glucose) just about 350 mM could be found in the supernatants (in form of the named products) at the end of the experiment.

To test intracellular accumulation of metabolites, the whole cultures were spun down at the end of the experiments. After discarding the supernatants, the cells were lysed in H_2SO_4 and the suspension was filtrated (0.2 µm). The final solution was then directly investigated via

high performance liquid chromatography (HPLC) for significant concentrations of fumarate or other organic acids. However, no conspicuous accumulation of a metabolite could be obtained.

Taken together, the attempt to produce fumarate under oxygen deprivation with *C. glutamicum* by interrupting the TCA cycle in *C. glutamicum* ELB-P was not successful, as no enhancement in fumarate accumulation was observed in the experiments with *C. glutamicum* ELB-P Δ*sdhCAB*. The accumulation of succinate in the supernatants indicates the existence of a pathway bypassing the *sdhCAB* deletion.

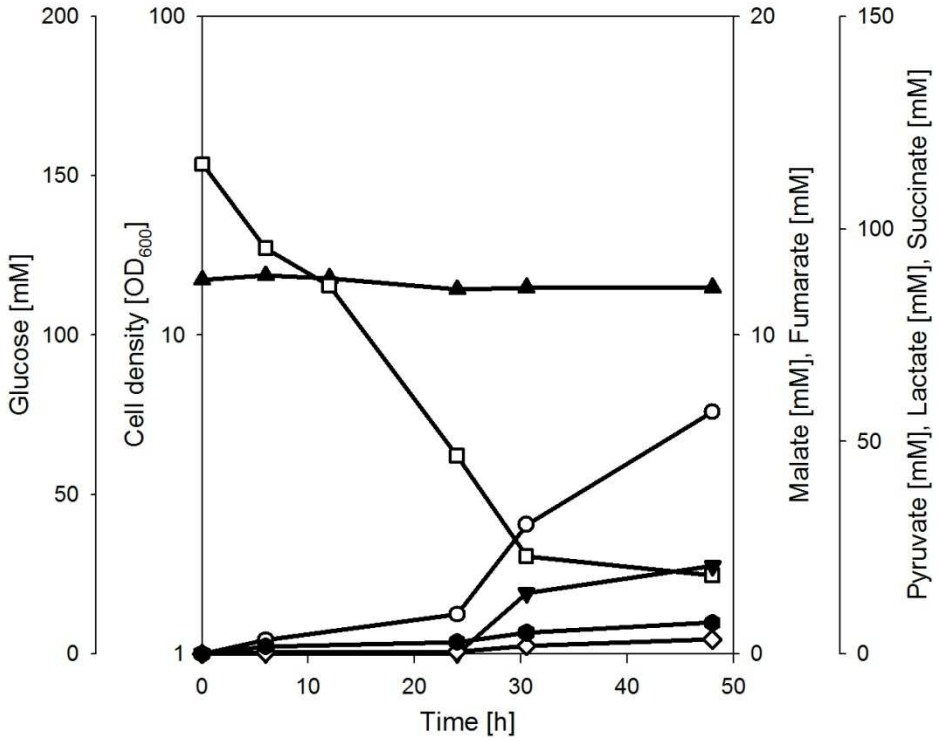

Figure 15: Cell density, substrate consumption and product formation during a representative anaerobic batch experiment of *C. glutamicum* ELB-P Δ*sdhCAB* in modified CGXII medium containing 2.5% (*w*/*v*) glucose. ▲ cell density; □ glucose; ● pyruvate; ○ succinate; ▼ malate; ◇ fumarate; ⬢ lactate. At least four independent fermentations were performed, all of them showing comparable results.

4.2 Engineering *C. glutamicum* for anaerobic malate production

Also for anaerobic malate production, the pyruvate-producing strain *C. glutamicum* ELB-P was used as basis. Malate is under oxygen deprivation conditions an intermediate of the reductive branch of the TCA cycle. To prevent conversion of malate to fumarate and consequently produce malate as end-product of anaerobic metabolism, the Fum (Figure 2, Table 1) should be inactivated in *C. glutamicum* ELB-P. Without Fum, the oxidative circuit of the TCA cycle is blocked, but as described above for *C. glutamicum* ELB-P Δ*sdhCAB*, growth under aerobic conditions on acetate is possible, bypassing the interruption via the glyoxylate shunt.

The corresponding gene *fum* (Table 1) was deleted, using the method described in "1. Construction of *C. glutamicum* mutant strains". Therefore, the suicide vector pK19*mobsacB*-Δ*fum* (pK19*mobsacB*, carrying a truncated *fum* gene; Marcus Persicke, Bielefeld University, 2010, unpublished) was transformed into *C. glutamicum* ELB-P. The successful deletion was proven by colony PCR. The resulting strain *C. glutamicum* ELB-P Δ*fum* was precultured in complex medium with 1% (*w*/*v*) acetate, but grew very slow (data not shown). The bacteria were inoculated to an OD_{600} of about 15 to Müller-Krempel bottles containing modified CGXII medium with 2% (*w*/*v*) glucose. Figure 16 shows that in contrast to experiments with the parental strain *C. glutamicum* ELB-P, impaired glucose uptake occurred (42.6 ± 0.76 mM in 48 h). In average just 7.5 ± 2.9 mM malate were produced, besides small amounts of pyruvate (8.6 ± 0.8 mM), lactate (4.0 ± 0.6 mM), and succinate (4.5 ± 2 mM). Fumarate levels were below the detection limit (experiments performed in collaboration with Stephanie Färber, 2011).

As surprisingly succinate accumulated in the supernatants, additionally a mutant devoid of Fum as well as SDH activity was developed. Since growth of the *C. glutamicum* ELB-P Δ*fum* mutant was very slow, the new strain was developed using the above described potential fumarate producer *C. glutamicum* ELB-P Δ*sdhCAB* as starting point. The *fum* gene was deleted in this strain as described above, using the suicide vector pK19*mobsacB*-Δ*fum*. The resulting mutant *C. glutamicum* ELB-P Δ*fum* Δ*sdhCAB* was inoculated to an OD_{600} of about 15 to Müller-Krempel bottles, containing modified CGXII medium with 2% (*w*/*v*) glucose, and incubated on a rotary shaker (120 rpm) at 30 °C. The results obtained with *C. glutamicum* ELB-P Δ*fum* Δ*sdhCAB* (data not shown) were similar to those shown for *C. glutamicum* ELB-P Δ*fum*.

In comparison to the parental strain (*C. glutamicum* ELB-P) both *C. glutamicum* ELB-P Δ*fum* and *C. glutamicum* ELB-P Δ*fum* Δ*sdhCAB* showed under the tested conditions a drastically lower succinate productivity of 0.1 $mmol \cdot g_{(CDW)}^{-1} \cdot h^{-1}$, in contrast to *C. glutamicum* ELB-P with 0.81 $mmol \cdot g_{(CDW)}^{-1} \cdot h^{-1}$, and a drastically lower succinate $Y_{P/S}$ of 0.1 mol succinate per mol of glucose, in contrast to *C. glutamicum* ELB-P with 0.78 mol succinate per mol of glucose (Table 7).

Table 7: $Y_{P/S}$ and productivities for succinate and malate produced by *C. glutamicum* ELB-P, *C. glutamicum* ELB-P Δ*fum* and *C. glutamicum* ELB-P Δ*fum* Δ*sdhCAB* during anaerobic incubation in Müller-Krempel bottles

***C. glutamicum* strain**	**product $Y_{P/S}$ [mol succinate /mol glucose]**	**production rate succinate [mmol·$g_{(DCW)}^{-1}$·h^{-1}]**	**product $Y_{P/S}$ [mol malate /mol glucose]**	**production rate malate [mmol·$g_{(DCW)}^{-1}$·h^{-1}]**
ELB-P	0.78 ± 0.08	0.81 ± 0.06	0.06 ± 0.00	0.04 ± 0.02
ELB-P Δ*fum*	0.12 ± 0.03	0.11 ± 0.01	0.25 ± 0.05	0.23 ± 0.02
ELB-P Δ*fum* Δ*sdhCAB*	0.10 ± 0.02	0.10 ± 0.02	0.23 ± 0.03	0.24 ± 0.05

It is noteworthy to mention that in the first 6 h of the fermentations indeed malate productivity increased for both mutants six-fold to about 0.24 from 0.04 mmol·$g_{(CDW)}^{-1}$·h^{-1}, while the malate $Y_{P/S}$ increased fourfold to about 0.24 from 0.06 mol malate per mol of glucose, when compared to the parental strain (Table 7; performed in collaboration with Stephanie Färber, 2011).

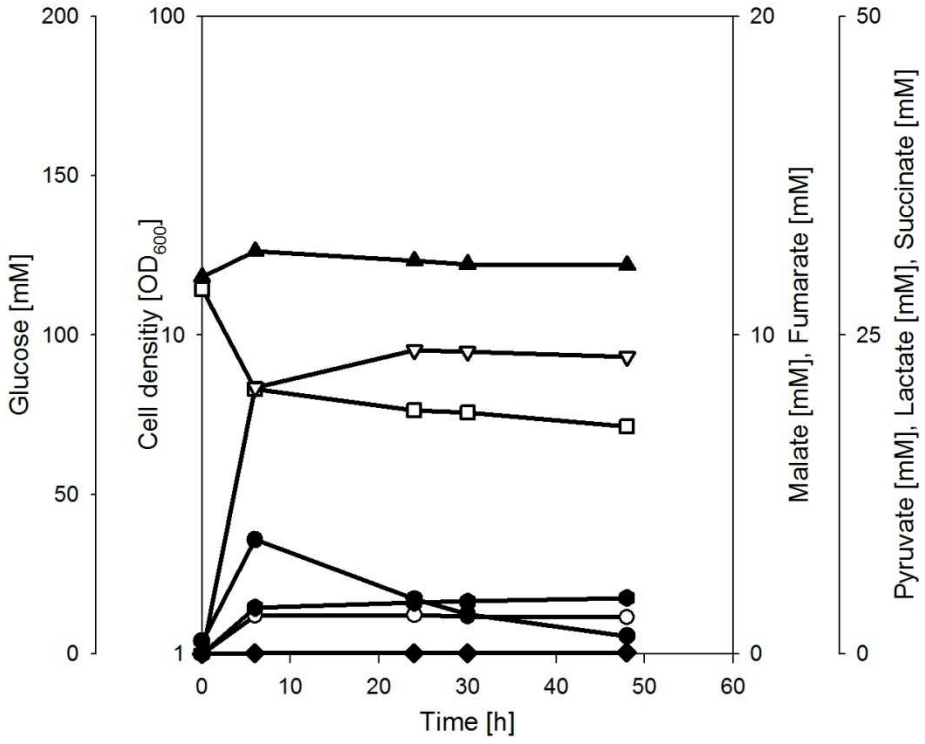

Figure 16: Cell density, substrate consumption and product formation during a representative anaerobic batch experiment of *C. glutamicum* ELB-P Δ*fum* in modified CGXII medium, containing 2% (*w*/*v*) glucose. ▲ cell density; □ glucose; ● pyruvate; ○ succinate; ▽ malate; ◆ fumarate; ⬢ lactate. At least three independent fermentations were performed, all of them showing comparable results.

As for fumarate production with *C. glutamicum* ELB-P mutants, also for malate production impaired glucose consumption seemed to be the major problem. In comparison to the parental strain *C. glutamicum* ELB-P, both *C. glutamicum* ELB-P Δ*fum* and *C. glutamicum* ELB-P Δ*fum* Δ*sdhCAB* have just one NADH-dependent reaction towards the desired product malate, catalysed by Mdh. Probably low Mdh activity led under the tested conditions to less efficient regeneration of NAD^+ and therefore, to a lowered glucose consumption after the first 6 h of incubation. To enhance the reductive flux, the plasmid pBB1-*cg2613*, overexpressing *mdh*, was transformed into these strains (plasmid and procedure are described in chapter "3 Tailoring *C. glutamicum* as a designer bug for the production of succinate"; p. 25ff). But no beneficial effect in any of the mentioned experimental setups occurred (data not shown).

C. glutamicum ELB-P Δ*fum* and *C. glutamicum* ELB-P Δ*fum* Δ*sdhCAB* showed a strong increase of the $Y_{P/S}$ for malate under oxygen deprivation conditions in comparison to the parental strain *C. glutamicum* ELB-P. This is the first report of targeted malate production with *C. glutamicum*. However, the final titre for malate was very low with less than 10 mM. Furthermore, the appearance of succinate in supernatants of all *fum* and *sdhCAB* single and double mutants constructed during this work indicates the existence of pathways bypassing these deletions and leading to succinate formation.

Investigation of an alternative succinate forming route in *C. glutamicum* ELB-P derivatives, which is mediated by aspartase

During the course of developing fumarate and malate producing *C. glutamicum* mutants, the reductive TCA cycle was interrupted at the reactions mediated by Fum, SDH or both Fum and SDH. All these strains were expected to be unable to produce an intermediate of the TCA cycle downstream of the interrupted reactions under oxygen deprivation conditions. However, *C. glutamicum* ELB-P Δ*sdhCAB*, *C. glutamicum* ELB-P Δ*fum*, as well as *C. glutamicum* ELB-P Δ*fum* Δ*sdhCAB* accumulated significant amounts of succinate in the supernatants. At least a pathway exists, which might bypass a deletion of *fum*, resulting in formation of succinate. This pathway leads from oxaloacetate to aspartate, which is then converted to fumarate, using aspartase (AspA) for deamination (Figure 2). This pathway might be an overflow valve for carbon flux, when the reductive TCA cycle is interrupted.

To verify this hypothesis, the *aspA* gene, encoding AspA (Table 1) should be deleted in *C. glutamicum* ELB-P Δ*fum*. After the method described in "1. Construction of *C. glutamicum* mutant strains", the suicide vector pK18*mobsacB*-Δ*aspA* (pK18*mobsacB*, carrying a truncated *aspA* gene; Christian Ziert, Bielefeld University, 2011, unpublished) should be transformed into *C. glutamicum* ELB-P Δ*fum*. However, growth of *C. glutamicum* ELB-P Δ*fum* was very weak. Therefore, the additional gene deletion in this strain was complicated, and only a mutant *C. glutamicum* ELB-P Δ*aspA* with active Fum could be developed. The successful deletion was proven by colony PCR.

It was investigated if there is already a reduction of succinate as product during fermentation under oxygen deprivation conditions with *C. glutamicum* ELB-P Δ*aspA* in comparison to the parental strain *C. glutamicum* ELB-P. The AspA bypass might also function in the mother strain as overflow valve for the reductive TCA cycle branch. During incubation at an initial OD_{600} of about 15 in 100 ml screw cap bottles, containing modified CGXII medium with 3% (*w*/*v*) glucose and 200 mM $NaHCO_3$, no difference was obtained concerning succinate or by-product formation, except a small increase in fumarate production to more than 2 mM (Figure 17). Up to now, the double mutant *C. glutamicum* ELB-P Δ*fum* Δ*aspA* could not be successfully constructed. Therefore, up to now, an impact of AspA on succinate production could not be verified.

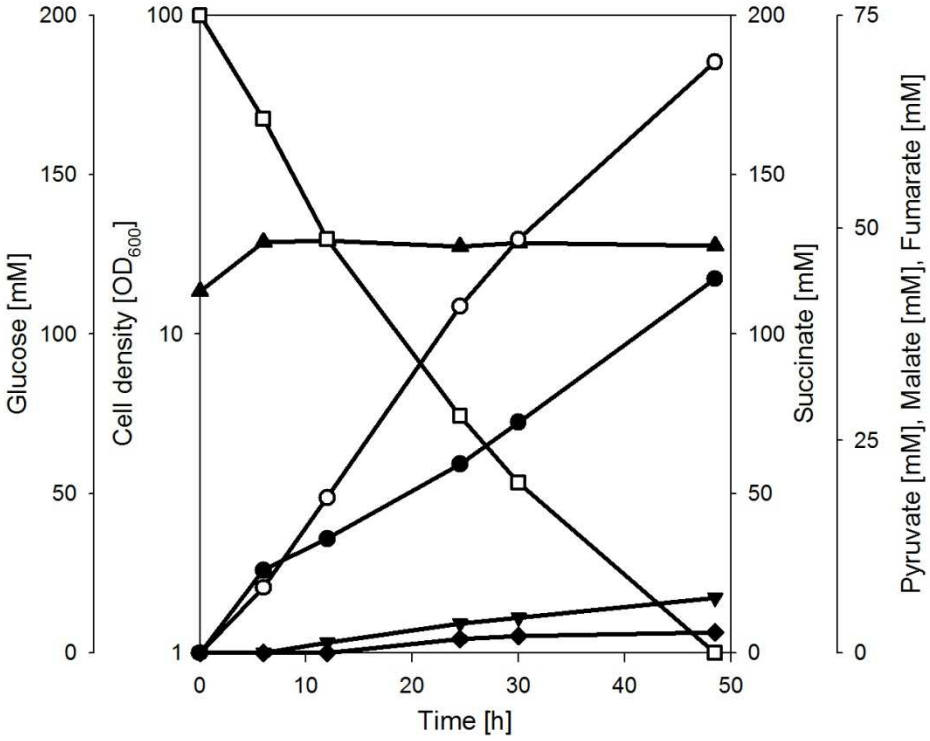

Figure 17: Cell density, substrate consumption and product formation during a representative anaerobic batch experiment of *C. glutamicum* ELB-P Δ*aspA* in modified CGXII medium, containing 3% (*w*/*v*) glucose and 200 mM $NaHCO_3$. ▲ cell density; □ glucose; ● pyruvate; ○ succinate; ▼ malate, ◆ fumarate. At least two independent fermentations were performed, all of them showing comparable results.

5. *C. glutamicum* tailored for efficient isobutanol production

In a further project of this work, *C. glutamicum* was tailored by metabolic engineering for efficient isobutanol production under oxygen deprivation conditions. This chapter describes briefly all efforts and achievements made during the construction and investigation of isobutanol-producing *C. glutamicum* mutants, which have been published in Blombach et al. (2011).

An important precursor for isobutanol production with *C. glutamicum* is KIV. Therefore, as mother strain a *C. glutamicum* mutant, tailored for KIV production from glucose (Krause et al. 2010), was used. Since KIV derives from two molecules of pyruvate this strain was PDHC-deficient. To avoid transamination of KIV the *ilvE* gene, encoding transaminase B (TA) was additionally deleted, leading to auxotrophy for branched chain amino acids, but also to accumulation of KIV. Overexpression of the *ilvBNCD* genes, encoding AHAS, AHAIR and DHAD (Figure 2, Figure 18), respectively, further shifted the product spectrum towards KIV. Although the PQO has been found to be dispensable for growth, and a deletion was just slightly beneficial on L-valine production (Schreiner et al. 2006, Blombach et al. 2008), its inactivation had a highly beneficial effect on KIV production (Krause et al. 2010).

This efficient KIV-producing strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* (pJC4*ilvBNCD*) (further denoted as *C. glutamicum* Iso1) was further metabolically engineered for isobutanol production by introduction of a biosynthetic "Ehrlich pathway" (Figure 18). Since the formation of 1 mol of isobutanol from 1 mol of glucose requires 1 mol of NADH and 1 mol of NADPH (Figure 18), isobutanol fermentations were performed under oxygen deprivation conditions to increase NADH availability. Incubation of *C. glutamicum* Iso1 to an OD_{600} of about 15 in Müller-Krempel bottles containing modified CGXII medium with 2% (*w*/*v*) glucose resulted in significant production of L-lactate (~122 mM) and succinate (~29 mM). No isobutanol was detected, showing that *C. glutamicum* naturally is not able to produce isobutanol (Blombach et al. 2011). Therefore, a synthetic pathway was implemented by expressing *kivd*, encoding the 2-ketoacid decarboxylase (KIVD) from *Lactococcus lactis* and *adh2*, encoding the ADH from *S. cerevisiae* (Figure 18), resulting in *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* (pJC4*ilvBNCD*) (pBB1*kivd-adh2*) (further denoted as *C. glutamicum* Iso2). However, *C. glutamicum* Iso2 showed under oxygen deprivation conditions similar results to those obtained with *C. glutamicum* Iso1. Therefore, by deletion of *ldhA*, the LdhA was inactivated, preventing L-lactate formation as major product, resulting in *C. glutamicum* Iso3 (*C. glutamicum* Iso2 Δ*ldhA*). *C. glutamicum* Iso3 was incubated under the same conditions as the previous strains. The mutant consumed glucose within 48 h and produced succinate (~69 mM) and isobutanol (~26 mM), but no L-lactate. These results showed the functionality of the synthetic isobutanol pathway (Figure 18) in *C. glutamicum*, as well as the necessity to inactivate LdhA for isobutanol production under the tested conditions.

To get rid of the side-product succinate, *C. glutamicum* Iso3 was further optimized by interrupting the reductive branch of the TCA cycle. The *mdh* gene (Table 1), encoding Mdh was deleted, resulting in *C. glutamicum* Iso4 (*C. glutamicum* Iso3 Δ*mdh*). When the Mdh was inactivated to avoid succinate formation, a severe reduction of glucose consumption occurred during anaerobic experiments, probably due to an unbalanced redox state of the cells. Consequently, the *E. coli* genes *pntAB*, coding for the membrane-bound ATP-dependent TH of *E. coli*, were expressed in the mutant. It could be shown that *C. glutamicum* Iso5 (*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* (pJC4*ilvBNCD-pntAB*) (pBB1*kivd-adh2*)) in contrast to *C. glutamicum* Iso4 possessed TH activity (0.20 $U \cdot mg^{-1}_{(protein)}$) and regained the ability to metabolize glucose efficiently. Hence, *C. glutamicum* Iso5 formed 42 ± 1 mM isobutanol with a $Y_{P/S}$ of 0.6 ± 0.02 mol isobutanol per mol of glucose, and showed with about 10 mM 86% reduced production of succinate (Blombach et al. 2011).

As a next step, it was investigated whether *C. glutamicum* exhibits native isobutyraldehyde-dependent ADH activity and therefore produces isobutanol without expressing the *adh2* gene from *S. cerevisiae*. Thus, two new mutants were constructed: *C. glutamicum* Iso6 (*C. glutamicum* Iso3 without plasmid-borne *adh2* gene) and *C. glutamicum* Iso7 (*C. glutamicum* Iso5 with plasmid-borne overexpression of *adhA* from *C. glutamicum* instead of *adh2* from *S. cerevisiae*). *C. glutamicum* Iso6 formed the same amounts of isobutanol as *C. glutamicum* Iso3, indicating that not the ADH from *S. cerevisiae*, but the endogenous ADH of *C. glutamicum* is responsible for isobutanol production. *C. glutamicum* Iso7, showed in comparison to all other strains enhanced isobutanol production of 82 ± 1 mM with a $Y_{P/S}$ of 0.77 ± 0.01 mol isobutanol per mol of glucose (Blombach et al. 2011).

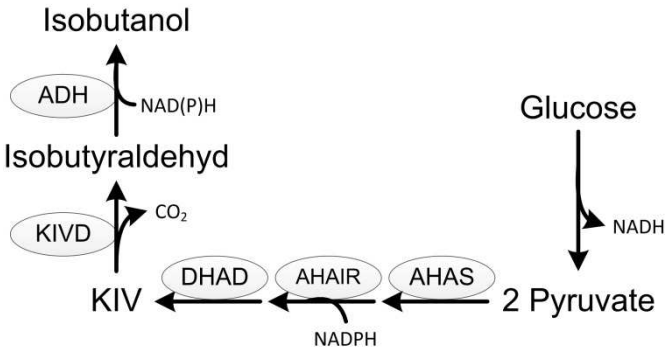

Figure 18: Biosynthetic "Ehrlich pathway" to form isobutanol from glucose. Abbreviations: ADH, alcohol dehydrogenase; AHAIR, acetohydroxyacid isomeroreductase; AHAS, acetohydroxyacid synthase; DHAD, dihydroxyacid dehydratase; KIV, 2-ketovaleric acid; KIVD, 2-ketoacid decarboxylase

The improvement of isobutanol production by *pntAB* expression (*C. glutamicum* Iso5 and *C. glutamicum* Iso7) indicated that NADPH availability is a critical factor for isobutanol production. *C. glutamicum* Iso3 produced isobutanol without PntAB, pointing to another reaction in *C. glutamicum* which converts NADH to NADPH. Sauer and Eikmanns (2005) sketched out a metabolic cycle, causing such a conversion by combining the reactions of PCx and/or PEPCx, Mdh and MalE. By deletion of the MalE gene *malE* in *C. glutamicum* Iso3, yielding *C. glutamicum* Iso8, it could be shown that in absence of MalE isobutanol production completely stopped. Deletion of *malE* in *C. glutamicum* Iso7 (resulting in *C. glutamicum* Iso9), led to a reduction of isobutanol by 50% (~24 mM) in Müller-Krempel bottle experiments. This shows the importance of MalE, even in presence of the TH from *E. coli*.

Finally, biphasic fed-batch fermentations were performed with *C. glutamicum* Iso7 in 300 ml cultures, with an aerobic growth phase, followed by an anaerobic production phase, which was established by stopping aeration and stirring at 300 rpm. In this experiment *C. glutamicum* Iso7 produced about 175 mM isobutanol at a volumetric productivity of 4.4 $mM \cdot h^{-1}$. The $Y_{P/S}$ was with 0.48 mol isobutanol per mol of glucose lower, in comparison to batch fermentations.

Altogether, *C. glut*amicum was successfully engineered for the production of isobutanol. It could be shown that native isobutyraldehyde-dependent ADH activity alone is responsible for isobutanol formation and that MalE plays a major role to supply the cell with NADPH under the conditions tested. The highest titre of 175 mM isobutanol was obtained with *C. glutamicum* Iso7 in fed-batch fermentations. However, with a 38% lower $Y_{P/S}$ when compared to batch fermentations.

C) DISCUSSION

C. glutamicum has been used in the past to produce a large variety of products. Besides classical formation of amino acids, the general need for chemical building blocks and fuel from biomass led to an expansion of the directed production of chemical compounds, which may serve as substitute or precursor for different commodity or specialty chemicals. White biotechnology is a promising field for future industrial production, with regard to the restriction of fossil resources (Lorenz and Zinke, 2005). The technical breakthroughs in the last decade on metagenomics, *in vitro* evolution, functional genomics, transcriptomics and metabolomics, as well as *in situ* process design opened the gate to countersteer a global social and economic crisis, by industrial onset of white biotechnology (Lorenz and Zinke, 2005). Being a well investigated model-organism for Gram-positive bacteria with a completely sequenced genome, *C. glutamicum* represents a promising workhorse for the production of desired compounds from biomass. The tools for engineering *C. glutamicum* are well-established and easy to handle. The present work deals *inter alia* with the exploitation of *C. glutamicum* for the production of the 1,4-dicarboxylic acids malate, fumarate and succinate. For that purpose, the central metabolite pyruvate was recognized as a precursor for the production of these acids. Therefore, the starting point of this work was the construction of a mutant strain, efficiently producing pyruvate with high yield and productivity. The stepwise development of the pyruvate producer *C. glutamicum* ELB-P is described and discussed in detail in Wieschalka et al. 2012a.

Deletion of the genes *aceE*, *pqo*, *ldhA*, and additional attenuation of the AHAS led step by step to an efficient aerobically pyruvate-producing *C. glutamicum* mutant. Additionally, during the stages of developing the mutant *C. glutamicum* ELB-P, *alaT* and *avtA* were sequentially deleted to decrease L-alanine as side-product. This resulted in a gradual reduction of growth, which could be bypassed by addition of 2 mM L-alanine at the beginning of each experiment (Figure 4). Though, *C. glutamicum* ELB-P produced significant amounts of L-alanine during production phase. It can be concluded that unspecific activity or an unknown transaminase compensates the lack of AlaT and AvtA. At the beginning of growth phase, the pyruvate-pool probably is not high enough to activate an unspecific transamination. Therefore, the whole growth process within this strain background might need a kick off to cross a certain alanine level. A self-balancing of this level is imaginable during growth, by release of D-alanine during cell wall synthesis. A conversion between the L- and D-form of alanine is anytime possible due to alanine racemase (Tauch et al. 2002).

The maximal $Y_{P/S}$ of 1.49 mol pyruvate per mol of glucose, obtained with *C. glutamicum* ELB-P in aerobic batch experiments, left room for optimization of this strain, as the theoretical $Y_{P/S}$ for pyruvate production can be calculated to be 2 mol per mol with a C_6 carbon as substrate. The need of a microaerobic phase during growth, accompanied by a decreased maximum $Y_{P/S}$ of 0.97 mol pyruvate per mol of glucose in fed-batch fermentations,

pointed to an impact of reductive TCA cycle pathways on pyruvate production. The Mdh is an important enzyme in *C. glutamicum*, exclusively responsible for the reduction of OAA to malate (Molenaar et al. 2000). It was shown previously that *mdh* transcription is 2.7-fold up-regulated under oxygen deprivation conditions (Inui et al. 2007). As also in shake flasks, at least at higher cell densities (OD_{600} of > 10), the cells face oxygen limitation (Zimmermann et al. 2006), an up-regulation of *mdh* transcription can be expected during batch fermentations of *C. glutamicum* ELB-P. Enhanced *mdh* transcription might enable flux towards the reductive branch of the TCA cycle, also during the aerobic production phase of *C. glutamicum* ELB-P. This leads in combination with a high availability of pyruvate possibly to a drain of carbon towards TCA cycle and aspartate-pathway intermediates. To avoid such a reductive flux, the *mdh* gene was deleted. However, *C. glutamicum* ELB-P Δ*mdh* showed reduced pyruvate production in comparison to the parental strain, and instead, formation of about 10 mM glutamate (Figure 5). These results suggest that an active Mdh seems to be beneficial for pyruvate production. It is possible that the reaction from OAA to malate represents some kind of excess valve for the fast pyruvate production and concomitant formation of NADH during glycolysis. Malate can afterwards be reconverted to pyruvate, raising the final pyruvate titre. Throughout the reactions back to pyruvate, either electrons are transferred to the quinone-pool by MQO, or to $NADP^+$ by MalE. The latter case would explain the appearance of the amino-donor glutamate in the supernatants when *mdh* is deleted, as stopping the NADPH supply may inhibit transamination reactions with glutamate as amino-donor.

With the characteristics described here and in Wieschalka et al. (2012a), *C. glutamicum* ELB-P not only is a useful platform for pyruvate production, but can be exploited for the production of pyruvate-derived products. The next topic in this project was the development of conditions enabling succinate production with this strain. The first attempt was the plasmid-borne overproduction of PCx and PEPCx to enhance the flux from pyruvate to OAA under aerobic conditions. This idea was not successful as under aerobic conditions *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc* still accumulated pyruvate and did not produce succinate at all. This effect can be explained by the energetic advantage of the respiratory chain. The cells transfer electrons from reduction equivalents gained by conversion of glucose to pyruvate to the electron carriers in the membrane. Thus, proton motif force is generated, leading to ATP formation. In contrast, the reactions in the reductive branch of the TCA cycle just restore redox-balance.

Consequently, the cells were pressured to restore their redox-balance by reductive TCA cycle activity. In sealed bottles the bacteria were incubated in absence of oxygen as external electron acceptor. The accumulation of lactate, succinate and acetate, when oxygen becomes limiting, was first shown by Dominguez et al. in 1993. Later, Inui et al. (2004a) used this fact for the production of organic acids with *C. glutamicum* WT under oxygen deprivation

conditions. In the following decade, different setups and strain modifications were reported, showing positive effects on succinate production. The major targets were the enhancement of the succinate production ratio in comparison to the other products, which can be achieved by addition of bicarbonate (Okino et al. 2005) and blocking of side-product forming reactions. The latter was achieved by deletion of the *ldhA* gene, stopping the production of L-lactate under oxygen deprivation conditions and leading to the formation of 1.24 M succinate with a $Y_{P/S}$ of 1.4 mol succinate per mol of glucose (Okino et al. 2008a). However, up to 0.3 M acetate accumulated as side-product (Okino et al. 2008a). More recently, Litsanov et al. (2012b) could produce succinate with lower by-product concentrations, additionally decreasing acetate formation by deletion of *cat*, *pqo*, and the *pta-ack* operon.

In the present work, L-lactate formation was already blocked by deletion of the *ldhA* gene in *C. glutamicum* ELB-P. The strain therefore was used directly for succinate production under oxygen deprivation conditions (Figure 6). In 125 ml Müller-Krempel bottles, a concentration of up to 100 mM succinate from 2% (*w*/*v*) glucose was obtained ($Y_{P/S}$ 0.78 mol succinate per mol of glucose). Besides, small amounts of the TCA cycle intermediates fumarate (~1 mM) and malate (~7 mM) were produced. Major side-product was pyruvate (~20 mM), leading to the assumption that there is a limitation in the reaction steps below the level of pyruvate. Noteworthy to mention is the fact that in comparison to all other published strains, which were metabolically engineered for the production of succinate, no acetate occurred as by-product with *C. glutamicum* ELB-P. This can be explained by the deletion of the *pqo* gene and especially by the inactivity of the PDHC, preventing the formation of acetyl-CoA as precursor for all other known acetate-forming pathways in *C. glutamicum*. Lower acetate formation under oxygen deprivation conditions by deleting the *aceE* gene was also reported previously by Yasuda et al. (2007). Although the $Y_{P/S}$ obtained with *C. glutamicum* ELB-P and *C. glutamicum* ELB-P pAN6*pyc*^{P458S}*ppc* was not very high under oxygen deprivation conditions, a limitation in succinate export was excluded since overproduction of the succinate exporter SucE (Huhn et al. 2011) had no effect on succinate production. Though, recent *in vivo* NMR studies showed that succinate accumulates in *C. glutamicum* cells after reaching a certain extracellular level (Radoš, ITQB Oeiras; unpublished data).

Also the influence of bicarbonate on anaerobic succinate production with *C. glutamicum* ELB-P was investigated. Inui et al. (2004a) stated the positive influence of bicarbonate on succinic acid production. The same group afterwards demonstrated that the predominant bicarbonate-dependent enzyme in *C. glutamicum* is under oxygen deprivation conditions the PEPCx (Inui et al. 2004b). Enhanced activity of PEPCx and consequent positive influence on succinate production by high CO_2 concentrations has also been reported for *A. succiniproducens* (Samuelov et al. 1991). Figure 7 shows for the experimental setup used with *C. glutamicum* ELB-P that addition of bicarbonate not only has an influence on the $Y_{P/S}$

of succinate, which increased from 0.78 to 0.91 mol succinate per mol of glucose (Table 3, Table 4), but also negated a limitation of the bicarbonate pool for the carboxylation step towards OAA, leading to complete consumption of 3% (*w*/*v*) glucose (Figure 7B). The effect of bicarbonate on the $Y_{P/S}$ disappeared by introduction of pAN6*pyc*^{P458S}*ppc* into the mutant, probably due to enhanced carboxylation activity (Table 3, Table 4). Although productivity slightly decreased by addition of $NaHCO_3$ in all cases (Table 3, Table 4), the effect to keep glucose consumption and succinate production stable during feed of higher substrate (glucose) amounts, showed the crucial role of bicarbonate availability for the carboxylation step towards OAA.

In connection with CO_2 supply to the medium, the origin of bicarbonate in the medium was investigated, which enabled *C. glutamicum* ELB-P to convert 2% (*w*/*v*) of glucose to succinate in absence of an additional CO_2 source. It turned out that urease, which is able to degrade urea to ammonia and CO_2 (Siewe et al. 1998) seemed to be responsible for CO_2 availability in modified CGXII medium (Figure 8). Experiments with higher urea concentrations showed the complete consumption of glucose even with initial concentrations above 2% (*w*/*v*) glucose, leading to higher final succinate titres. In experiments with lower concentrations or no urea in the medium glucose consumption decreased, resulting in lowered succinate accumulation (Figure 8).

For further improvement of the succinate production conditions, the influence of volume and composition of the gas phase in the headspace of the sealed bottles was investigated. It was presumed that the cultures became rapidly anaerobic, consuming the oxygen in the headspace (Blombach et al. 2011). However, the short aerobic phase led to less efficient succinate yields in all different bottle types tested, since exchanging the air gas phase in the headspace by nitrogen in 125 ml Müller-Krempel as well as in 100 ml screw-cap bottles had a beneficial effect (Table 5). The highest $Y_{P/S}$ was obtained by incubation of *C. glutamicum* ELB-P in 100 ml screw cap bottles, replacing the air in the headspace by nitrogen in presence of $NaHCO_3$ (Table 5). The advanced effect of $NaHCO_3$ on the $Y_{P/S}$ obtained in the 100 ml bottles can be explained by a lower eruption of CO_2 into the gas phase. In the smaller bottles the pressure is higher and increases the concentration of dissolved CO_2. A higher concentration of dissolved CO_2 in the medium supports diffusion of CO_2 into the cells. Therefore, these conditions were used for all further experiments. In presence of $NaHCO_3$, the positive effect of pAN6*pyc*^{P458S}*ppc* was surprisingly low (Table 3, Table 4). This is in contrast to other succinate producing *C. glutamicum* strains, in which additional expression of *pyc* showed a beneficial effect on succinate production (Inui et al. 2004b; Litsanov et al. 2012b), and in contrast to the postulated importance of PEPCx under oxygen deprivation (Inui et al. 2007). However, also the succinate producer *C. glutamicum* Δ*ldhA*-pCRA717 did not show an improvement in succinate production by overexpression of the *pyc* gene (Okino et al. 2008a).

Although, this effect could be a consequence of ATP-limitation during anaerobic experiments, the authors of this publication suggested that the rate-limiting step is the provision of bicarbonate for the carboxylase reaction (Okino et al. 2008a). CO_2 and not HCO_3^- crosses the membrane, meaning that the transport becomes rate-limiting in dependence on carbonic anhydrase activity, since CO_2 diffusion is decelerated when the intracellular CO_2 concentration is high (Gutknecht et al. 1977). For *E. coli* it was calculated that the demand for bicarbonate during growth is 10^3- to 10^4-fold greater than would be provided by uncatalyzed intracellular hydration, leading to the assumption that carbonic anhydrase activity is essential (Merlin et al. 2003). These suggestions are fortified for succinate production with *C. glutamicum* ELB-P, as the results without addition of $NaHCO_3$ show a higher productivity in comparison to those with $NaHCO_3$ supply (Table 3, Table 4). The enhanced productivity in absence of $NaHCO_3$ is probably due to the reaction catalysed by urease, which already forms carbonate ions as a product in aqueous solution, leading to easier accessible bicarbonate, without dependency on CO_2 diffusion. Of course the medium which is additionally supplied with $NaHCO_3$ contains also urea, but the intracellular urease reaction might be lowered by saturation of the medium with carbonate ions or by other, probably pH-dependent effects. Carbonic anhydrases are not well investigated in *C. glutamicum*. In 2004, Mitsuhashi et al. identified the genes *bca* and *gca* coding for putative *β*-type and *γ*-type carbonic anhydrases. It could be shown that the *bca* gene product was essential for growth under aerobic conditions, but the overexpression of the identified genes had no beneficial effect on L-lysine production, during which HCO_3^- availability is also a limiting step at the level of the PEP-pyruvate-OAA-node (Mitsuhashi et al. 2004).

It was decided to investigate the impact of a carboxylation-independent reaction on succinate production, by plasmid-borne overexpression of *mdh*. The aim was to increase the reductive flux by higher Mdh availability. Due to the expression on pBB1, transcriptional regulation of *mdh* was additionally bypassed. Such a regulation is stated for the native *mdh* gene, which is activated under oxygen deprivation conditions (Inui et al. 2007). The absence of an effect on succinate production under usage of the *mdh* overexpressing plasmid pBB1-*cg2613* eliminated the Mdh as bottleneck in the reductive TCA cycle branch.

Throughout the anaerobic experiments with *C. glutamicum* ELB-P, the carbon balance was not even, adding the measured products in the supernatants in comparison to the glucose consumed. HPLC analysis revealed that no considerable amounts of amino acids were produced (L-alanine and L-valine < 7 mM, L-isoleucine and L-lysine < 2 mM, all others below detection limit; Böhm, 2012). This fact led to the assumption that intracellular storage substances, such as glycogen, are formed. However, measurements of the intracellular glycogen-levels in *C. glutamicum* ELB-P, after incubation under the for succinate production established conditions, revealed very low values of 12-15 $mg \cdot g_{(CDW)}^{-1}$, which is just 10% of those obtained for *C. glutamicum* wildtype, grown under the same conditions (Philipp von

Zaluskowski, Institut für Mikrobiologie und Biotechnologie der Universität Ulm; unpublished data). Therefore, the lacking carbon (approximately 10-20%) went into unknown by-products.

To study the relevance for industrial applications, one-stage fed-batch experiments for succinate production with *C. glutamicum* ELB-P were established on the basis of the experiences made with this strain during pyruvate fed-batch fermentations. *C. glutamicum* ELB-P showed low glucose consumption and minor succinate production when growth was performed completely under oxygen surplus (30% DO). When the cells could enter a self-induced microaerobic phase after the second batch of acetate (Figure 9B), they started in the following anaerobic production phase to consume glucose efficiently and they produced up to 350 mM succinate with a $Y_{P/S}$ of 1.02 mol succinate per mol of glucose (Figure 9A, Table 6). Although the productivity was slightly lower (0.58 $mmol \cdot g_{(CDW)}^{-1} \cdot h^{-1}$) in comparison to sealed bottle experiments (see Table 5), the $Y_{P/S}$ remained at a similar level in fed-batch fermentations. The beneficial effect of introducing such a microaerobic phase before entering the oxygen deprivation stage was shown recently for several production strains of *E. coli*. Martínez et al. (2010) reported of improvement of $Y_{P/S}$, titre and productivity for succinate production with *E. coli* SBS550MG (pHL413), introducing a microaerobic phase at the end of growth before switching to anaerobic conditions. This could be improved with the same strain by Zhu et al. (2011), using an experimental setup with non-controlled agitation and non-controlled pH during growth. Under these conditions, a self-induced microaerobic phase was established during growth, accompanied by self-regulated pH conditions, resulting in an about 3-fold higher succinate titre in comparison to the previous work. Wu et al. (2011) did not run a microaerobic step for succinate production at the end of growth, but a low-growth-rate adjusted setup with a special agitator recycling the used air from the headspace back into the medium, leading to significant succinate production with *E. coli* NZN111. Combining these reports with the findings of this work in conjunction with dual phase and tri-phasic fed-batch fermentations of strains producing pyruvate (Wieschalka et al. 2012a), succinate (see above) and isobutanol (Blombach et al. 2011), the results point to an important and complex impact of the physiological state of the cell on the respective production processes. Up to now, the knowledge of transcriptional, translational and enzymatic connections within the cells is not sufficient to understand the complex background of cell adaption to the different conditions. However, better understanding of the physiological reactions on changing conditions, such as transition from aerobic to oxygen deprivation conditions, will help to optimize process settings and may significantly improve different production systems used within this work.

Effective fermentation systems for the production of succinate had been established for sealed bottle batch cultures as well as for fed-batch fermentations in the 400 ml fermenter system. As a next step, the mutant strain should be further improved to compete with other *C. glutamicum* succinate producers (reviewed in Wieschalka et al. 2012b), such as the above

mentioned *ldhA* negative and *pyc* overexpressing metabolic engineered *C. glutamicum* R strain *C. glutamicum* Δ*ldhA*-pCRA717 (1,240 mM, 1.4 mol succinate per mol of glucose, 27 $mM \cdot h^{-1}$; Okino et al. 2008a). More recently, Litsanov et al. (2012b) engineered *C. glutamicum* ATCC 13032 for succinate production by further improving the attempts shown by Okino et al. (2008a). By deletion of the *ldhA* gene, chromosomal integration of pyc^{P458S} and deletion of the genes responsible for acetate synthesis (Δ*cat*, Δ*pqo*, Δ*pta-ack*), strain *C. glutamicum* BOL-2 was obtained, which produced up to 120 mM succinate with a $Y_{P/S}$ of 1.03 mol succinate per mol of glucose. As in the case of *C. glutamicum* ELB-P (see below), the relatively low $Y_{P/S}$ for succinate revealed an impact of reduction equivalent limitation. Therefore, by addition of formate to the medium and genomic integration of the *fdh* gene from *Mycobacterium vaccae*, coding for a NAD^+-coupled formate dehydrogenase, NADH and CO_2 availability were increased. The resulting strain *C. glutamicum* BOL-3 showed impaired glucose uptake. To increase the glycolytic flux, the GAPDH was additionally overproduced. The final strain, *C. glutamicum* BOL-3/pAN6-*gap*, produced 1,134 mM succinate with a $Y_{P/S}$ of 1.67 mol succinate per mol of glucose during fed-batch fermentations on a substrate mixture of glucose and formate, complemented with bicarbonate (Litsanov et al. 2012b). However, the need of formate as additional carbon source for production complicates medium composition in comparison to the succinate production conditions established for *C. glutamicum* ELB-P.

The same group reported of aerobic succinate production with *C. glutamicum* for the first time. Deletion of the genes coding for the SDH initiated aerobic succinate production via the oxidative branch of the TCA cycle, but especially due to glyoxylate shunt and concomitant isocitrate lyase activity. Additional shut-down of the pathways for acetate synthesis (Δ*cat*, Δ*pqo*, Δ*pta-ack*) led to the mutant *C. glutamicum* BL-1 (Litsanov et al. 2012a). Introduction of pAN6-pyc^{P458S}*ppc* into this mutant resulted under nitrogen-limited growth conditions in final succinate titre and $Y_{P/S}$ of 90 mM and 0.45 mol succinate per mol of glucose, respectively (Litsanov et al. 2012a). More recently, *C. glutamicum* BL-1 was used to produce succinate on glycerol as sole carbon source, by introduction of the glycerol utilizing genes *glpFKD* from *E. coli* (Litsanov et al. 2012c). The plasmid pVWEx1-*glpFKD* was previously shown to enable growth and amino-acid production of *C. glutamicum* on glycerol as sole carbon source (Rittmann et al. 2008). *C. glutamicum* BL-1 pVWEx1-*glpFKD* aerobically produced 79 mM succinate with a $Y_{P/S}$ of 0.21 mol per mol of glycerol (Litsanov et al. 2012c). However, all attempts to produce succinate aerobically resulted in lower yields, productivities and titres than reported for production under oxygen deprivation.

Also *E. coli* was used in the past for directed production of succinate; e.g. *E. coli* SBS550MG (pHL413) produced under oxygen deprivation 350 mM with a $Y_{P/S}$ of 1.6 mol succinate per mol of glucose and a productivity of 4 $mM \cdot h^{-1}$ (Martínez et al. 2010). The most efficient anaerobic succinate producing *E. coli* strain *E. coli* SBS550MG/pHL413 (Sánchez et

al. 2005) yielded 1.6 mol succinate per mol of glucose and produced with almost 350 mM succinate the same concentration of succinate as *C. glutamicum* ELB-P, however with formate and acetate as by-products.

Aside from *C. glutamicum* and *E. coli*, there are natural succinate producing bacteria, such as *A. succiniproducens* ATCC53488 (180 mM, 1.37 mol succinate per mol of glucose, 9 $mM \cdot h^{-1}$; Glassner and Datta, 1992), *Actinobacillus succinogenes* (897 mM, 1.26 mol succinate per mol of glucose, 12 $mM \cdot h^{-1}$; Guettler et al. 1996), and *M. succiniproducens* (444 mM, 1.16 mol succinate per mol of glucose, 15 $mM \cdot h^{-1}$; Lee et al. 2006). However, these bacteria all have in contrast to *C. glutamicum* ELB-P the disadvantage to need complex medium for growth and production, resulting in higher production costs. Furthermore, *C. glutamicum* ELB-P possesses not only potential for further improvement of succinate production from glucose as sole carbon source, but represents also a promising platform for the production of other pyruvate-derived products.

All mentioned natural and non-natural succinate producers have in common that their $Y_{P/S}$ is above 1 mol succinate per mol of glucose under oxygen deprivation conditions. This fact is not astonishing as, taking the known carbon metabolism pathways into consideration, also *C. glutamicum* ELB-P theoretically should be able to form more than 1 mol succinate (C_4) out of 1 mol glucose (C_6), due to additional carbon fixation (Figure 2). Rate-limiting was the supply of reduction equivalents, leading to a redox imbalance during succinate production from glucose under oxygen deprivation conditions (Figure 10). As a solution a concept was intended to provide more reduction equivalents for the reactions catalysed by Mdh and SDH. By redirection of the carbon flux through the PPP, additional reduction equivalents should be generated in the reactions catalysed by G6PDH and 6PGDH. In *C. glutamicum* ATCC 13032 WT, a carbon flux of 69% through the PPP was observed (Bartek et al. 2011). The flux was increased in PDHC-deficient mutants (*C. glutamicum* Δ*aceE*) to 78%, when grown on glucose and acetate as carbon sources (Bartek et al. 2011). For L-valine production optimized daughter strains of *C. glutamicum* Δ*aceE* showed an even more increased flux through the PPP, of up to 113% (Bartek et al. 2011). This effect was explained by the high NADPH demand for L-valine formation. However, preliminary *in vivo* studies with *C. glutamicum* ELB-P revealed a reduced impact of the PPP of approximately 5% for this mutant (Radoš, ITQB Oeiras; unpublished data). The differences, in comparison to the L-valine producers, are a highly optimized flux of the branched-chain amino acid pathway in the L-valine producers, enforcing the demand for NADPH, and the usage of oxygen deprivation conditions for succinate production with *C. glutamicum* ELB-P. The latter difference is in line with the findings of Dominguez et al. (1993), describing a greatly reduced flux through the PPP under oxygen limitation. Albeit, it is theoretically possible to enhance the PPP flux in *C. glutamicum* ELB-P by metabolic engineering, in order to achieve enhanced reductive TCA

cycle flux. Such interference offers a wider range of reduction equivalents, like shown for L-lysine producers (Ohnishi et al. 2005; Becker et al. 2007).

One target for further modifications was the G6PDH, which is inhibited in its activity by NADPH, GAP, ATP, Fructose-1,6-bisphosphate (F1,6P), erythrose-4-phosphate, ribulose-5-phosphate, and PEP (Moritz et al. 2000; Becker et al. 2007). An amino acid exchange of Ala243 → Thr in the G6PDH led to a higher affinity of the enzyme to its substrate $NADP^+$ and a stronger resistance to its inhibitors ATP and PEP (Becker et al. 2007), albeit not to higher G6P affinity and not to diminished inhibition by NADPH (Becker et al. 2007). The latter point caused probably the inefficiency of a sole G6PDH deregulation in *C. glutamicum* ELB-P. An amino acid exchange of Ser361 → Phe in the 6PGDH led to a diminished allosteric regulation and diminished competitive inhibition of the enzyme by NADPH, ATP, GAP and F1,6P (Ohnishi et al. 2005). The double chromosomal exchange of *zwf* and *gnd* with the genes encoding the deregulated derivatives of G6PDH and 6PGDH led just to a slight increase of succinate accumulation with *C. glutamicum* ELB-P (Figure 11). It is noteworthy that in all experiments with the double deregulation mutant, the $Y_{P/S}$ (average of 1.07 mol succinate per mol of glucose) and the productivity (average of 0.79 $mmol \cdot g_{(CDW)}^{-1} \cdot h^{-1}$) were increased in comparison to the parental strain (Table 5). However, with p-values of 0.28 and 0.3, respectively, the enhanced $Y_{P/S}$ and productivity could not be verified as significant. Nevertheless, the results suggest a stronger impact of 6PGDH deregulation on the redirection of the carbon flux into the PPP than the deregulation of G6PDH, reflecting the results shown for L-lysine producing strains of *C. glutamicum*. *C. glutamicum* AHP-3, containing the GndS361F derivative, displayed increased L-lysine production by approximately 15% (Ohnishi et al. 2005), while *C. glutamicum* ATCC 13032 $lysC^{fbr}$, containing the ZwfA243T derivative, showed no significant increase in L-lysine production (Becker et al. 2007). However, by overproducing ZwfA243T on a plasmid under control of the *sod* promoter, a 40% increased L-lysine production could be obtained (Becker et al. 2007). The same plasmid was recently tested for succinate production with *C. glutamicum* ELB-P, showing no effects (Böhm, 2012). The expression of the genes *zwf* and *gnd*, as well as of other PPP genes, is repressed by the two transcriptional regulators GntR1 and GntR2 (Frunzke et al. 2008). Gluconate and glucono-δ-lactone interfere with the binding of these regulators (Frunzke et al. 2008). Since these effectors are not present in the absence of gluconate as carbon source, a repression of the PPP by GntR1 and GntR2 is conceivable.

By deletion of the *pgi* gene the carbon flux was forced completely into the PPP. Such a metabolic modification has already been shown to increase the $Y_{P/S}$ for L-valine with *C. glutamicum*, decreasing pyruvate as by-product due to the enhanced availability of NADPH for the reactions of the branched-chain amino acid pathway (Bartek et al. 2010). The same effect was reported for product and by-product titres in L-lysine producers (Marx et al.

2003). In *E. coli* a *pgi* deletion leads to the use of the PPP for glucose metabolism (Hua et al. 2003). Interestingly, in such a mutant the amounts of PEPCx-derived OAA are decreased, while the usage of the glyoxylate shunt increases for the formation of sufficient amounts of OAA (Hua et al. 2003). The PGI negative mutant designed during this work, *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F, had a peculiar phenotype, as it formed clumps in liquid cultures, which could be unclenched by addition of glucose or fructose to the medium. Furthermore, *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F hardly consumed glucose in the established oxygen deprivation system, leading to the production of negligible amounts of succinate (Figure 12).

The surprising observation that *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F, although growing well in aerobic complex medium precultures, hardly consumed glucose under oxygen deprivation was investigated. It was hypothesized that glucose metabolism was lowered due to a redox imbalance within the cells under oxygen deprivation conditions. Performing batch experiments with *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F in presence of an external electron acceptor to an OD_{600} of about 15, either in baffled Erlenmeyer flasks (Figure 13A) or in sealed bottles supplemented with 50 mM nitrate (Figure 13B), should compensate such an imbalance. Actually, resting *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F cells were able to consume glucose as sole carbon source in presence of an external electron acceptor, forming pyruvate as major product (Figure 13). It was concluded that a surplus of NADPH, generated during PPP, probably blocks metabolism of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F under oxygen deprivation conditions, since glucose consumption in presence of an external electron acceptor indicates oxidation of reduction equivalents. Indeed, there are reports on NADPH dehydrogenase activity. Matsushita et al. described in 2001 NADPH dehydrogenase activity by an unknown NADH dehydrogenase II homolog at acidic pH. In contrast, side-effects of the type II NADH dehydrogenase were reported to be responsible for NADPH oxidation even under neutral pH (Molenaar et al. 2000). Such type II NADH dehydrogenases lack energy coupling sites, bear flavins but no iron-sulphur clusters (Yagi et al. 2001) and were shown to transfer electrons intensively from NADPH to oxygen instead of quinones in *C. glutamicum*, resulting in reactive oxygen species (Nantapong et al. 2005). Regardless which enzyme is in charge for NADPH oxidation, the lowered carbon consumption under oxygen deprivation conditions by *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F indicated a severe redox imbalance, either by the inability to regenerate $NADP^+$, or by regulatory effects due to a high $NAD(P)H/NAD(P)^+$ ratio.

The enzymes requiring reduction equivalents in the pathway of anaerobic succinate production are Mdh and SDH. The Mdh uses *in vitro* NADPH as well as NADH as cofactor (Molenaar et al. 2000; Genda et al. 2003), but is possibly not able to oxidise NADPH *in vivo*.

Deletion of *mdh* probably improved NADH availability and led to a severe redox imbalance in isobutanol producing *C. glutamicum* mutants under oxygen deprivation conditions (Blombach et al. 2011), disclosing the possibility to circumvent the impaired glucose consumption of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F by conversion of NADPH to NADH. Such a conversion can be carried out by heterologous overproduction of a TH (Figure 14). *E. coli* possesses two THs: the membrane-bound ATP-dependent PntAB and the soluble UdhA (Sauer et al. 2004). UdhA was shown to be essential for growth of a *pgi* null *E. coli* mutant, for which reason overexpression of *udhA* could significantly improve growth of such a mutant (Sauer et al. 2004; Canonaco et al. 2001). Furthermore, in *E. coli* Δ*pgi* mutants, *udhA* was up-regulated, while *pntAB* was down regulated after a 50-day adaptive evolution period, probably due to the higher need of NADH instead of NADPH (Charusanti et al. 2010). Plasmid-borne overproduction of UdhA in *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F indeed improved growth in aerobic cultures (data not shown), but had no beneficial effect on the situation under oxygen deprivation conditions, although low TH activity could be detected in *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F pBB1-*udhA*. Probably, the specific activity obtained was too low to enhance glucose consumption under the established succinate production conditions.

Another possibility, explaining the lowered carbon consumption of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F, was found to be inhibition of the GAPDH by the excess of reduction equivalents. *C. glutamicum* possesses two GAPDH isoforms: GapA and GapB (Omumasaba et al. 2004). GapA is indispensable for growth on glucose, while GapB has greater influence on growth under glyconeogenetic conditions (Eikmanns, 1992; Omumasaba et al. 2004). Recently, Litsanov et al. (2012b) reported of a carbon metabolism blockage with a metabolically engineered succinate producer of *C. glutamicum* (see above). In this publication the GAPDH activity was decreased by an excess of NADH. Such an inhibition by NADH has been proven by several groups (Dominguez et al. 1998; Omumasaba et al. 2004). Litsanov et al. (2012b) solved the problem successfully by overproduction of GapA, probably due to increased total availability this enzyme. Consequently, the plasmid was introduced to *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F, expecting a similar effect during NADPH excess. However, pAN6-*gap* had no beneficial effect on succinate production with *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F. Anyway, the impact of GapA overproduction under these conditions might not be of importance for the *pgi* negative mutant. It was shown in the past that *in vitro* relative GapA activity is not inhibited during NADPH excess, but is even enhanced to 122% for the substrate NAD^+ (Omumasaba et al. 2004). This implies for *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F pAN6-*gap* either another regulatory effect due to additional enhanced NADH formation by GAPDH, or that the bottle-neck, lowering carbon consumption, has to be found elsewhere.

Reflecting all the observations made under succinate producing conditions with the derivatives of *C. glutamicum* ELB-P and concerning current publications, there is still potential to improve succinate production by further strain development. Considering the studies of *pgi* negative *E. coli* mutants, in which PEPCx activity is decreased and glyoxylate shunt activity increased (Hua et al. 2003), interference at these points might be beneficial. Overproduction of PEPCx, PCx, or unlocking of the glyoxylate shunt by introduction of a leaky variant of the PDHC, is imaginable. For the latter, a promoter exchange construct for the *aceE* gene has already been constructed and tested (Brunnenkan, 2010). Furthermore, a leaky PDHC has the advantage to bypass acetate auxotrophy during growth. However, an active PDHC enables formation of acetate as anaerobic by-product. As a solution for this problem a deletion of the *pta-ack* operon is recommended, possibly by exchanging the operon with the *pyc*^{P458S} gene, like done by Litsanov et al. (2012b). A combination of different approaches, e.g. subsequent overproduction of UdhA and Mdh, also might enhance glucose consumption of *C. glutamicum* ELB-P Δ*pgi* ZwfA243T GndS361F under oxygen deprivation conditions.

Biotechnological fumarate production in relevant amounts is up to now just known for filamentous fungi and biotechnological engineered yeasts (Roa Engel et al. 2008; Xu et al. 2012). The production of fumarate with *C. glutamicum* ELB-P, using the reductive branch of the TCA cycle and disrupting the genes encoding SDH was not successful. In comparison to succinate production from glucose, the closed redox balance towards fumarate seemed to be a promising approach. However, fumarate was produced in very low amounts, while succinate accumulated as major product (Figure 15). These findings point to a potential toxicity of fumarate to the cell, probably due to the inability to actively export this metabolite. A similar observation was made with *E. coli*. The deletion of the genes coding for fumarate reductase led to the production of malate, but not fumarate (Zhang et al. 2011). The results obtained with *C. glutamicum* ELB-P Δ*sdhCAB* also point to an alternative reductive succinate producing pathway, since the deletion of *sdhCAB* could not block succinate formation completely. The existence of such a pathway is most likely, as a deletion of *mdh* was also not sufficient to completely block succinate production under oxygen deprivation conditions (Blombach et al. 2011).

Additionally, the approaches to produce malate with *C. glutamicum* by deletion of either *fum* or both *fum* and *sdhCAB* in *C. glutamicum* ELB-P were accompanied by succinate production (Figure 16). One alternative succinate producing pathway, at least in *mdh* and *fum* negative mutants, is a route from OAA to aspartate followed by deamination via AspA. Up to now, just a deletion of *aspA* in the parental strain *C. glutamicum* ELB-P was investigated, leading to no difference in succinate production. To investigate this in detail, a mutant devoid of Fum, SDH and AspA should be investigated in future. A potential impact of the glyoxylate

shunt as source for succinate can literally be excluded as this pathway cannot be sustained in PDHC negative mutants on glucose as sole carbon source, due to the lack of acetyl-CoA. However, side-activity of the oxoglutarate dehydrogenase complex (ODHC) is conceivable, converting pyruvate to acetyl-CoA. The ODHC subunits most likely form a supercomplex with the subunits of the PDHC (Hoffelder et al. 2010) and may have the ability to bypass PDHC deficiency when the pressure on metabolism is high enough. In malate-producing *E. coli* mutants, an aspartase and glyoxylate shunt bypass, as well as activity of an unknown fumarate reductase or spontaneous dehydration of malate, could be experimentally excluded as reason for unexpected succinate formation (Zhang et al. 2011).

Regarding malate production obtained with *C. glutamicum* ELB-P Δ*fum* and the double mutant *C. glutamicum* ELB-P Δ*fum* Δ*sdhCAB*, the $Y_{P/S}$ and the productivity increased significantly in comparison to the parental strain (Table 7). However, glucose consumption decreased intensely after 6 h, leading to a final malate titre of only about 8 mM (Figure 16). The weak production phase was more or less comparable to that obtained during pyruvate and succinate fed-batch fermentations when growth was performed under oxygen surplus. This indicates that due to the weak growth of mutants lacking the *fum* gene, the bacteria do not undergo the low oxygen tension during growth in baffled Erlenmeyer flaks, which was postulated by Zimmermann et al. (2006), and do not up-regulate *mdh* transcription. Therefore, the reductive branch is most likely not activated properly. However, plasmid-borne overproduction of Mdh in these strains did not further improve malate production. The final titre obtained, is by far not enough to compete with other malate-producing microorganisms. Amongst these are natural malate producers to be found, e.g. *Aspergillus flavus* (843 mM malate with a $Y_{P/S}$ of 1.26 mol per mol of glucose and a volumetric productivity of 4.4 $mmol \cdot l^{-1} \cdot h^{-1}$; Battat et al. 1991), as well as biotechnological engineered *E. coli* strains, e.g. *E. coli* XZ658 (253 mM malate with a $Y_{P/S}$ of 1.42 mol per mol of glucose and a volumetric productivity of 3.5 $mmol \cdot l^{-1} \cdot h^{-1}$; Zhang et al. 2011). Nevertheless, this was the first time and attempt to engineer *C. glutamicum* for the directed production of malate. Improvement might be possible by introducing a leaky variant of the PDHC as described for succinate production (see above). Such interference probably will enable malate accumulation from two directions, namely the reductive branch of the TCA cycle as well as the glyoxylate shunt, though also increasing the by-product succinate. Furthermore, combining metabolic evolution with classical strain design appeared to be a promising tool for the enhanced production of malate, as described for *E. coli* (Jantama et al. 2008). Such an approach is also conferrable to *C. glutamicum*, starting with the already modified strains for malate production.

Besides production of chemical building blocks from biomass, also the microbial production of biofuels is of great economical and industrial interest. *C. glutamicum* was already exploited in the past to produce ethanol (Inui et al. 2004b). During this work, in addition to the

production of the 1,4-dicarboxylic acids, *C. glutamicum* was engineered to produce the higher alcohol isobutanol from glucose. In the last decade, it was shown for *E. coli*, *B. subtilis* and *C glutamicum* that the production of isobutanol with these bacteria needs the implementation of a biosynthetic "Ehrlich pathway" (reviewed in Blombach and Eikmanns, 2011). For that purpose the genes *kivd* and *adh2* encoding KIVD from *L. lactis* and ADH from *S. cerevisiae* had to be expressed in *C. glutamicum*, to complement the already existing pathway mediated by the reactions of AHAS, AHAIR and DHAD (Figure 18). As KIV is an important precursor of isobutanol, as a starting point the KIV producer *C. glutamicum* Iso1 (*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* (pJC4*ilvBNCD*); Krause et al. 2010) was used as a basis. The beneficial effect of AHAS, AHAIR and DHAD overproduction on isobutanol production was also shown by Atsumi et al. (2008) with *E. coli* and in parallel to the present work, by Smith et al. (2010) with *C. glutamicum*. To prevent L-lactate formation as predominant product under oxygen deprivation conditions (Inui et al. 2004a), additionally to *kivd* and *adh2* expression, the LdhA had to be inactivated, leading to a redirection of carbon flux towards KIV and isobutanol (Blombach et al. 2011). The resulting strain *C. glutamicum* Iso3 produced isobutanol (~26 mM) for the first time, but no L-lactate. Such a beneficial effect on isobutanol formation was observed before by Smith et al. (2010). However, succinate accumulated as by-product. The Mdh was inactivated to prevent succinate formation and to increase pyruvate as well as reduction equivalent availability. But the Mdh negative mutant *C. glutamicum* Iso4, showed a severe reduction of glucose consumption, probably due to a redox imbalance, unless the *pntAB* genes of *E. coli* were expressed within the strain (*C. glutamicum* Iso5). PntAB uses the electrochemical proton gradient across the membrane to reduce $NADP^+$ by NADH oxidation (Kabus et al. 2007), thus enhancing NADPH availability under oxygen deprivation conditions.

Investigation of the mutant *C. glutamicum* Iso6, which harboured all modifications made within *C. glutamicum* Iso3 but was devoid of the ADH of *S. cerevisiae* revealed that solely the native ADH of *C. glutamicum* was responsible for the reduction of isobutyraldehyde. Therefore, the mutant *C. glutamicum* Iso7 was generated, overexpressing *adhA* instead of *adh2* within the strain background of *C. glutamicum* Iso5. The mutant showed an increased $Y_{P/S}$ of 0.77 mol isobutanol per mol of glucose in line with observations of Smith et al. (2010), which revealed also improved isobutanol formation of *C. glutamicum* by overexpression of *adhA*. Atsumi et al. (2010) investigated the role of different ADHs on isobutanol production with *E. coli* and showed that also in *E. coli* the ADH of *S. cerevisiae* plays a minor role on converting isobutyraldehyde in comparison to the native YqhD of *E. coli*.

Finally, a production process was developed, combining biomass formation and isobutanol production in a single reactor (one-stage fermentation). *C. glutamicum* Iso7 grew aerobically and produced isobutanol in a growth-decoupled manner when aeration was

stopped. The application of oxygen deprivation conditions and low level stirring not only should enhance reduction equivalent availability, but also prevent a loss of isobutanol by gas stripping. The reduction of the $Y_{P/S}$ during fed-batch fermentation to 0.48 mol isobutanol per mol of glucose indicated again the severe impact of the physiological state of the cells during transition from aerobic to oxygen deprivation conditions, as already discussed for succinate production (see above). However, *C. glutamicum* Iso7, was competitive in comparison to other isobutanol producer strains, such as *E. coli* JCL260/pSA55/pSA69 ($Y_{P/S}$ of 0.86 mol per mol of glucose; Atsumi et al. 2008), *E. coli* 1993 (pGVferm6) ($Y_{P/S}$ of 1.03 mol per mol of glucose; Bastian et al. 2011), or *B. subtilis* UL03 ($Y_{P/S}$ of 0.2 mol per mol of glucose; Li et al. 2011).

Since *C. glutamicum* does not possess a chromosomally encoded TH (Kabus et al. 2007), a native TH-like route could be identified in *C. glutamicum*, which was sketched out already by Sauer and Eikmanns (2005) as an ATP-dependent metabolic cycle, consisting of pyruvate and/or PEP carboxylase, Mdh and MalE, causing a conversion of NADH and $NADP^+$ to NADPH and NAD^+. Inactivation of the MalE in *C. glutamicum* Iso3 abolished isobutanol production completely. Deletion of *malE* in *C. glutamicum* Iso7 was partially bypassed by PntAB activity, however leading to a reduction of isobutanol production by NADPH limitation, suggesting that such a TH-like route exists.

For future strain improvement, achievements made for L-valine production can be exploited. The “Ehrlich pathway” uses almost completely the same reactions. For example NADPH availability can be increased by enhanced PPP flux (Bartek et al. 2010). However, the results obtained for succinate production during this work, indicate that such interference might cause problems under oxygen deprivation conditions.

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E) ABBREVIATIONS

6PG:	6-phosphogluconate
6PGDH:	6PG dehydrogenase
AHAIR:	acetohydroxyacid isomeroreductase
AHAS:	acetohydroxyacid synthase
AK:	acetate kinase
ADH:	alcohol dehydrogenase
AlaT:	alanine aminotransferase
ALDH:	acetaldehyde dehydrogenase
AspA:	aspartase
ATP:	adenosine triphosphate
AvtA:	valine-pyruvate aminotransferase
BCAAs:	branched-chain amino acids
CDW:	cell dry weight
Cm^R:	chloramphenicol resistance cassette
CtfA:	CoA transferase
CS:	citrate synthase
Dld:	quinone-dependent D-lactate dehydrogenase
DHAD:	dihydroxyacid dehydratase
DO:	dissolved oxygen
DOE:	U.S. Department of Energy
e.g.:	exempli gratia (= for example)
et al.:	et alii (= and others)
F1,6P:	fructose-1,6-bisphosphate
F6P:	fructose-6-phosphate
FAD:	flavine adenine dinucleotide
Fum:	fumarase
GAP:	glyceraldehyde-3-phosphate
GAPDH:	GAP dehydrogenase
G6P:	glucose-6-phosphate
G6PDH:	G6P dehydrogenase
g:	gram
h:	hour
HPLC:	high performance liquid chromatography
i.e.:	id est (= that is)
ICD:	isocitrate dehydrogenase
ICL:	isocitrate lyase
IPTG:	Isopropyl-β-D-thiogalactopyranoside
Kan^R:	kanamycin resistance cassette
KMV:	2-keto-3-methylvalerate
l:	litre

LdhA: NAD^+-dependent L-lactate dehydrogenase
LldD: quinone-dependent L-lactate dehydrogenase
M: molar
M-Krempel: Müller-Krempel
MalE: malic enzyme
min: minute
mM: millimolar
mg: milligram
MS: malate synthase
MSC: multiple cloning site
MctC: monocarboxylic acid transporter
Mdh: malate dehydrogenase
MQ: menaquinone (oxidised)
MQH_2: menaquinone (reduced)
MQO: malate:quinone oxidoreductase
NAD^+: nicotinamide adenine dinucleotide (oxidised)
NADH: nicotinamide adenine dinucleotide (reduced)
$NADP^+$: nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH: nicotinamide adenine dinucleotide phosphate (reduced)
OAA: oxaloacetate
ODHC: oxoglutarate dehydrogenase complex
OD_{600}: optical density at a wavelength of 600 nm
ODx: oxaloacetate decarboxylase
P: phosphate
P_i: inorganic phosphate
PCx: pyruvate carboxylase
PDHC: pyruvate dehydrogenase complex
PEP: phosphoenolpyruvate
PEPCk: PEP carboxykinase
PEPCx: PEP carboxylase
PGI: phosphoglucoisomerase
PPP: pentose phosphate pathway
PQO: pyruvate:quinone oxidoreductase
PTA: phosphotransacetylase
P_{tac}: *tac* promoter
PTS: phosphotransferase system
Pyk: pyruvate kinase
Ribu5P: ribulose-5-phosphate
rpm: rounds per minute
SD: standard deviation
SDH: succinate dehydrogenase
SucE: succinate exporter

TA:	transaminase B
TCA:	tricarboxylic acid
TH:	transhydrogenase
T_{trp}:	tryptophane terminator
U:	unit
vvm:	gas volume flow per unit of liquid volume per minute
w/*v*:	weight per volume
WT:	wild-type
$Y_{P/S}$:	substrate specific product yield
µmol:	micromol

F) PUBLICATIONS

1. Publications within the framework of this dissertation

All publications which resulted out of this dissertation are attached in the following chapter.

1.1 Chronological list of publications

(1) Blombach B, Riester T, Wieschalka S, Ziert C, Youn JW, Wendisch VF, Eikmanns BJ (2011) *Corynebacterium glutamicum* tailored for efficient isobutanol production. Appl Environ Microbiol 77:3300-3310

(2) Wieschalka S, Blombach B, Eikmanns BJ (2012a) Engineering *Corynebacterium glutamicum* for the production of pyruvate. Appl Microbiol and Biotechnol 94:449-459

(3) Wieschalka S, Blombach B, Bott M, Eikmanns BJ (2012b) Bio-based production of organic acids with *Corynebacterium glutamicum*. Submitted to Microb Biotechnol (14/09/2012)

1.2 Personal contribution within the scientific publications

(1) My contribution to the publication "*Corynebacterium glutamicum* tailored for efficient isobutanol production" included the construction of the *ldhA*, *mdh* and *malE* negative mutants. Furthermore, I was involved in the establishment of the shake-flask experiments under oxygen deprivation conditions. During the terminal fermentations I was involved in process management, as well as analytics.

(2) My contribution to the publication "Engineering *Corynebacterium glutamicum* for the production of pyruvate" included the construction of all mutant strains created in this work and the execution of the corresponding growth experiments and analytics. Furthermore, I established the analytical procedures for measurement of organic acids via HPLC, and fed-batch fermentation processes, especially under low oxygen conditions, with the new mutant strains. In addition, I wrote the manuscript.

(3) My contribution to the publication "Bio-based production of organic acids with *Corynebacterium glutamicum*" included summarization and discussion of current achievements on the production of pyruvate, lactate, 2-ketoisovalerate and succinate with *C. glutamicum*, as well as describing the expansion of feedstocks for organic acid production with *C. glutamicum*.

2. Scientific attendance on conferences and symposia

2.1 Poster presentations

(1) Wieschalka S, Blombach B, Eikmanns BJ (2009) *Corynebacterium glutamicum* engineered as a designer bug for the production of pyruvate and its derivates. International Summer School on Advanced Techniques in Bacterial Genome Research, September 28th – October 3rd 2009, D-Bielefeld

(2) Wieschalka S, Blombach B, Eikmanns BJ (2011) *Corynebacterium glutamicum* ATCC 13032 engineered as a designer bug for the production of pyruvate. Jahrestagung VAAM, April 3rd – April 6th 2011, D-Karlsruhe

(3) Wieschalka S, Blombach B, Eikmanns BJ (2012) *Corynebacterium glutamicum* engineered as a designer-bug for the anaerobic production of succinate. Symposium on Bio-based Production of Organic Acids, May 10th – May 11th 2012, D-Frankfurt

2.2 Talks

(1) Wieschalka S, Blombach B, Eikmanns BJ (2009) Engineering *Corynebacterium glutamicum* as a designer bug for the production of organic acids derived from the citric acid cycle. 2nd ERA-IB symposium, BioProChemBB, October 19th 2009, D-Berlin

(2) Wieschalka S, Blombach B, Eikmanns BJ (2010a) Engineering *Corynebacterium glutamicum* as a designer bug for the production of organic acids derived from the citric acid cycle - progress. 3rd ERA-IB symposium, BioProChemBB, March 22nd 2010, PT-Lisbon

(3) Wieschalka S, Blombach B, Eikmanns BJ (2010b) Engineering *Corynebacterium glutamicum* as a designer bug for the production of organic acids derived from the citric acid cycle – progress II. 4th ERA-IB symposium, BioProChemBB, September 14th 2010, I-Rimini

(4) Wieschalka S, Blombach B, Eikmanns BJ (2011a) Engineering *Corynebacterium glutamicum* as a designer bug for the production of organic acids derived from the citric acid cycle –progress III. 5th ERA-IB symposium, BioProChemBB, May 6th 2011, ES-Leon

(5) Wieschalka S, Blombach B, Eikmanns BJ (2011b) Engineering *Corynebacterium glutamicum* as a designer bug for the production of organic acids derived from the citric acid cycle – progress IV. 6th ERA-IB symposium, BioProChemBB, December 2nd 2011, F-Paris

(6) Wieschalka S, Blombach B, Eikmanns BJ (2012) Engineering *Corynebacterium glutamicum* for pyruvate and succinate production. Final ERA-IB symposium, BioProChemBB, August 30th 2012, D-Frankfurt

G) APPENDIX

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Corynebacterium glutamicum Tailored for Efficient Isobutanol Production▽†

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We recently engineered *Corynebacterium glutamicum* for aerobic production of 2-ketoisovalerate by inactivation of the pyruvate dehydrogenase complex, pyruvate:quinone oxidoreductase, transaminase B, and additional overexpression of the *ilvBNCD* genes, encoding acetohydroxyacid synthase, acetohydroxyacid isomeroreductase, and dihydroxyacid dehydratase. Based on this strain, we engineered *C. glutamicum* for the production of isobutanol from glucose under oxygen deprivation conditions by inactivation of L-lactate and malate dehydrogenases, implementation of ketoacid decarboxylase from *Lactococcus lactis*, alcohol dehydrogenase 2 (ADH2) from *Saccharomyces cerevisiae*, and expression of the *pntAB* transhydrogenase genes from *Escherichia coli*. The resulting strain produced isobutanol with a substrate-specific yield ($Y_{P/S}$) of 0.60 ± 0.02 mol per mol of glucose. Interestingly, a chromosomally encoded alcohol dehydrogenase rather than the plasmid-encoded ADH2 from *S. cerevisiae* was involved in isobutanol formation with *C. glutamicum*, and overexpression of the corresponding *adhA* gene increased the $Y_{P/S}$ to 0.77 ± 0.01 mol of isobutanol per mol of glucose. Inactivation of the malic enzyme significantly reduced the $Y_{P/S}$, indicating that the metabolic cycle consisting of pyruvate and/or phosphoenolpyruvate carboxylase, malate dehydrogenase, and malic enzyme is responsible for the conversion of $NADH+H^+$ to $NADPH+H^+$. In fed-batch fermentations with an aerobic growth phase and an oxygen-depleted production phase, the most promising strain, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*), produced about 175 mM isobutanol, with a volumetric productivity of 4.4 mM h^{-1}, and showed an overall $Y_{P/S}$ of about 0.48 mol per mol of glucose in the production phase.

The shortage of oil resources and steadily rising oil prices result in the necessity to develop safe and efficient bioprocesses for the production of biofuels from renewable biomass. Great efforts have been made for the successful improvement of ethanol production. However, higher alcohols, like isobutanol, possess several advantages, such as a lower hygroscopicity, vapor pressure, and corrosivity, full compatibility with existing engines and pipelines, and a higher energy density, allowing safer handling and more efficient use than ethanol (15). Furthermore, isobutanol can serve as a precursor for the production of isobutene (34), which nowadays is exclusively produced in large scale by petroleum refining and is used as a gasoline additive and for the production of butyl rubber and specialty chemicals (20).

Corynebacterium glutamicum is a Gram-positive, facultatively anaerobic organism that grows on a variety of sugars and organic acids and is the workhorse for the production of a number of amino acids (32, 33, 36, 50). Recent studies also showed the successful employment of *C. glutamicum* for the production of putrescine and cadaverine (26, 27, 45) and of organic acids, ethanol, and xylitol under oxygen deprivation conditions (24, 38, 39, 42).

Under anaerobiosis, *C. glutamicum* ferments glucose via glycolysis. The major fermentation products are L-lactate, succinate, and small amounts of acetate (37). While L-lactate is formed from pyruvate by the $NADH+H^+$-dependent L-lactate dehydrogenase (LdhA; *ldhA* gene product), succinate is formed via the reductive branch of the tricarboxylic acid (TCA) cycle from either phosphoenolpyruvate (PEP) or pyruvate (37) (Fig. 1). The acetate formed under anaerobic conditions derives from acetyl coenzyme A (acetyl-CoA) (53). Deletion of the *aceE* gene, encoding the E1p subunit of the pyruvate dehydrogenase complex (PDHC), in *C. glutamicum* R almost completely abolished acetate formation, indicating a carbon flux over the PDHC and additional provision of $NADH+H^+$ under anaerobiosis (53) (Fig. 1).

Recently, we identified and functionally characterized the E1p subunit of the PDHC in *C. glutamicum* and showed that the activity of this complex is essential for growth of this organism on glucose, pyruvate, or L-lactate (46). A PDHC-deficient *C. glutamicum* strain required either acetate or ethanol as an additional carbon source for growth (9, 46). Further characterization of the PDHC-deficient *C. glutamicum* Δ*aceE* strain showed that the mutant, under aerobic conditions, forms significant amounts of L-valine, L-alanine, and pyruvate from glucose when acetate was exhausted from the medium and growth was stopped (6). Plasmid-bound overexpression of the *ilvBNCE* L-valine biosynthesis genes, encoding acetohydroxy-

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† Dedicated to our colleague, partner, and friend Jean-Louis Goergen, who unexpectedly died in December 2010.

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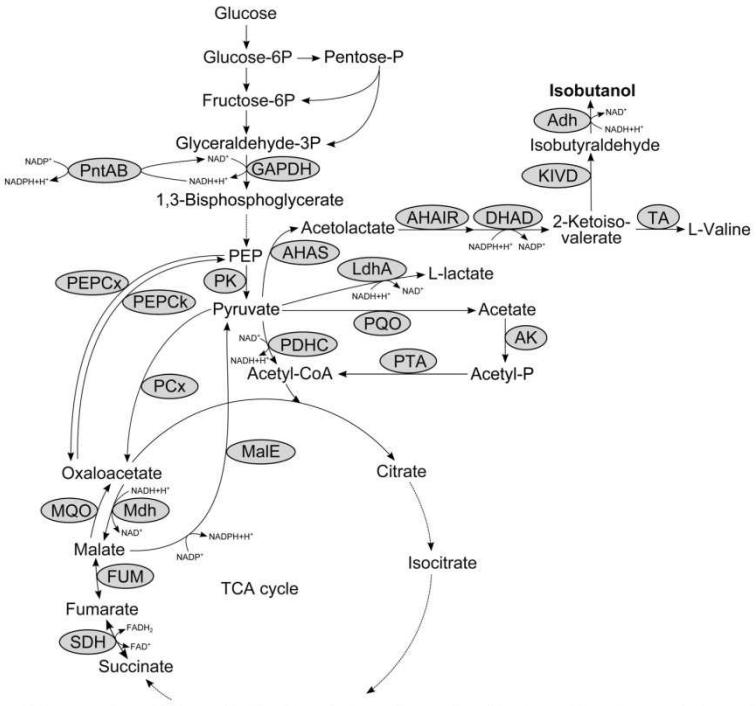

FIG. 1. Enzymes of the central metabolism with the biosynthetic pathway of L-valine in *C. glutamicum* and the synthetic pathway from ketoisovalerate to isobutanol. Abbreviations: Adh, alcohol dehydrogenase; AHAIR, acetohydroxyacid isomeroreductase; AHAS, acetohydroxyacid synthase; AK, acetate kinase; DHAD, dihydroxyacid dehydratase; FUM, fumarase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KIVD, 2-ketoacid decarboxylase from *L. lactis*; LdhA, L-lactate dehydrogenase; MalE, malic enzyme; Mdh, malate dehydrogenase; MQO, malate:quinone oxidoreductase; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; PEPCk, PEP carboxykinase; PEPCx, PEP carboxylase; PK, pyruvate kinase; PntAB, membrane bound transhydrogenase from *E. coli*; PTA, phosphotransacetylase; PQO, pyruvate:quinone oxidoreductase; SDH, succinate dehydrogenase; TA, transaminase B; TCA, tricarboxylic acid.

acid synthase (AHAS; *ilvBN* gene product), acetohydroxyacid isomeroreductase (AHAIR; *ilvC* gene product), and transaminase B (TA; *ilvE* gene product) (Fig. 1) shifted the product spectrum toward L-valine (6), and inactivation of pyruvate:quinone oxidoreductase (PQO; *pqo* gene product) (Fig. 1) and of phosphoglucose isomerase (PGI; *pgi* gene product) in *C. glutamicum* Δ*aceE*(pJC4*ilvBNCE*) resulted in even more efficient L-valine production, i.e., up to 410 mM, with a maximum yield of 0.86 mol per mol of glucose in the production phase (8). Based on these results, we engineered the wild type of *C. glutamicum* for the aerobic, growth-decoupled production of 2-ketoisovalerate (KIV) from glucose by deletion of the *aceE* and *ilvE* genes and additional overexpression of the *ilvBNCD* genes (the *ilvD* gene encodes dihydroxyacid dehydratase [DHAD]) (Fig. 1) (28). KIV production was further improved by deletion of the *pqo* gene. In fed-batch fermentations at high cell densities, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE*(pJC4*ilvBNCD*) produced up to 188 mM KIV and showed a volumetric productivity of about 4.6 mM KIV per h in the overall production phase (28). Since KIV is a precursor for isobutanol (Fig. 1), *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* (pJC4*ilvBNCD*) seems to be an ideal basis for the production of isobutanol.

Atsumi et al. (2) engineered *Escherichia coli* for the production of isobutanol from glucose under microaerobic conditions, by inactivation of competing pathways, overexpression of the *alsS* gene (encoding AHAS from *Bacillus subtilis*) and the *ilvCD* gene from *E. coli*, and implementation of a synthetic pathway, including a 2-ketoacid decarboxylase (KIVD; *kivd* gene product) from *Lactococcus lactis* and an alcohol dehydrogenase (ADH2; *adh2* gene product) from *Saccharomyces cerevisiae*. KIVD catalyzes the reaction from KIV to isobutyraldehyde, which is finally converted to isobutanol by the NADH-dependent ADH2 (Fig. 1). Further studies showed that this synthetic pathway can also be used for the production of other higher alcohols, e.g., isopropanol (23), 3-methyl-1-butanol (11, 12), 1-butanol and 1-propanol (3, 47), and 2-methyl-1-butanol (10). More recently, Smith et al. (49) engineered also *C. glutamicum* for the production of isobutanol, since they found that this organism possesses an increased tolerance against isobutanol toxicity compared to that of *E. coli*. However, the final titer and the yield of the best producing strain,

TABLE 1. Bacterial strains used in this study[a]

Strain	Relevant characteristic(s)	Source or reference
E. coli DH5α	F$^-$ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (rk$^-$, mk$^+$) *supE44 thi-1 gyrA96 relA1 phoA*	22
C. glutamicum WT	WT strain ATCC 13032, biotin auxotrophic	American Type Culture Collection
C. glutamicum Δ*aceE* Δ*pqo* Δ*ilvE*	*C. glutamicum* WT with deletion of *aceE*, *pqo*, and *ilvE* genes, encoding the E1p subunit of the pyruvate dehydrogenase complex, the pyruvate:quinone oxidoreductase, and transaminase B, respectively	28
C. glutamicum Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA*	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* with an additional deletion of the *ldhA* gene, encoding L-lactate dehydrogenase	This work
C. glutamicum Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* with an additional deletion of the *mdh* gene, encoding malate dehydrogenase	This work
C. glutamicum Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*malE*	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* with an additional deletion of the *malE* gene, encoding malic enzyme	This work
C. glutamicum Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* Δ*malE*	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* with an additional deletion of the *malE* gene, encoding malic enzyme	This work
C. glutamicum Iso1	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE*(pJC4*ilvBNCD*)	This work
C. glutamicum Iso2	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE*(pJC4*ilvBNCD*)(pBB1*kivd-adh2*)	This work
C. glutamicum Iso3	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA*(pJC4*ilvBNCD*) (pBB1*kivd-adh2*)	This work
C. glutamicum Iso4	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD*) (pBB1*kivd-adh2*)	This work
C. glutamicum Iso5	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*) (pBB1*kivd-adh2*)	This work
C. glutamicum Iso6	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA*(pJC4*ilvBNCD*)(pBB1*kivd*)	This work
C. glutamicum Iso7	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*)	This work
C. glutamicum Iso8	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*malE*(pJC4*ilvBNCD*)(pBB1*kivd-adh2*)	This work
C. glutamicum Iso9	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* Δ*malE*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*)	This work

[a] WT, wild type.

C. glutamicum Δ*pyc* Δ*ldhA*(pKS167), were suboptimal (66 mM isobutanol and 23% of the theoretical maximal yield) and certainly improvable.

Based on our results for KIV production (28) and also inspired by the results of Atsumi et al. (2), we used in this study a straightforward and iterative engineering approach for the efficient production of isobutanol with *C. glutamicum* under oxygen deprivation conditions. Thereby, we constructed an efficient isobutanol production strain and found strong indications for a significant contribution of the transhydrogenase-like metabolic cycle consisting of pyruvate carboxylase (PCx) and/or PEP carboxylase (PEPCx), malate dehydrogenase (Mdh), and malic enzyme (MalE) (Fig. 1) to the overall NADPH+H$^+$ supply, even in the presence of the transhydrogenase PntAB of *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides. All bacterial strains used and their relevant characteristics and sources are listed in Table 1. The plasmids and oligonucleotides (primers) used, their characteristics or sequences, and their sources or purpose are listed in Table 2.

DNA preparation and transformation. Isolation of plasmids from *E. coli* was performed as described previously (17). Plasmid DNA transfer into *C. glutamicum* was carried out by electroporation, and recombinant strains were selected on Luria-Bertani brain heart infusion (LB-BHI) agar plates containing 0.5 M sorbitol, 85 mM potassium acetate, and appropriate concentrations of antibiotics (kanamycin, 50 μg ml^{-1}; chloramphenicol, 6 μg ml^{-1}]) (51). Isolation of chromosomal DNA from *C. glutamicum* was performed as described previously (17). Electroporation of *E. coli* was carried out with competent cells according to the method described by Dower et al. (14).

Conditions for growth and isobutanol formation. *E. coli* was grown aerobically in 2× tryptone-yeast (TY) complex medium (41) at 37°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Precultures of the different *C. glutamicum* strains were grown in 2× TY medium containing 0.5% (wt/vol) potassium acetate. For isobutanol fermentations, cells of an overnight preculture were washed with 0.9% (wt/vol) NaCl and inoculated into CGXII minimal medium (pH 7.4) (16) with 2% (wt/vol) glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-leucine, and L-isoleucine (2 mM each), to give an optical density at 600 nm (OD_{600}) of about 15. The cells were cultivated for 4 h at 30°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. The cells were then washed with 0.9% (wt/vol) NaCl, inoculated into the same medium, and incubated at 30°C as 50-ml cultures in 125-ml Müller-Krempel (Müller+Krempel AG, Bülach, Switzerland) bottles on a rotary shaker at 120 rpm. Initially, the gas phase in these bottles was aerobic; however, the cultures became anaerobic by rapidly consuming the oxygen in the gas phase. Antibiotics were added appropriately (kanamycin, 25 μg ml^{-1}; chloramphenicol, 6 μg ml^{-1}). Samples were taken using a needle and syringe to inhibit the penetration of oxygen into the culture. The number of grams of cells (dry weight) was calculated from the OD_{600}, using a ratio of 0.3 g of cells (dry weight) liter^{-1} per OD_{600} (7).

Fed-batch fermentations were performed at 30°C in 300-ml cultures in a fed-batch Pro fermentation system from DASGIP (Jülich, Germany). The pH was maintained at 7.4 by online measurement using a standard pH electrode (Mettler Toledo, Giessen, Germany) and the addition of 4 M KOH and 4 M H_2SO_4. Foam development was prohibited by manual injection of about 20 μl of 1:5-diluted Struktol 674 antifoam (Schill und Seilacher, Hamburg, Germany). Dissolved oxygen was measured online using an oxygen electrode (Mettler Toledo, Giessen, Germany) and adjusted in the growth phase to 30% of saturation in a cascade by stirring at 300 to 1,000 rpm and aeration with 1 volume of air per volume of medium per minute (vvm). After complete consumption of acetate, aeration was completely switched off, and the stirring speed was reduced to 300 rpm. The fermentations were carried out in CGXII minimal medium (pH 7.4) (16) initially containing 4% (wt/vol) glucose, 1% (wt/vol) acetate, 0.5% (wt/vol) yeast extract, and L-valine, L-leucine, and L-isoleucine (2 mM each). Antibiotics

TABLE 2. Plasmids and oligonucleotides used in this study

Plasmid or oligonucleotide	Relevant characteristic(s) or sequence	Source, reference, or purpose
Plasmids		
pK19*mobsacB*	Km^r, mobilizable (carrying *oriT* gene), carrying *oriV* gene	44
pK19*mobsacB*-Δ*ldhA*	pK19*mobsacB* carrying a truncated *ldhA* gene	This work
pK19*mobsacB*-Δ*mdh*	pK19*mobsacB* carrying a truncated *mdh* gene	This work
pK19*mobsacB*-Δ*malE*	pK19*mobsacB* carrying a truncated *malE* gene	This work
pJC4*ilvBNCD*	Kan^r; plasmid carrying the *ilvBNCD* genes, encoding the L-valine biosynthetic enzymes acetohydroxyacid synthase, isomeroreductase, and dihydroxyacid dehydratase	40
pJC4*ilvBNCD-pntAB*	Kan^r; plasmid pJC4 carrying the *ilvBNCD* genes and additionally carrying the *pntAB* genes from *E. coli*, encoding the membrane-bound transhydrogenase PntAB; carrying *pntAB* genes under the control of P_{tac}	This work
pBB1	Cm^r; pBB1 is compatible to pJC4*ilvBNCD* and harbors the P_{tac} promoter and the T_{rrnB} terminator; *lacI* negative	29
pEKEx2-*pntAB*	Plasmid pEKEx2 carrying the *pntAB* genes from *E. coli*	25
pBB1*pntAB*	Cm^r; plasmid pBB1 carrying the *pntAB* genes from *E. coli*; *pntAB* under the control of P_{tac}	This work
pSA55	Plasmid for expression of the *adh2* gene (encoding alcohol dehydrogenase 2) from *Saccharomyces cerevisiae* and the *kivd* gene (encoding 2-ketoacid decarboxylase) from *Lactococcus lactis*	2
pBB1*kivd*	Cm^r; plasmid pBB1 expressing the *kivd* gene from *L. lactis*; carrying *kivd* gene under the control of P_{tac}	This work
pBB1*adh2*	Cm^r; plasmid pBB1 expressing the *adh2* gene from *S. cerevisiae*; carrying *adh2* gene under the control of P_{tac}	This work
pBB1*kivd-adh2*	Cm^r; plasmid pBB1 expressing the *kivd* gene from *L. lactis* and the *adh2* gene from *S. cerevisiae*; carrying *kivd* and *adh2* genes under the control of P_{tac}	This work
pBB1*kivd-adhA*	Cm^r; plasmid pBB1 expressing the *kivd* gene from *L. lactis* and the *adhA* gene from *C. glutamicum*; carrying *kivd* gene under the control of P_{tac} and *adhA* gene under the control of the native promoter	This work
Oligonucleotides		
adh4fow	5′-AACTGCAGAACCAATGCATTGGAGGAGACACAACATGTCTATTCCAGAA ACTCAAAAAG-3′	Amplification of the *adh2* gene
adh2rev	5′-CCGCTCGAGCGGTTATTTAGAAGTGTCAACAACGTAT-3′	Amplification of the *adh2* gene
kivdfow	5′-AACTGCAGAACCAATGCATTGGAGGAGACACAACATGTATACAGTAGG AGATTACCTAT-3′	Amplification of the *kivd* gene
kivd2rev	5′-CCAATGCATTGGTTCTGCAGTTTTATGATTTATTTTGTTCAGCAAAT-3′	Amplification of the *kivd* gene
Ptaccheck	5′-CACTCCCGTTCTGGATAATG-3′	Primer to verify orientation of the *kivd* gene
kivdchkrevec	5′-CTGAGAGTGTACCATTATAG-3′	Primer to verify orientation of the *kivd* gene
adhAfowsalI	5′-ACGCGTCGACGGGAATTGTGTGAATCTTGAAAAG-3′	Amplification of *adhA* gene; primer to verify orientation of *adhA* gene
adhArevsalI	5′-GCTATGGCCGACGTCGACCAAAGGTCATGCCTTAAGCAGC-3′	Amplification of the *adhA* gene
pMM36rev	5′-ACTACCGGAAGCAGTGTG-3′	Primer to verify orientation of the *adhA* gene
pntABfow	5′-CATGCCTGCAGTCATCAATAAAACCG-3`	Amplification of the *pntAB* genes
pntABrev	5`-GTACGCTGCAGTCTTACAGAGCTTTCAGG-3`	Amplification of the *pntAB* genes
transfow2	5′-CTAACATGTATACCCCGCGAATTGCAAGCTGATCCGGGC-3′	Amplification of the *pntAB* genes
transrev2	5′-CTAACATGTATACAAAAAAAAGCCCGCTCATTAGGCGGGCTGGATGCTC TTACAGAGCTTTCAGGATTGCATCC-3′	Amplification of the *pntAB* genes
ldhA1	5′-CGCCCGGGTTCGGCAACAATGACGGCGAGA-3′	Primer for deletion of the *ldhA* gene
ldhA2	5′-CCCATCCACTAAACTTAAACAGACGGTTTCTTTCATTTTCGATCC-3′	Primer for deletion of the *ldhA* gene
ldhA3	5′-TGTTTAAGTTTAGTGGATGGGAAGCAGTTCTTCTAAATCTTTGGCG-3′	Primer for deletion of the *ldhA* gene
ldhA4	5′-CGCCCGGGGGCATCGACGACATCTGAG-3′	Primer for deletion of the *ldhA* gene
ldhfow	5′-TGATGGCACCAGTTGCGATGT-3′	Primer to verify deletion of the *ldhA* gene
ldhrev	5′-CCATGATGCAGGATGGAGTA-3′	Primer to verify deletion of the *ldhA* gene
mdh1	5′-CCCAAGCTTGTTGCCAGGTCCAGACCTCG-3′	Primer for deletion of the *mdh* gene
mdh2	5′-CGTCACCGGCGCAGCTGGTCCGAATGCTCAGGAATTGCAGG-3′	Primer for deletion of the *mdh* gene
mdh3	5′-GACCAGCTGCGCCGGTGACGGTGACCTTCTTGGTGGAGACG-3′	Primer for deletion of the *mdh* gene
mdh4	5′-CGCGGATCCCGCTTGGACATGCCAGATGC-3′	Primer for deletion of the *mdh* gene
mdhcheckfow	5′-CCTGATTCCAGGAACGCATC-3′	Primer to verify deletion of the *mdh* gene
mdhcheckrev	5′-CCTAACATCTTGCAGGTGAG-3′	Primer to verify deletion of the *mdh* gene
malE1	5′-CGGGATCCTTGCTGCCTACACCTACCTTG-3′	Primer for deletion of the *malE* gene
malE2	5′-CCCATCCACTAAACTTAAACACTGCAGGTCGATGGTCATATC-3′	Primer for deletion of the *malE* gene
malE3	5′-TGTTTAAGTTTAGTGGATGGGGTCGCCGAAGCGCAAAACGCTTAA-3′	Primer for deletion of the *malE* gene
malE4	5′-CGGGATCCGAAGTGCTGATCCGCGAACC-3′	Primer for deletion of the *malE* gene
Co-malE1	5′-CTTCCAGACACGGAATCAGAG-3′	Primer to verify deletion of the *malE* gene
Co-malE2	5′-GTGATCCTTCCGAGCGTTCC-3′	Primer to verify deletion of the *malE* gene

were added at the appropriate concentrations (kanamycin, 25 μg ml^{-1}; chloramphenicol, 6 μg ml^{-1}). During the fed-batch processes, adequate amounts of 50% (wt/vol) glucose and 50% (wt/vol) potassium acetate were injected.

Analytics. 1 ml of the culture was harvested by centrifugation (13,000 rpm, 10 min, room temperature [RT]) and the supernatant was used for determination of alcohols and glucose and/or organic acid concentrations in the culture fluid. Glucose, acetate, L-lactate, and succinate concentrations were determined by enzymatic tests (Roche Diagnostics, Penzberg, Germany). The pyruvate concentrations were determined enzymatically according to Lamprecht and Heinz (30). Alcohols in the culture fluid were quantified with a gas chromatograph (GC; PerkinElmer Clarus 600) equipped with a flame ionization detector. Separation of the alcohol compounds was carried out by using a Chromosorb 101 glass column (2-m length, 80/100 mesh) at 130°C, with 10 mM acetone as the internal standard. N_2 was used as the carrier gas. The injector temperature was 200°C,

and the detector temperature was 300°C. Analysis of the chromatographic data was done with PerkinElmer software (TotalChrom chromatography data system [CDS] software).

Construction of expression plasmids. For construction of plasmid pBB1*adh2*, the *adh2* gene from *S. cerevisiae* was amplified from plasmid pSA55 by PCR with primer pair adh4fow/adh2rev. The resulting fragment was digested with PstI/XhoI and ligated into PstI/XhoI-restricted plasmid pBB1. For construction of plasmids pBB1*kivd* and pBB1*kivd-adh2*, the *kivd* gene from *L. lactis* was amplified from plasmid pSA55 by PCR with primer pair kivdfow/kivd2rev. The resulting fragment was digested with PstI and ligated into PstI-restricted plasmids pBB1 and pBB1*adh2*, yielding plasmids pBB1*kivd* and pBB1*kivd-adh2*. The correct orientation of the *kivd* gene was verified via PCR with the primer pair Ptaccheck/kivdcheckrev. For construction of plasmid pBB1*kivd-adhA*, the *adhA* gene from *C. glutamicum* was amplified from chromosomal DNA by PCR with the primer pair adhAfowsalI/adhArevsalI. The resulting fragment was digested with SalI and ligated into the SalI-restricted plasmid pBB1*kivd*. The correct orientation of the *adhA* gene was verified via PCR with the primer pair adhAfowsalI/pMM36rev. For construction of plasmid pBB1*pntAB*, a 2,985-bp fragment containing the *pntAB* genes from *E. coli* was amplified from plasmid pEKEx2-*pntAB* via PCR using the primer pair pntABfow/pntABrev. The resulting fragment was cut with PstI and cloned into the PstI-restricted plasmid pBB1. The correct orientation of the *pntAB* genes was verified via restriction with XhoI. For construction of plasmid pJC4*ilvBNCD-pntAB*, the *pntAB* genes (under the control of the P_{tac} promoter) were amplified from plasmid pBB1*pntAB* by PCR with the primer pair Transfow2/Transrev2. The resulting fragment was digested with Bst1107I and ligated into the Bst1107I-restricted plasmid pJC4*ilvBNCD*. All cloned fragments were checked by sequencing (MWG Biotech).

Construction of *C. glutamicum* deletion mutants. Chromosomal inactivation of the *ldhA* L-lactate dehydrogenase gene in *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* strain and of the *malE* malic enzyme gene in *C. glutamicum aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* and *C. glutamicum aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* were performed using crossover PCR and the suicide vector pK19*mobsacB*. DNA fragments were generated using the primer pairs ldhA1/ldhA2 and ldhA3/ldhA4 or primer pairs malE1/malE2 and malE3/malE4, respectively. The two fragments were purified, mixed in equal amounts, and subjected to crossover PCR using primer pairs ldhA1/ldhA4 and malE1/malE4, respectively. The resulting fusion products (containing the *ldhA* gene shortened by 917 bp and the *malE* gene shortened by 1,137 bp) were ligated into SmaI-restricted plasmid pK19*mobsacB* and transformed into *E. coli*. After isolation and sequencing (MWG Biotech), the recombinant plasmids were introduced by electroporation into the respective *C. glutamicum* strains. By application of the method described by Schäfer et al. (44), the intact chromosomal *ldhA* and *malE* genes were replaced by the truncated genes via homologous recombination (double crossover). The screening of the deletion mutants was performed with 2× TY agar plates containing 10% (wt/vol) sucrose and 0.5% (wt/vol) potassium acetate. The replacements at the chromosomal loci were verified by PCR using primers ldhfow/ldhrev and Co-malE1/Co-malE2, respectively.

Chromosomal inactivation of the *mdh* malate dehydrogenase gene in *C. glutamicum aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* was performed accordingly. DNA fragments were generated using the primer pairs mdh1/mdh2 and mdh3/mdh4, respectively. The two fragments were purified, mixed in equal amounts, and subjected to crossover PCR using primers mdh1 and mdh4. The resulting fusion product (containing the *mdh* gene shortened by 876 bp) was ligated into the BamHI/HindIII-restricted plasmid pK19*mobsacB* and transformed into *E. coli*. After isolation and sequencing, the recombinant plasmid was introduced by electroporation into *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA*. Double crossover and screening for the correct mutants were performed as described above. The replacement at the chromosomal locus was verified by PCR using the primer pair mdhcheckfow/mdhcheckrev.

Determination of enzyme activities. For determination of enzyme activities, the relevant strains were cultivated aerobically in shake flasks to an OD_{600} of about 5 (for measurement of Mdh, PntAB, and Adh activities). Adh activities were also determined under oxygen deprivation conditions. For this purpose, the cells were cultivated for 6 h with an OD_{600} of about 15 in Müller-Krempel bottles. For both conditions, 50 ml CGXII medium (pH 7.4) (16) with 2% (wt/vol) glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-leucine, and L-isoleucine (2 mM each) was used (see culture conditions). The cells were harvested by centrifugation for 10 min at 4,500 × *g*, washed once with 25 ml of 0.2 M Tris-HCl (pH 7.4), centrifuged again and resuspended in 1 ml of the same buffer. The cell suspension was transferred into 2-ml screw-cap vials together with 250 mg of glass beads (diameter, 0.1 mm; Roth) and subjected to mechanical disruption four times for 30 s each at speed 6.5 with a RiboLyser (Hybaid) at 4°C with intermittent cooling on ice for 5 min. Intact cells and cell debris were removed by centrifugation for 15 min at 4,500 × *g* and 4°C.

For determination of transhydrogenase activity, the resulting cell extract was subjected to ultracentrifugation for 45 min at 45,000 × *g* and 4°C. The sedimented membranes were resuspended in 0.5 ml of 10 mM Tris-HCl (pH 8.0) and used for measurement of transhydrogenase activity, which was performed according to Kabus et al. (25). One unit of activity is defined as 1 μmol of 3-acetylpyridine-NADH formed per min.

Determination of the reductive alcohol dehydrogenase (Adh) activity was performed using cell extracts with isobutyraldehyde as the substrate according to Smith et al. (49). One unit of activity is defined as 1 μmol of NADH consumed per min.

Malate dehydrogenase (Mdh) activity in cell extracts was determined by measuring NAD^+ reduction at 30°C at 365 nm in 1 ml of 100 mM phosphate buffer (pH 9.2), 4.5 mM $MgCl_2$, 3 mM NAD^+, and 25 mM malate, according to Smith (48; modified). One unit of activity is defined as 1 μmol NADH formed per min.

For all tested strains, three biological and two technical replicates were performed. The protein concentration was quantified with a BCA protein assay (Pierce) with bovine serum albumin as the standard. Assays were linear over time and proportional to the protein concentration.

RESULTS

Inactivation of LdhA is essential for isobutanol production with *C. glutamicum*. Previously, we demonstrated the ability of *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE*(pJC4*ilvBNCD*) (referred to here as *C. glutamicum* Iso1) to produce KIV under aerobic conditions from glucose (28). As KIV is a precursor for isobutanol production, this strain seemed to be ideally suited for production of this alcohol with *C. glutamicum*. Since the formation of 1 mol of isobutanol from 1 mol of glucose requires 1 mol of $NADH+H^+$ and 1 mol of $NADPH+H^+$ (Fig. 1), we performed the isobutanol fermentations under oxygen deprivation conditions with the aim of increasing $NADH+H^+$ availability. We inoculated *C. glutamicum* Iso1 to an OD_{600} of about 15, which remained almost constant in the course of the fermentations. After 48 h, the glucose was completely consumed, and under these conditions *C. glutamicum* Iso1 produced no isobutanol but did produce significant amounts of L-lactate (122 ± 23 mM) and succinate (29 ± 3 mM) as major fermentation products (Fig. 2A). These results show that *C. glutamicum* is naturally not able to produce isobutanol and underline the necessity of implementing a synthetic pathway. Therefore, we cloned the *kivd* gene from *L. lactis* and the *adh2* gene from *S. cerevisiae* on plasmid pBB1, constructed *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE*(pJC4*ilvBNCD*)(pBB1*kivd-adh2*), *C. glutamicum* Iso2, and performed isobutanol fermentations under oxygen deprivation conditions. Within 48 h, *C. glutamicum* Iso2 consumed the glucose completely, but again produced no isobutanol and formed significant amounts of L-lactate and succinate (data not shown). To avoid L-lactate formation and to increase pyruvate and $NADH+H^+$ availability, we additionally eliminated LdhA activity by deletion of the corresponding gene in *C. glutamicum* Iso2. The resulting strain, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA*(pJC4*ilvBNCD*)(pBB1*kivd-adh2*), or *C. glutamicum* Iso3, metabolized the glucose within 48 h completely and produced no L-lactate anymore, but formed 69 ± 8 mM succinate, which is about two times more than that formed by *C. glutamicum* Iso1 and Iso2. Furthermore, *C. glutamicum* Iso3 formed 26 ± 4 mM isobutanol with a substrate-specific yield ($Y_{P/S}$) of 0.22 ± 0.05 mol per mol of glucose (Fig. 2B). These results

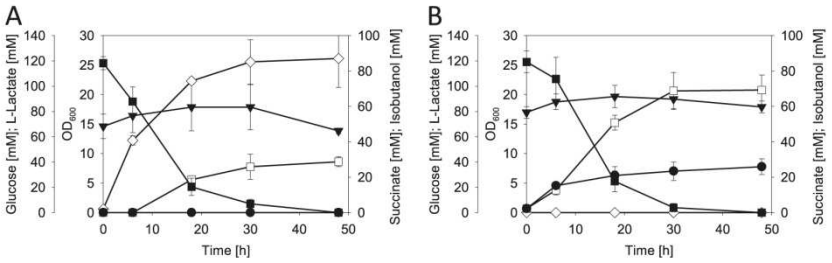

FIG. 2. OD, glucose consumption, and L-lactate, succinate, and isobutanol formation of (A) *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE*(pJC4*ilvBNCD*) (*C. glutamicum* Iso1) and (B) *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA*(pJC4*ilvBNCD*)(pBB1*kivd-adh2*) (*C. glutamicum* Iso3) cultivated in Müller-Krempel bottles filled with CGXII medium containing about 100 mM glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-isoleucine, and L-leucine (2 mM each). ▼, OD_{600}; ■, glucose; □, succinate; ◇, L-lactate; ●, isobutanol. Three independent fermentations were performed. Error bars show standard deviations.

show that inactivation of LdhA is essential for isobutanol production with *C. glutamicum* under the conditions tested.

Deletion of the *mdh* Mdh gene in combination with the expression of the *pntAB* transhydrogenase genes further improves isobutanol production. To eliminate succinate as a by-product and to further increase the availability of pyruvate and $NADH+H^+$, we eliminated Mdh activity by deletion of the *mdh* gene in *C. glutamicum* Iso3. The resulting strain, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* (pJC4*ilvBNCD*) (pBB1*kivd-adh2*), or *C. glutamicum* Iso4, showed no detectable specific Mdh activity, whereas the parental strain *C. glutamicum* Iso3 exhibited 0.46 ± 0.03 U per mg protein. However, in Müller-Krempel bottles, *C. glutamicum* Iso4 consumed only small amounts of glucose (26 mM in 48 h) and produced neither L-lactate nor succinate or isobutanol in significant amounts (Fig. 3A). We speculated that the low glucose consumption is due to a redox imbalance under oxygen deprivation conditions and therefore ligated the *pntAB* operon, encoding the membrane-bound transhydrogenase from *E. coli*, into plasmid pJC4*ilvBNCD*, and constructed *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adh2*), or *C. glutamicum* Iso5. To verify the successful expression of *pntAB*, we determined the specific transhydrogenase activities in the membrane fraction of *C. glutamicum* Iso5. Whereas *C. glutamicum* Iso4 showed no detectable transhydrogenase activity, *C. glutamicum* Iso5 possessed 0.20 ± 0.03 U per mg protein. Isobutanol fermentations of *C. glutamicum* Iso5 under oxygen deprivation conditions revealed that this strain regained the ability to metabolize glucose efficiently and that the cells produced 2 ± 0.1 mM pyruvate (not shown) and 10 ± 1 mM succinate, which is 86% less than that for *C. glutamicum* Iso3. Furthermore, within 48 h, *C. glutamicum* Iso5 produced 42 ± 1 mM isobutanol with a $Y_{P/S}$ of 0.60 ± 0.02 mol per mol of glucose (Fig. 3B), which is about 3-fold higher than that for *C. glutamicum* Iso3. These results demonstrate that, on the one hand, inactivation of Mdh reduces succinate formation and therefore obviously increases pyruvate and/or $NADH+H^+$ availability. On the other hand, expression of the *pntAB* transhydrogenase genes probably results in a more balanced redox state, with a regaining of efficient glucose utilization of *C. glutamicum* Iso4, and improves isobutanol production with *C. glutamicum* under oxygen deprivation conditions.

AdhA of *C. glutamicum* is a bottleneck for isobutanol production. To investigate whether *C. glutamicum* possesses isobutyraldehyde-dependent Adh activity, we constructed *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* (pJC4*ilvBNCD*)(pBB1*kivd*), or *C. glutamicum* Iso6 (without

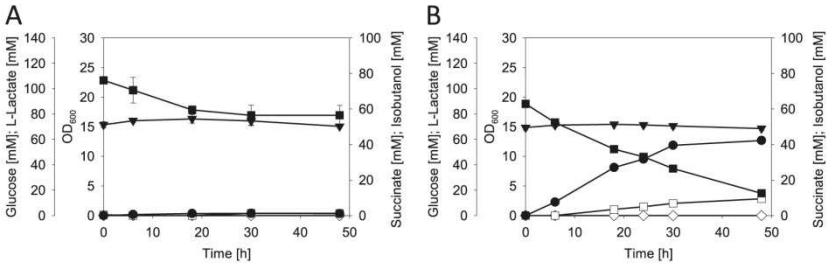

FIG. 3. OD, glucose consumption, and L-lactate, succinate, and isobutanol formation of (A) *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD*)(pBB1*kivd-adh2*) (*C. glutamicum* Iso4) and (B) *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adh2*) (*C. glutamicum* Iso5) cultivated in Müller-Krempel bottles filled with CGXII medium containing about 100 mM glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-isoleucine, and L-leucine (2 mM each). ▼, OD_{600}; ■, glucose; □, succinate; ◇, L-lactate; ●, isobutanol. Three independent fermentations were performed. Error bars show standard deviations.

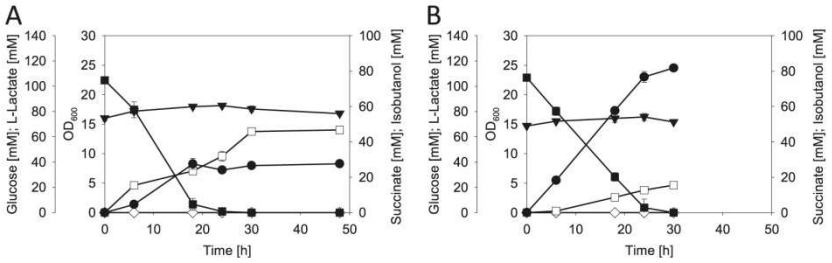

FIG. 4. OD, glucose consumption, and L-lactate, succinate, and isobutanol formation of (A) *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA*(pJC4*ilvBNCD*)(pBB1*kivd*) (*C. glutamicum* Iso6) and (B) *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*) (*C. glutamicum* Iso7) cultivated in Müller-Krempel bottles filled with CGXII medium containing about 100 mM glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-isoleucine, and L-leucine (2 mM each). ▼, OD_{600}; ■, glucose; □, succinate; ◇, L-lactate; ●, isobutanol. Three independent fermentations were performed. Error bars show standard deviations.

the plasmid-bound *adh2* gene), and analyzed substrate utilization and the product spectrum of this strain under oxygen deprivation conditions. As shown in Fig. 4A, *C. glutamicum* Iso6 consumed the glucose completely within 24 h, produced 47 ± 2 mM succinate, and formed 28 ± 1 mM isobutanol. Since *C. glutamicum* Iso6 (without the plasmid-bound *adh2* gene) produced about as much isobutanol as *C. glutamicum* Iso3 (with plasmid-bound *kivd* and *adh2* genes), these results indicated that ADH2 from *S. cerevisiae* does not significantly contribute to isobutanol formation in *C. glutamicum.* This hypothesis was corroborated by determination of the specific isobutyraldehyde-dependent Adh activities in *C. glutamicum* Iso6 and Iso3, which were nearly identical under oxygen deprivation conditions (0.40 ± 0.03 and 0.35 ± 0.04 U per mg of protein, respectively) and slightly lower in aerobically grown cells (0.17 ± 0.02 and 0.25 ± 0.06 U per mg of protein, respectively). Although transcription of the *adh2* gene in *C. glutamicum* Iso3 was verified by reverse transcription-PCR (data not shown), the specific ADH activities indicate that ADH2 is not functionally expressed in *C. glutamicum*. This result is in accordance with recent findings for *E. coli* (4). These data, in combination with those of Smith et al. (49), indicate that one of the endogenous Adh enzymes of *C. glutamicum* is responsible for isobutanol formation from isobutyraldehyde. Furthermore, oxygen deprivation conditions obviously increase *adhA* expression in *C. glutamicum*, since the specific isobutyraldehyde-dependent Adh activities of *C. glutamicum* Iso6 under oxygen-deprived conditions were more than twice as high than those under aerobic conditions.

Smith et al. (49) already observed that overexpression of the *adhA* gene is favorable for isobutanol production with *C. glutamicum.* Therefore, we cloned the *adhA* gene of *C. glutamicum* on plasmid pBB1*kivd* and constructed *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*), or *C. glutamicum* Iso7. To verify successful expression of the *C. glutamicum adhA* gene, we determined the specific isobutyraldehyde-dependent Adh activity of *C. glutamicum* Iso7. *C. glutamicum* Iso7 showed 0.94 ± 0.11 U per mg protein, which is about 3-fold higher than that for *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adh2*) (0.33 ± 0.04 U per mg protein). To test for the effect of *adhA* gene overexpression on isobutanol formation, we cultivated *C. glutamicum* Iso7 in Müller-Krempel bottles. As shown in Fig. 4B, *C. glutamicum* Iso7 consumed the glucose rapidly within 30 h and produced 16 ± 1 mM succinate and 82 ± 1 mM isobutanol, with a $Y_{P/S}$ of 0.77 ± 0.01 mol per mol of glucose. Taken together, these results show that plasmid-encoded ADH2 from *S. cerevisiae* does not contribute to isobutanol production with *C. glutamicum*. In accordance with the results by Smith et al. (49), we show that AdhA of *C. glutamicum* is a bottleneck and that plasmid-bound overexpression of the *adhA* gene significantly improves isobutanol production with *C. glutamicum*.

The role of malic enzyme (MalE) for isobutanol production with *C. glutamicum*. The improvement of isobutanol production by expression of the *pntAB* transhydrogenase genes (*C. glutamicum* Iso5 and *C. glutamicum* Iso7) (Fig. 3B and 4B) indicated that $NADPH+H^+$ supply might be a critical factor for isobutanol production with *C. glutamicum*. However, *C. glutamicum* Iso3 produced isobutanol without expression of transhydrogenase genes; thus, this strain should have the ability to convert $NADH+H^+$ to $NADPH+H^+$. As outlined in a review by Sauer and Eikmanns (43), one proposed transhydrogenase-like route consists of the combined reactions of PCx and/or PEPCx, Mdh, and MalE (Fig. 1). To test this hypothesis, we inactivated MalE by deletion of the corresponding gene in *C. glutamicum* Iso3, yielding *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*malE*(pJC4*ilvBNCD*)(pBB1*kivd-adh2*), here called *C. glutamicum* Iso8. Under oxygen deprivation conditions, *C. glutamicum* Iso8 consumed only about half of the glucose within 48 h and produced 59 ± 3 mM succinate; however, no isobutanol was observed (Fig. 5A). This result suggests that inactivation of MalE interrupts the transhydrogenase-like cycle consisting of PCx/PEPCx, Mdh, and MalE, and therefore is essential for providing $NADPH+H^+$ for isobutanol production. We also investigated the role of MalE in a *pntAB* gene-expressing strain and deleted the mal*E* gene in *C. glutamicum* Iso7 to obtain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* Δ*malE*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*), *C. glutamicum* Iso9. Within 48 h, *C. glutamicum* Iso9 consumed the glucose almost completely and produced 15 ± 2 mM succinate and 24 ± 4 mM isobutanol (Fig. 5B), which is about half of the concentration of isobutanol observed with *C. glutamicum* Iso7. These results again underline the importance of the transhy-

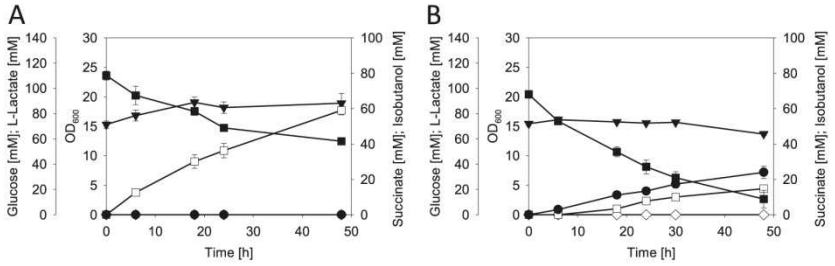

FIG. 5. OD, glucose consumption, and L-lactate, succinate, and isobutanol formation of (A) *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*malE*(pJC4*ilvBNCD*)(pBB1*kivd-adh2*) (*C. glutamicum* Iso8) and (B) *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* Δ*malE*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*) (*C. glutamicum* Iso9) cultivated in Müller-Krempel bottles filled with CGXII medium containing about 100 mM glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-isoleucine, and L-leucine (2 mM each). ▼, OD_{600}; ■, glucose; □, succinate; ◇, L-lactate; ●, isobutanol. Three independent fermentations were performed. Error bars show standard deviations.

drogenase-like cycle for the $NADPH+H^+$ supply for isobutanol production with *C. glutamicum*, even in the presence of transhydrogenase. The fact that *C. glutamicum* Iso9 and also *C. glutamicum* Iso5, in spite of the inactivation of Mdh, still produced succinate is surprising and indicates the presence of an alternative route for succinate formation in *C. glutamicum*.

Fed-batch fermentations with *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*). To test the suitability of *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*), or *C. glutamicum* Iso7, for an improved isobutanol production process, we established a fed-batch fermentation based on mixed substrate divided in an aerobic growth phase and a production phase under oxygen deprivation conditions (Fig. 6). These fermentations were carried out using CGXII medium initially containing 4% (wt/vol) glucose, 1% (wt/vol) acetate, 0.5% (wt/vol) yeast extract, and L-valine, L-leucine, and L-isoleucine (2 mM each). To allow growth to a high cell density, after 6.5 h, an adequate amount of a 50% (wt/vol) acetate stock solution was added to the growing cells, resulting in an OD_{600} of about 45 after 9.5 h (Fig. 6). During the growth period, about 60 mM glucose were consumed in addition to acetate; however, no isobutanol, pyruvate, L-lactate, or succinate was excreted into the medium. After complete consumption of acetate (at 9.5 h), we added about 330 mM glucose (applied as 50% [wt/vol] stock solution) into the medium, switched off aeration, and reduced the stirring speed to 300 rpm. The pO_2 dropped to 0% within less than 1 min (and remained at 0% during the rest of the experiment), and the cells started to excrete isobutanol into the medium. As shown in Fig. 6, the cells accumulated about 175 mM isobutanol within 39.5 h with a volumetric productivity of 4.4 mM h^{-1} and an overall yield in the production phase

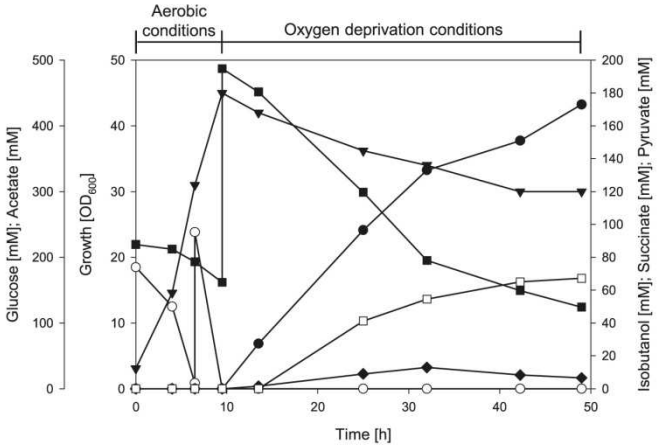

FIG. 6. Isobutanol accumulation during a representative fed-batch fermentation of *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*) (*C. glutamicum* Iso7) on CGXII medium initially containing 4% (wt/vol) glucose, 1% (wt/vol) acetate, 0.5% (wt/vol) yeast extract, and 2 mM L-valine, L-isoleucine, and L-leucine, respectively. After 9.5 h, the aeration was switched off and the stirring speed was reduced to 300 rpm. ▼, OD_{600}; ■, glucose; ○, acetate; □, succinate; ◇, pyruvate; ●, isobutanol. Three independent fed-batch fermentations were performed, all three showing comparable results.

(between 9.5 h and 49 h) of about 0.48 mol of isobutanol per mol of glucose. In addition to isobutanol, the cells excreted about 7 mM pyruvate and 67 mM succinate into the medium, indicating that isobutanol production by *C. glutamicum* Iso7 can be further increased. Taken together, these results demonstrate that *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*) is a very useful platform for optimizing isobutanol production with *C. glutamicum* under oxygen deprivation conditions.

DISCUSSION

Recently, we engineered *C. glutamicum* for efficient aerobic production of KIV from glucose (28). Atsumi et al. (2) showed with *E. coli* that KIV can serve as a precursor for isobutanol production and that implementation of a synthetic pathway, consisting of the broad-range 2-ketoacid decarboxylase from *L. lactis* and ADH2 from *S. cerevisiae* in combination with the expression of the *alsS* gene (encoding AHAS) from *B. subtilis* and the *ilvCD* gene from *E. coli* results in efficient isobutanol production from glucose. More recently, Smith et al. (49) used a similar approach with *C. glutamicum.* The authors showed that this organism possesses a higher isobutanol tolerance than *E. coli* and concluded that *C. glutamicum* might be the superior host for isobutanol production. In growth experiments with CGXII minimal medium with 2% (wt/vol) glucose and increasing isobutanol concentrations, we also found that the *C. glutamicum* wild type tolerates about 1% (vol/vol; i.e., 108 mM) isobutanol, yielding a growth rate (μ) of about 0.35 h^{-1}, which is slightly reduced compared to growth without isobutanol (0.40 h^{-1}). Furthermore, we observed that the addition of 0.5% (wt/vol) yeast extract in the medium promotes the tolerance of isobutanol up to 2% (vol/vol; i.e., 216 mM), yielding a μ of about 0.28 h^{-1}, which is two times higher than that for medium without yeast extract (data not shown). The reasons for this effect remain unclear so far; however, we characterized our producer strains by using CGXII minimal medium with 0.5% (wt/vol) yeast extract, and to improve $NADH+H^+$ availability for isobutanol formation, we additionally applied oxygen deprivation conditions.

C. glutamicum Δ*aceE* Δ*pqo* Δ*ilvE*(pJC4*ilvBNCD*)(pBB1*kivd-adh2*) excreted significant amounts of L-lactate and succinate, but no isobutanol, indicating that pyruvate was not effectively directed toward KIV and isobutanol. Consequently, we inactivated the LdhA gene in this strain, resulting in isobutanol production with a $Y_{P/S}$ of about 0.22 ± 0.05 mol per mol glucose. A beneficial effect on isobutanol formation by inactivation of LdhA was observed before by Smith et al. (49). To further increase pyruvate and/or $NADH+H^+$ availability and to avoid succinate formation, we additionally inactivated Mdh. Interestingly, the resulting strain showed a severe reduction of glucose consumption, possibly due to an unbalanced redox state of the cell under the oxygen deprivation conditions applied. One possibility to regenerate NAD^+ and simultaneously increase $NADPH+H^+$ availability in *C. glutamicum* would be the expression of the *pntAB* genes encoding the membrane-bound transhydrogenase from *E. coli.* This enzyme uses the proton gradient across the cytoplasmic membrane to drive the reduction of $NADP^+$ by oxidizing $NADH+H^+$ (Fig. 1) and previously was shown to improve L-lysine production with *C. glutamicum* under aerobic conditions (25). Expression of the *pntAB* genes in *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD*)(pBB1*kivd-adh2*) in fact recovered efficient glucose utilization, led in combination with the inactivation of Mdh to efficient reduction of succinate formation, and strongly improved isobutanol production ($Y_{P/S}$ of 0.60 ± 0.02 mol per mol of glucose). The results indicate that under oxygen deprivation conditions, the expression of the *pntAB* genes results in the conversion of $NADH+H^+$ to $NADPH+H^+$ and therefore contributes to maintaining a balanced redox state for isobutanol production. Also, Smith et al. (49) tried to increase $NADPH+H^+$-availability for isobutanol production, by redirecting the carbon flux in the *C. glutamicum* Δ*aceE* Δ*ldhA* strain (pKS167) through the pentose phosphate pathway by inactivation of PGI. Unfortunately, this attempt to increase $NADPH+H^+$ availability did not improve isobutanol production, probably generating an imbalance in the redox state of the cell (49).

Since *C. glutamicum* possesses no chromosomally encoded transhydrogenases (25), we speculated that in *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA*(pJC4*ilvBNCD*)(pBB1*kivd-adh2*), a transhydrogenase-like route consisting of the enzymes PCx/PEPCx, $NADH+H^+$-dependent Mdh, and $NADP^+$-dependent MalE (Fig. 1) is responsible for $NADPH+H^+$ supply. Such a cycle was previously assumed to play a role in $NADPH+H^+$ supply for aerobic L-lysine production (13). In fact, inactivation of MalE in our strain led to a complete inability to form isobutanol and, thus, gives further indication of the functionality of a transhydrogenase-like cycle in *C. glutamicum.* Even in the *pntAB* gene-expressing strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*), inactivation of MalE reduced the $Y_{P/S}$ for isobutanol about 2-fold. This finding is surprising, since inactivation of the Mdh should interrupt the proposed transhydrogenase-like route. However, since *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*) still produced succinate, the presence of malate as substrate for MalE is likely. Therefore, these results indicate that MalE is an important enzyme for $NADPH+H^+$ generation. This, in consequence, means that MalE does not work in the reverse (malate-forming) direction under these conditions, as was previously proposed (5, 21), and indicates the existence of an alternative route for the formation of succinate and/or malate, as proposed by Inui et al. (24). However, due to the finding that MalE plays a crucial role for the generation of $NADPH+H^+$, it is obvious that overexpression of the *malE* gene might be an opportunity to replace expression of the *pntAB* genes, thereby reducing the amount of the undesired by-product succinate and improving isobutanol production with *C. glutamicum*.

Atsumi et al. (4) investigated the role of different Adhs on isobutanol production with *E. coli* and showed that the chromosomally encoded YqhD is the major isobutyraldehyde-converting enzyme, and ADH2 from *S. cerevisiae* contributes only to a minor extent to isobutanol production with *E. coli*. We found that under aerobic and also under oxygen deprivation conditions, ADH2 does not contribute at all to the isobutyraldehyde-dependent Adh activity in *C. glutamicum*. *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE*(pJC4*ilvBNCD*)(pBB1*kivd*) produced as much isobutanol as the same strain additionally expressing

the *adh2* gene, showing that an Adh enzyme must be the predominant enzyme for the last step in isobutanol production with *C. glutamicum*. Smith et al. (49) showed that overexpression of the *adhA* gene, encoding the $NADH+H^+$-dependent AdhA (1), increases isobutanol production with *C. glutamicum*. Consequently, we overexpressed the *adhA* gene and found that the resulting strain with the plasmid-bound *adhA* gene showed an improved $Y_{P/S}$ of 0.77 ± 0.01 mol isobutanol per mol of glucose, which is as high as that previously reported for the optimally isobutanol-producing *E. coli* strain (2).

A suitable production process on the industrial scale might be the combination of biomass formation and isobutanol production in a single reactor. Therefore, we established a fed-batch fermentation with *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*). During the aerobic growth phase, neither isobutanol nor pyruvate or succinate were formed. This is in accordance with previous results obtained with PDHC-deficient *C. glutamicum* L-valine producer strains, which also did not secrete L-valine during growth (6, 8). The nonproduction phenotype is due to reduced glucose uptake in the presence of acetate, mediated by the global regulator SugR (9, 18). Inactivation of SugR or replacement of acetate by ethanol resulted in L-valine production during growth (9) and might be also useful to improve isobutanol production with PDHC-deficient *C. glutamicum* strains. However, production during the aerobic growth phase probably is not very useful, since aeration would result in a loss of isobutanol by gas stripping. Furthermore, we applied oxygen deprivation conditions to improve $NADH+H^+$ availability. The presence of succinate as a (major) by-product gives evidence for a surplus of pyruvate and of $NADH+H^+$ and indicates that isobutanol production with *C. glutamicum* can be further improved by, e.g., overexpression of the *malE* gene (see above). Between 9.5 h and 32 h, the *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* strain (pJC4*ilvBNCD-pntAB*) (pBB1*kivd-adhA*) showed a volumetric productivity of about 5.9 mM h^{-1}, which is similar to that of 1-butanol production with different *Clostridium* stains (31). After 32 h of fermentation, the glucose consumption rate dropped from 1.1 to 0.4 mmol of glucose h^{-1} (g of cells [dry weight])$^{-1}$, and the volumetric productivity decreased to about 4.4 mM h^{-1} (between 9.5 and 49 h) (Fig. 6). However, the $Y_{P/S}$ remained constant in the course of the whole fermentation. The reason for this behavior remains unclear but might be attributed to isobutanol toxicity for the cells. Cell toxicity might be avoided by integrated product removal by gas stripping with N_2 and product recovery by continuous condensation, which was successfully applied for 1-butanol production with *Clostridium beijerinckii* (19). Such a process will probably allow the system to maintain its high productivity.

The fact that the fed-batch fermentations of *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh*(pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*) showed a significantly reduced $Y_{P/S}$ compared to that from the Müller-Krempel bottles indicates that the physiological state of the cells during the transition from aerobic to oxygen-deprived conditions may have an impact on the overall production behavior. In favor of this hypothesis, Vemuri et al. (52) found in a combined (consecutive) aerobic/anaerobic succinate production process with *E. coli* that $Y_{P/S}$ for succinate changed in response to altered culture conditions in the growth phase. The authors attributed this observation to the physiological state of the cells entering the transition from aerobic to anaerobic conditions. Recently, Martínez et al. (35) investigated more precisely the role of the physiological state of the cell in a similar approach for succinate production with *E. coli*. These authors found that the introduction of a microaerobic phase at the end of the aerobic growth phase led to an adjusted enzymatic machinery for the anaerobic production phase, which resulted in increased succinate yields, and they concluded that besides the genetic modification of a strain, process optimization is crucial for reaching high yields in such a system. This, in consequence, opens the possibility of improving our *C. glutamicum* production process, e.g., by the introduction of oxygen-limited conditions at the end of the growth phase.

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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Engineering *Corynebacterium glutamicum* for the production of pyruvate

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Abstract A *Corynebacterium glutamicum* strain with inactivated pyruvate dehydrogenase complex and a deletion of the gene encoding the pyruvate:quinone oxidoreductase produces about 19 mM L-valine, 28 mM L-alanine and about 55 mM pyruvate from 150 mM glucose. Based on this double mutant *C. glutamicum* Δ*aceE* Δ*pqo*, we engineered *C. glutamicum* for efficient production of pyruvate from glucose by additional deletion of the *ldhA* gene encoding NAD^+-dependent L-lactate dehydrogenase (LdhA) and introduction of a attenuated variant of the acetohydroxyacid synthase (ΔC–T IlvN). The latter modification abolished overflow metabolism towards L-valine and shifted the product spectrum to pyruvate production. In shake flasks, the resulting strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC–T *ilvN* produced about 190 mM pyruvate with a $Y_{P/S}$ of 1.36 mol per mol of glucose; however, it still secreted significant amounts of L-alanine. Additional deletion of genes encoding the transaminases AlaT and AvtA reduced L-alanine formation by about 50%. In fed-batch fermentations at high cell densities with adjusted oxygen supply during growth and production (0–5% dissolved oxygen), the newly constructed strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC–T *ilvN* Δ*alaT* Δ*avtA* produced more than 500 mM pyruvate with a maximum yield of 0.97 mol per mole of glucose and a productivity of 0.92 mmol $g_{(CDW)}^{-1}$ h^{-1} (i.e., 0.08 g $g_{(CDW)}^{-1}$ h^{-1}) in the production phase.

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Introduction

Pyruvate is widely used, e.g., for the synthesis of various chemicals and polymers or as ingredient or additive in food, cosmetics, and pharmaceuticals (Li et al. 2001; Zhu et al. 2008). Furthermore, it is used for other applications, e.g., weight loss diets (Stanko et al. 1992a; Stanko et al. 1992b; Roufs 1996), exercise endurance (Stanko et al. 1990), anti-acne and anti-ageing skin treatment (Cotellessa et al. 2004; Ghersetich et al. 2004), and it serves as antioxidant (DeBoer et al. 1993) and acts positively on graft tolerance (Cicalese et al. 1997; Cicalese et al. 1999). Chemical production of pyruvate is realized by dehydration and decarboxylation of tartaric acid (Howard and Fraser 1932). However, this process is not very cost efficient (Li et al. 2001) and therefore, fermentative production of pyruvate is of strong interest and has been implemented successfully with metabolically engineered *Escherichia coli* strains and with multi-auxotrophic yeasts (Li et al. 2001, Wendisch et al. 2006).

Corynebacterium glutamicum is a facultatively anaerobic Gram-positive rod-shaped soil bacterium that grows on a variety of sugars and organic acids and is widely used for the production of various amino acids, such as L-glutamate and L-lysine (Eggeling and Bott 2005; Leuchtenberger et al. 2005; Liebl 2006; Nishimura et al. 2007; Takors et al. 2007, Stansen et al. 2005). In recent years, the organism has been engineered to widen the biotechnological product spectrum, e.g., to produce organic acids, such as lactic and succinic

acid (Inui et al. 2004a; Okino et al. 2008a; Okino et al. 2008b, Litsanov et al. 2011), 2-ketoisovalerate, ethanol, isobutanol, or xylitol (Krause et al. 2010; Inui et al. 2004b; Smith et al. 2010; Blombach et al. 2011; Sasaki et al. 2010) or putrescine and cadaverine (Schneider and Wendisch 2010; Mimitsuka et al. 2007, Schneider and Wendisch 2011). However, to our knowledge, the targeted production of pyruvate with *C. glutamicum* has not been reported and so far there were no efforts to produce pyruvate with this organism.

The phosphoenolpyruvate-pyruvate-oxaloacetate node in *C. glutamicum* displays a tangled network, connecting major pathways of carbon and energy metabolism (for a review see Sauer and Eikmanns, 2005) and serving precursors for synthesis of the pyruvate and aspartate family of amino acids (Fig. 1). In an approach to metabolically engineer *C. glutamicum* for L-valine production, we found that the pyruvate dehydrogenase complex- (PDHC-) deficient strain *C. glutamicum* ▵*aceE* shows a relatively high intracellular concentration of pyruvate (when compared with the parental wildtype strain) and aside from L-valine secretes significant amounts of L-alanine and pyruvate from glucose (Blombach et al. 2007b). This phenotype already indicated an intracellular surplus of pyruvate. In further approaches, we additionally observed a positive effect on L-valine production by the inactivation of the pyruvate:quinone oxidoreductase (PQO, Fig. 1) gene *pqo* in *C. glutamicum* ▵*aceE*, probably again due to increased pyruvate availability (Blombach et al. 2008). Having these results in mind, *C. glutamicum* ▵*aceE* ▵*pqo* (Schreiner et al. 2005) seemed to be an ideal basis to develop efficient pyruvate overproducing *C. glutamicum* strains.

In this study, we constructed efficient pyruvate producing derivatives from *C. glutamicum* ▵*aceE* ▵*pqo* by introduction of a leaky (low activity) variant of the acetohydroxyacid synthase (AHAS) and additional deletion of the LdhA gene *ldhA* to prevent L-lactate formation. L-alanine formation was reduced by additional inactivation of the transaminases AlaT and AvtA. Finally, we established an industrially feasible production process with relevant final pyruvate titers, yields, and productivities.

Materials and methods

Bacterial strains and plasmids

All bacterial strains and plasmids and their relevant characteristics and sources are given in Table 1. The oligonucleotides used and their sequences are also listed in Table 1.

DNA preparation and transformation

The isolation of plasmids from *E. coli* was performed as described before (Eikmanns et al. 1994). Plasmid DNA transfer into *C. glutamicum* was carried out by electroporation and the recombinant strains were selected on Brain Heart Infusion (BHI) agar plates containing 0.5 M sorbitol, 83 mM potassium acetate [corresponds to 0.5% (*w*/*v*)] and kanamycin (50 μg ml^{-1}) (van der Rest et al. 1999). Electroporation of *E. coli* was performed with competent cells according to the method of Dower et al. (1988).

Construction of deletion mutants

C. glutamicum strains with chromosomal deletions of *ldhA*, *alaT*, and *avtA* as well as inactivation of the C-terminal (regulatory) domain of IlvN were constructed using the suicide vector pK19*mobsacB*. The recombinant plasmids were isolated from *E. coli* and introduced by electroporation into *C. glutamicum*. Using the method described by Schäfer et al. (1994), the chromosomal *ldhA*, *alaT*, *avtA*, and *ilvN* genes were replaced by truncated versions via homologous recombination (double crossover). Screening of the respective mutants was performed on 2xTY agar plates containing 10% (*w*/*v*) sucrose and 0.5% (*w*/*v*) potassium acetate. The replacement of the native genes was verified in sucrose-resistant, kanamycin-sensitive clones by colony-PCR using the primer pairs ldhAFow/ldhARev, PD1/PD2, alaTFow/alaTRev, and avta1fow/avta1rev (see Table 1).

Culture conditions

C. glutamicum was grown aerobically at 30°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm or as 300-ml cultures in a glass bioreactor (see below). Precultures were grown on 2xTY medium containing 0.5% (*w*/*v*) potassium acetate. For pyruvate fermentations in shake flasks, sedimented cells (4,500×*g*; 10 min; 4°C) of an overnight preculture were resuspended in 0.9% (*w*/*v*) saline and inoculated into 50 ml modified CGXII minimal medium (pH 6.8; adjusted with 5 M KOH) (Eikmanns et al. 1991), with $(NH_4)_2SO_4$ (5 g/l) and $MnSO_4 \times H_2O$ (0.1 mg/l), supplied with 3% (*w*/*v*) glucose and 0.5% (*w*/*v*) potassium acetate as carbon sources. For growth of Δ*alaT* and Δ*alaT* Δ*avtA* mutant strains, the minimal medium was additionally supplied with 2 mM L-alanine. Cell dry weight (CDW) was calculated from the OD_{600} using a ratio of 0.3 $g_{(CDW)}$ l^{-1} per OD_{600} (Blombach et al. 2007a).

For fed-batch fermentations, 400 ml bioreactors were used in a fed-batch Pro fermentation system from DASGIP (Jülich, Germany). The initial working volume was 300 ml. The temperature was kept at 30°C, and the pH was

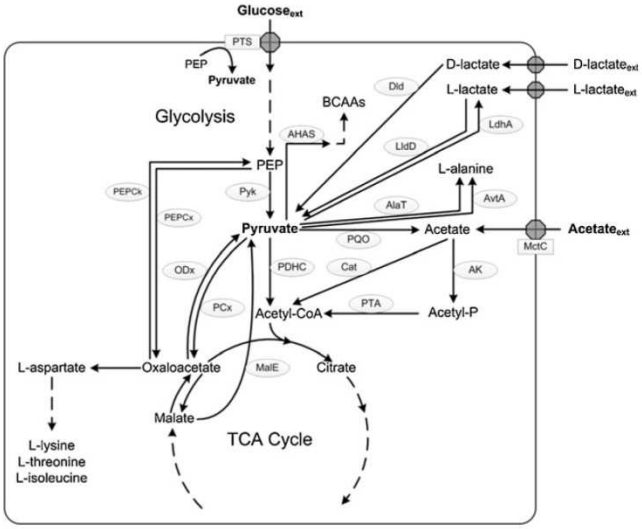

Fig. 1 The phosphoenol–pyruvate–oxaloacetate node in *C. glutamicum* with the corresponding enzymes for major pathways of carbon metabolism (Sauer and Eikmanns, 2005). Abbreviations: *AHAS* acetohydroxyacid synthase, *AK* acetate kinase, *AlaT* alanine aminotransferase and AvtA, valine-pyruvate aminotransferase (Marienhagen and Eggeling 2008); *BCAAs* branched-chain amino acids, *Cat* CoA transferase (Veit et al. 2009), *CoA* Coenzyme A, *Dld* quinone-dependent D-lactate dehydrogenase (Kato et al. 2010), *LdhA* NAD^+-dependent L-lactate dehydrogenase, *LldD* quinone-dependent L-lactate dehydrogenase (Stansen et al. 2005), *MalE* malic enzyme, *MctC* monocarboxylic acid transporter (Jolkver et al. 2009), *ODx* oxaloacetate decarboxylase, *PCx* pyruvate carboxylase, *PDHC* pyruvate dehydrogenase complex, *PEP* phosphoenolpyruvate, *PEPCk* PEP carboxykinase, *PEPCx* PEP carboxylase, *PQO* pyruvate:quinone oxidoreductase, *PTA* phosphotransacetylase, *PTS* phosphotransferase system, *Pyk* pyruvate kinase, *TCA* tricarboxylic acid

maintained at 7.0 by online measurement using a standard pH electrode (Mettler Toledo, Giessen, Germany) and addition of 4 M H_2SO_4 and 4 M KOH, respectively. Diluted Struktol 674 antifoam (Schill und Seilacher, Hamburg, Germany) (1:20) was added manually dropwise (~20 μl) when necessary. Dissolved oxygen (DO) was measured online, using an oxygen electrode (Mettler Toledo, Giessen, Germany) and adjusted during growth to 30% of saturation by stirring at 400 to 1,040 rpm and aeration with 1 volume of air per volume of medium per minute (vvm). In experiments with low oxygen conditions, the DO was allowed to drop down to levels around 0–5% by stirring at 400 to 750 rpm, while gassing with 0.25 vvm. When acetate concentrations of the last batch drew near 0 mM, the agitation was set to 500 rpm. During the fed-batch process, adequate amounts of 50% (*w*/*v*) acetate (growth phase) and 50% (*w*/*v*) glucose (production phase) were injected.

Analytical procedures

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Ultrospec® 3000 pro, Amersham Pharmacia Biotech GmbH, Freiburg).

For quantification of substrate consumption and product formation, 1 ml samples (2 ml in fed-batch fermentations) of the culture were harvested and spinned down (17,000×*g*, 10 min, RT). The resulting supernatants were used for determination of glucose, organic acid, and amino acid concentrations in the culture fluid. Glucose and organic acids (acetate, pyruvate, lactate, malate, fumarate, succinate) were measured via high-pressure liquid chromatography on an Agilent 1100 LC system (Agilent Technologies, Waldbronn Germany) equipped with a 300×8 mm organic acid-resin column (polystrol-divinylbenzol copolymer; CS—Chromatographie Service GmbH, Langerwehe, Germany) and a corresponding guard cartridge (40×8 mm). Separation was carried out under isocratic conditions for 38 min at 40°C with 100 mM sulfuric acid at a flow rate of 0.4 ml/min. Organic acids were detected with an Agilent 1100 Variable Wavelength Detector at 215 nm and glucose with an Agilent 1100 Refractive Index Detector. Quantification was done by calculation of the peak area, using an 8-point calibration curve as external standard for each substance.

Table 1 Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence	Source, reference, or purpose
Strains		
E. coli DH5α	F$^-$ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (rk$^-$, mk$^+$) *supE44 thi-1 gyrA96 relA1 phoA*	Hanahan 1983
C. glutamicum Δ*aceE* Δ*pqo*	Derived from *C. glutamicum* ATCC13032 with deletion of *aceE*, encoding the E1p subunit of the PDHC and deletion of *pqo*, encoding the pyruvate:quinone oxidoreductase	Schreiner et al. 2006
C. glutamicum Δ*aceE* Δ*pqo* Δ*ldhA*	*C. glutamicum* Δ*aceE* Δ*pqo* with additional deletion of *ldhA*, encoding the L-lactic acid dehydrogenase	This study
C. glutamicum Δ*aceE* Δ*pqo* Δ*ldhA* ΔC–T *ilvN*	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* with additional deletion of the last 249 bp of the C-terminal domain of *ilvN*, encoding the small subunit of the acetohydroxyacid synthase (AHAS)	This study
C. glutamicum Δ*aceE* Δ*pqo* Δ*ldhA* ΔC–T *ilvN* Δ*alaT*	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC–T *ilvN* with additional deletion of *alaT*, encoding the alanine aminotransferase	This study
C. glutamicum Δ*aceE* Δ*pqo* Δ*ldhA* ΔC–T *ilvN* Δ*alaT* Δ*avtA*	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC–T *ilvN* Δ*alaT* with deletion of *avtA*, encoding the valine:pyruvate aminotransferase	This study
Plasmids		
pK19*mobsacB* Δ*ldhA*	pK19*mobsacB* carrying a truncated *ldhA* gene	Blombach et al. 2011
pK19*mobsacB* ΔC–T *ilvN*	pK19*mobsacB* carrying a truncated *ilvN* gene (shortened by 249 bp)	Blombach et al. 2009
pK19*mobsacB* Δ*alaT*	pK19*mobsacB* carrying a truncated *alaT* gene	Marienhagen et al. 2005
pK19*mobsacB* Δ*avtA*	pK19*mobsacB* carrying a truncated *avtA* gene	Marienhagen et al. 2005
Oligonucleotides		
ldhAFow	5′-TGTGGGTTGTCCGGTTAG-3′	Primer to verify *ldhA* deletion
ldhARev	5′-TGGTAGTCAAGCGGGTAG-3′	Primer to verify *ldhA* deletion
PD1	5′-CCAAGATGGCTAATTCTGACGTCACC-3′	Primer to verify ΔC–T *ilvN* deletion
PD2	5′-GACTAGTCACATTTATGCAGCAGGTGC-3′	Primer to verify ΔC–T *ilvN* deletion
alaTFow	5′-CGAGGAACGGCAATAATC-3′	Primer to verify *alaT* deletion
alaTRev	5′-AGCAAGACCTGACATACC-3′	Primer to verify *alaT* deletion
avta1fow	5′-TCCGATAGCTGCAACAACTG-3′	Primer to verify *avtA* deletion
avta1rev	5′-TACCGCACTCAATGCTGAAG-3′	Primer to verify *avtA* deletion

For some shake flask experiments, glucose, acetate, and pyruvate concentrations were alternatively determined by enzymatic test kits (Roche Diagnostics, Penzberg, Germany) or by the method of Lamprecht and Heinz (1983), respectively. For the latter, 500 µl of 100 mM Tris (pH 7,4), 100 µl of 3 mM NADH, 290 µl of H_2O, and 100 µl adequately diluted samples were mixed in a 1 ml-cuvette, and extinction was measured at 365 nm against an offset value to determine a blank value (E_1). Afterwards, 10 µl of L-lactic acid dehydrogenase from porcine heart (Sigma, Steinheim) diluted 1:10 in 2.5 M $(NH_4)_2SO_4$ were added to the cuvette. After 10 min of incubation at RT, extinction was measured again at 365 nm (E_2). The concentration of pyruvate was calculated by the law of Lambert and Beer, dividing the difference of E_2 and E_1 by the product of the linear extinction coefficient of NADH ε_{NADH} (3.4 mM^{-1} cm^{-1}) and the thickness of the cuvette (1 cm).

The amino acid concentrations were determined by reversed-phase high-pressure liquid chromatography as described previously (Blombach et al. 2007b).

Results

Pyruvate production by *C. glutamicum* ▵*aceE* ▵*pqo* and *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA*

To test *C. glutamicum* ▵*aceE* ▵*pqo* for its ability to form organic acids and/or amino acids from glucose and acetate, we carried out shake-flask fermentations in modified CGXII medium with 3% (*w*/*v*) glucose and 1% (*w*/*v*) potassium acetate. Growth, glucose, and acetate utilization as well as organic acid and amino acid accumulation were monitored over the course of the experiment. Within 10 h, *C. glutamicum* ▵*aceE* ▵*pqo* grew exponentially with a μ of about 0.40 h^{-1} to an OD_{600} of about 17, consuming acetate and glucose. After complete consumption of the acetate, the cells stopped growing but continued to metabolize glucose and produced 19 mM L-valine, 27 mM L-alanine, and 55 mM pyruvate within 40 h with a $Y_{P/S}$ of about 0.48 mol pyruvate per mole glucose (Fig. 2). The cells did not secrete any succinate, acetate, or lactate in the

course of the experiment. However, to prevent lactate formation in further optimized producer strains, we deleted the *ldhA* gene in *C. glutamicum* ▵*aceE* ▵*pqo*. In modified CGXII medium with 3% (*w*/*v*) glucose and 1% (*w*/*v*) potassium acetate, the resulting strain *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* showed similar growth and the comparable (by)product spectrum and $Y_{P/S}$ when compared to the parental strain *C. glutamicum* ▵*aceE* ▵*pqo* (data not shown).

Deletion of the C-terminal domain of the regulatory subunit IlvN of the AHAS increases pyruvate formation

As described above, *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* produced significant amounts of L-valine as by-product. To avoid L-valine production without generating an auxotrophy for L-valine, we replaced the AHAS with a leaky variant, lacking the C-terminal domain of the regulatory subunit IlvN and showing an about twofold lower K_m for the substrate pyruvate and an about fourfold lower V_{max} (Blombach et al. 2009). The AHAS is the key enzyme for the formation of branched-chain amino acids (BCAAs), consuming either two molecules of pyruvate for L-valine and L-leucine synthesis or one molecule of pyruvate and one molecule of ketobutyrate for L-isoleucine synthesis. We introduced the ▵C–T *ilvN* deletion into *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* and performed shake-flask fermentations in modified CGXII medium with 3% (*w*/*v*) glucose and 1% (*w*/*v*) potassium acetate with the resulting strain *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN*. Within 10 h this strain grew exponentially with a μ of about 0.40 h^{-1} to an OD_{600} of about 15. After complete consumption of the acetate, the cells stopped growing, consumed the glucose within the next 48 h completely, and produced about 9 mM L-alanine, 1 mM L-valine, and about 193 mM pyruvate, with a $Y_{P/S}$ of 1.36 mol pyruvate per mole of glucose (Fig. 3). Thus, *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* showed a more than 90% reduction of L-valine formation, a more than 70% reduction of L-alanine formation, and an about threefold increased substrate specific pyruvate yield, when compared with the parental strain.

Inactivation of the transaminases AlaT and AvtA further reduces by-product formation

Aside from pyruvate, *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* still secreted significant amounts of L-alanine as by-product. Marienhagen et al. (2005) and Marienhagen and Eggeling (2008) identified the transaminases AlaT and AvtA as major L-alanine supplying enzymes. To avoid or to reduce L-alanine formation, we constructed an AlaT− and an AlaT/AvtA− mutant by deletion of the respective genes in *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* and performed shake-flask cultivations with both *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* ▵*alaT* and *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C-T *ilvN* ▵*alaT* ▵*avtA* in modified CGXII medium with 3% (*w*/*v*) glucose and 1% (*w*/*v*) potassium acetate. Without supplementation of L-alanine to the minimal medium, both strains showed only very restricted growth [μ of about 0.12 h^{-1}, final OD_{600}s of about 5 (▵*alaT*) and 3 (▵*alaT* ▵*avtA*) (data not shown)]. When adding 2 mM L-alanine to the medium, both strains grew with a μ of about

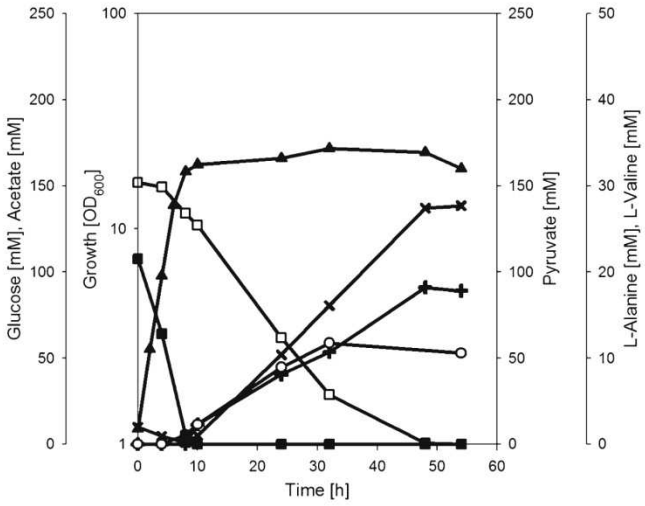

Fig. 2 Growth, substrate consumption, and product formation during a representative shake-flask batch cultivation of *C. glutamicum* ▵*aceE* ▵*pqo* on modified CGXII medium containing 3% (*w*/*v*) glucose and 1% (*w*/*v*) potassium acetate. (*Filled triangle*) growth; (*empty square*) glucose; (*filled square*) potassium acetate; (*multiplication symbol*) L-alanine; (*plus symbol*) L-valine; (*empty circle*) pyruvate. At least three independent fermentations were performed, showing comparable results

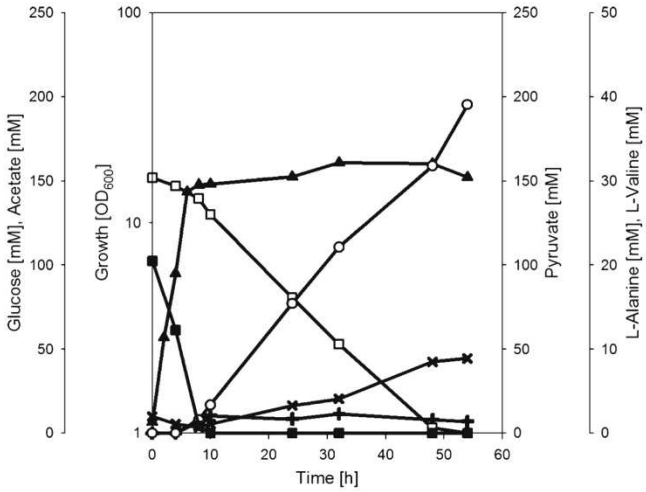

Fig. 3 Growth, substrate consumption, and product formation during a representative shake-flask batch cultivation of *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* on modified CGXII medium containing 3% (*w*/*v*) glucose and 1% (*w*/*v*) potassium acetate. (*Filled triangle*) growth; (*empty square*) glucose; (*filled square*) potassium acetate; (*multiplication symbol*) L-alanine; (*plus symbol*) L-valine; (*empty circle*) pyruvate. At least three independent fermentations were performed, showing comparable results

0.35 h^{-1} to an OD_{600} of about 12 after 8 h (Fig. 4, representative for both strains). Within 48 h, both strains completely consumed the glucose and produced nearly 200 mM pyruvate (Fig. 4) with a $Y_{P/S}$ of 1.49 mol pyruvate per mole of glucose. Furthermore, the inactivation of AlaT and AvtA resulted in a cumulative reduction of L-alanine formation by about 50%, when compared to the parental strain *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN*, and therefore might improve product purity in the subsequent downstream process.

Comparison of pyruvate accumulation, substrate-specific pyruvate yields, and by-product formation in shake-flask batch fermentations

Figure 5 summarizes the final pyruvate titers, the substrate-specific pyruvate yields and by-product (L-alanine and L-valine) formation during the production phases of *C. glutamicum* ▵*aceE* ▵*pqo* and its derivatives in shake-flask experiments. The results show that the critical step for pyruvate production is the disruption of the C-terminal domain of the regulatory subunit IlvN of the AHAS. Additional deletion of *alaT* or both *alaT* and *avtA* had a further beneficial effect on the reduction of the by-products L-alanine and L-valine.

Fed-batch fermentations with *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* ▵*alaT* ▵*avtA* in bioreactors

To study the relevance for an industrial application, we established fed-batch fermentations with *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* ▵*alaT* ▵*avtA* in modified CGXII medium, initially containing 4% (*w*/*v*) glucose, 1% (*w*/*v*) potassium acetate, and 6 mM L-alanine. To reach higher cell densities, we added adequate amounts of a 50% (*w*/*v*) acetate stock solution at given time points to the growing cells. When we cultivated *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* ▵*alaT* ▵*avtA* under oxygen surplus conditions, i.e., with a constant pO_2 of ≥30%, the cells grew with a μ of about 0.28 h^{-1} to an OD_{600} of 30, but in the production phase after complete consumption of the acetate, we observed a low glucose consumption rate of only 0.23 mmol $g_{(CDW)}^{-1}$ h^{-1}. Furthermore, *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C-T *ilvN* ▵*alaT* ▵*avtA* produced only 50 mM of pyruvate with a $Y_{P/S}$ of about 0.8 mol pyruvate per mole of glucose (data not shown), which is about two times lower when compared to that of the shake-flask fermentations with this strain. However, when we performed fed-batch fermentations, allowing oxygen deprivation conditions during mid- and late-growth phase (for details see "Materials and methods"), *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* ▵*alaT* ▵*avtA* grew with a μ of about 0.32 h^{-1} to a maximal OD_{600} of about 63 within 14 h (Fig. 6a). Within 5 h, the pO_2 dropped from about 100% to about 0% of saturation and remained at this value until the acetate was completely consumed and the production phase started (Fig. 6). In this phase, the cells continued to metabolize the glucose efficiently with a consumption rate of 0.81 mmol $g_{(CDW)}^{-1}$ h^{-1}, which is more than three times higher compared to that of the previous experiments with a constant pO_2 of about 30% of saturation during growth. Within 105 h, *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* ▵*alaT* ▵*avtA* produced about 15 mM malate, 18 mM L-valine, 20 mM L-alanine (data not shown), and up to 512 mM pyruvate (Fig. 6a) with a $Y_{P/S}$ of about 0.97 mol pyruvate per mole of glucose and a productivity of about 0.92 mmol $g_{(CDW)}^{-1}$ h^{-1}.

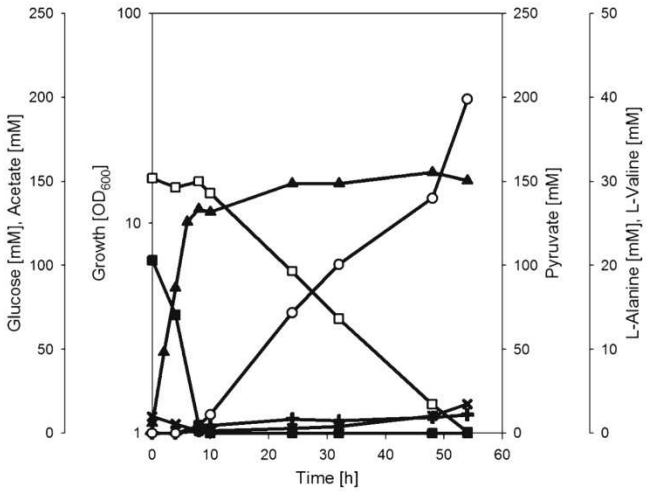

Fig. 4 Growth, substrate consumption, and product formation during a representative shake-flask batch cultivation of *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* ▵*alaT* ▵*avtA* on modified CGXII medium containing 3% (*w*/*v*) glucose, 1% (*w*/*v*) potassium acetate and 2 mM L-alanine. (*Filled triangle*) growth; (*empty square*) glucose; (*filled square*) potassium acetate; (*multiplication symbol*) L-alanine; (*plus symbol*) L-valine; (*empty circle*) pyruvate. At least six independent fermentations were performed, all of them showing comparable results

When comparing the results obtained during fed-batch fermentations in bioreactors with those obtained in shake-flask batch fermentations (Table 2), the growth rates as well as the biomass yields per mole carbon were slightly lower under fed-batch conditions. However, although the glucose consumption rate in the bioreactor was higher under fed-batch conditions, the production rate of pyruvate was slightly reduced and the $Y_{P/S}$ diminished to about 65% (Table 2).

Discussion

Based on previous experience on tailoring *C. glutamicum* for L-valine production and on the observation that L-valine-producing strains form pyruvate as an undesired by-product (Blombach et al. 2007b, Blombach et al. 2008), we constructed here for the first time *C. glutamicum* strains efficiently producing pyruvate with high yields and

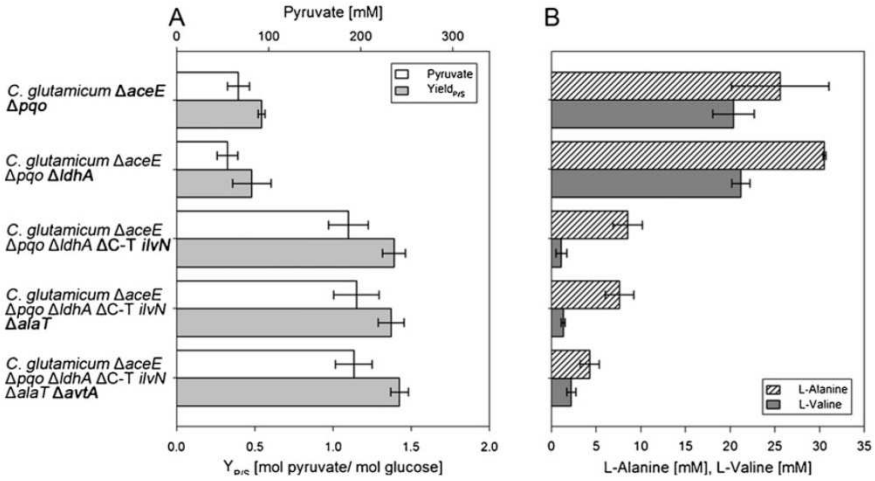

Fig. 5 **a** Comparison of final pyruvate concentrations and substrate specific yields (mole pyruvate per mole glucose) of pyruvate overproducing *C. glutamicum* strains at the end of production phase in shake-flask experiments. **b** Overview of the effects, resulting from stepwise inactivation of PDHC and PQO, LdhA, attenuation of the AHAS and additional deletion of *alaT* and *avtA*, on the formation of the byproducts L-alanine and L-valine

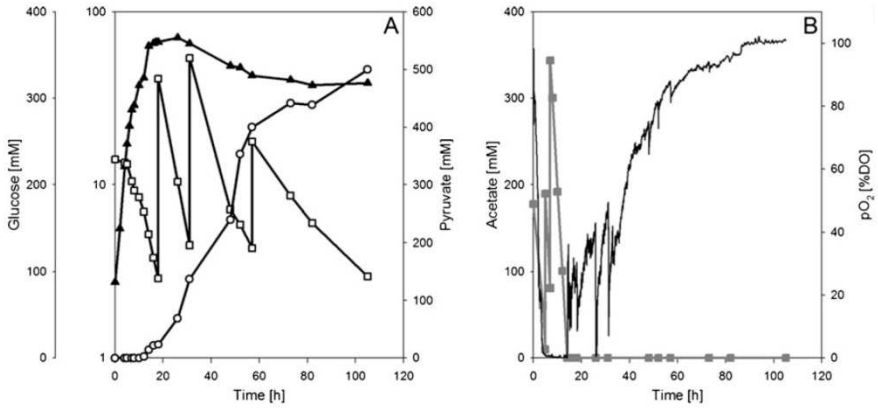

Fig. 6 **a** Growth, glucose consumption, and pyruvate formation, and **b** acetate consumption and the course of the pO_2 of *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C-T *ilvN* ▵*alaT* ▵*avtA* during a representative fed-batch cultivation with modified CGXII medium initially containing 4% (*w*/*v*) glucose, 1% (*w*/*v*) acetate, and 6 mM L-alanine. **a** (*Filled triangle*) growth; (*empty square*) glucose; (*empty circle*) pyruvate; **b** *gray line* acetate; *black line* pO_2. At least three independent fermentations were performed, showing comparable results

productivities. The most efficient pyruvate-producing strain was constructed stepwise from the PDHC- and PQO-deficient *C. glutamicum* ▵*aceE* ▵*pqo*, by deletion of the LdhA gene, exchange of the native AHAS by a truncated enzyme with lower activity and by deletion of the genes encoding the transaminases AlaT and AvtA. *C. glutamicum* ▵*aceE* ▵*pqo* already produced pyruvate but secreted significant amounts of L-valine and L-alanine (see Fig. 2). The introduction of the leaky AHAS variant ▵C–T *ilvN* into this *C. glutamicum* host almost completely abolished the carbon overflow towards L-valine, led to drastically increased pyruvate production and turned out to be highly beneficial for pyruvate overproduction without generating an auxotrophy for the BCAAs.

We previously replaced the native AHAS by the ▵C–T *ilvN* AHAS variant in L-lysine-producing strains of *C. glutamicum*, resulting in an efficient redirection of the carbon flux towards L-lysine and thus, in increased L-lysine formation by more than 40% (Blombach et al. 2009). L-lysine originates from pyruvate and oxaloacetate (which also is formed from pyruvate by the pyruvate caboxylase reaction; Peters-Wendisch et al. 1997 and 1998) and thus, the positive effect of decreased AHAS activity for pyruvate and for L-lysine production should be at least partially due to reduced carbon flow from pyruvate towards the BCCAs. However, the positive effect of the decreased AHAS activity on both pyruvate accumulation by *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* and on L-lysine accumulation by the L-lysine producer with the ▵C–T *ilvN* AHAS variant (Blombach et al. 2009) cannot be exclusively explained by diminishing the drain off of pyruvate in the direction of BCAAs, since the pyruvate and also the L-lysine titers

Table 2 Relevant process parameters of shake flask cultivations and fed-batch fermentations with *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* ▵*alaT* ▵*avtA*

Process parameters	Growth condition	
	500 ml shake flask	400 ml bioreactor (fed-batch)
Growth rate μ [h^{-1}]	0.35±0.01	0.32±0.01
Biomass yield [$g_{(CDW)}$ mol_C^{-1}]	11.6±1.82	9.97±0.8
Glucose consumption rate during production phase [mmol $g_{(CDW)}^{-1}$ h^{-1}]	0.58±0.09	0.81±0.05
Production rate [mmol $g_{(CDW)}^{-1}$ h^{-1}]	1.06±0.20	0.92±0.01
Product $Y_{P/S}$ [mole pyruvate/mole glucose]	1.49±0.20	0.97±0.10
By-product $Y_{P/S}$ [mole L-alanine/mole glucose]	0.03±0	0.11±0.03
By-product $Y_{P/S}$ [mole L-valine/mole glucose]	0.01±0	0.11±0.05

observed with the newly constructed strains exceeded by far the titers of pyruvate plus L-valine or of L-lysine plus BCAAs observed with the original strains with native AHAS.

The improvement of pyruvate production in *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* by substitution of the native AHAS was accompanied by a severe reduction of L-valine and L-alanine formation. The lower L-alanine formation was surprising, but might be explained by the action of the transaminases AlaT and AvtA, which are the major L-alanine supplying enzymes in *C. glutamicum* (Marienhagen et al. 2005; Marienhagen and Eggeling 2008). Marienhagen and Eggeling (2008) showed that inactivation of either AlaT or AvtA has no influence on growth of *C. glutamicum*, found that the double mutant is unable to grow without supplementation of L-alanine and postulated an amino group distribution system, due to overlapping substrate specificities of AlaT and AvtA. This system allows a cascade of amino transfers between L-glutamate, L-alanine, and L-valine in both directions using 2-oxoglutarate, pyruvate, and 2-ketoisovalerate as substrates, conferring flexibility to the cell (Marienhagen and Eggeling 2008). Since L-valine acts as amino donor for the AvtA reaction, a limitation in L-valine availability, due to introduction of the leaky AHAS, could be the reason for reduced L-alanine formation by *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN*. The reduced carbon flux towards the BCAAs and thus, the reduced availability of L-valine for AvtA also might explain our observation that inactivation of AlaT in *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* resulted in weak growth. Surprisingly, *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* ▵*alaT* ▵*avtA*, devoid of both aminotransferases AlaT and AvtA, still showed growth in minimal medium without L-alanine, although at a low μ of about 0.12 h^{-1}. This result indicates that a further transaminase in *C. glutamicum* is able to compensate the functions of AlaT and AvtA under these conditions. However, effective growth of *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* ▵*alaT* ▵*avtA* was restored by the addition of 2 mM L-alanine and deletion of the genes *alaT* and *avtA* significantly reduced L-alanine formation.

When we transferred *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C-T *ilvN* ▵*alaT* ▵*avtA* into a bioreactor and performed fed-batch fermentations under oxygen surplus, we observed significant lower glucose consumption rates and product-specific yields than in shake-flask cultivations. It can be assumed that in shake flasks, at least at higher cell densities (OD_{600} of >10), the cells face oxygen limitation (Zimmermann et al. 2006) and therefore, we tested the effect of low oxygen tension in the bioreactor from the middle to the end of the growth phase. Under these conditions, the cells showed slightly lower growth rates and biomass yields and in the production phase slightly lower production rates, significantly lower $Y_{P/S}$ and significantly higher glucose consumption rates (see Table 2). Recently, Martínez et al. (2010) reported a similar observation for a succinate production process with *E. coli*. This process was split into an aerobic growth phase and an anaerobic production phase (dual phase fermentation) and the authors found that the introduction of a microaerobic phase at the end of the growth phase improved yield, titer and volumetric productivity. More recently, we engineered *C. glutamicum* for the anaerobic production of isobutanol and also observed a reduced $Y_{P/S}$ in dual phase fermentations compared to cultivations in shaken bottles (Blombach et al. 2011). These findings demonstrate the importance and impact of the physiological state of the cell on the overall production behavior in such processes. However, a deeper understanding of the adaption of the cell's transcriptional, translational, and enzymatic machinery to changing conditions (such as a shift from growth to production or a shift from aerobic to oxygen deprivation conditions) is required. Such knowledge in consequence will help to optimize process conditions and may significantly improve our pyruvate production system.

Several studies focused on the biotechnological production of pyruvate with multi-auxotrophic yeasts and *E. coli* strains (reviewed in Li et al. 2001). Production processes with yeasts need a complex fermentation set-up, as maintenance of vitamin concentrations within the medium is crucial to direct carbon flux towards pyruvate; however, they led in 56 h to titers up to 784 mM (i.e., 69 g/l) (Li et al. 2001). The highest known $Y_{P/S}$ values with *E. coli* mutants in shake flasks were at about 1.9 mol pyruvate per mole glucose (Zelić et al. 2003). More recently, Zhu et al. (2008) engineered *E. coli* for the production of pyruvate and observed under optimized process conditions 1,022 mM (i.e., 90 g/l) with a $Y_{P/S}$ of 1.39 mol pyruvate per mole of glucose. These impressing production characteristics were based on the improvement of the glycolytic flux. Previous studies also showed successfully improved production of pyruvate by metabolic engineering strategies at glycolysis (Yokota et al. 1994a; Yokota et al. 1994b; Causey et al. 2004; Zhu et al. 2008). These strategies were also applied to *C. glutamicum*, leading to improved glutamate production with pyruvate as by-product (Sekine et al. 2001). However, under non-optimal process conditions *C. glutamicum* ▵*aceE* ▵*pqo* ▵*ldhA* ▵C–T *ilvN* ▵*alaT* ▵*avtA* showed a pyruvate production rate of more than 1 mmol $g_{(CDW)}^{-1}$ h^{-1} and a $Y_{P/S}$ of 1.49 mol per mole of glucose, and in non-optimized fed-batch fermentations the cells produce final titers of more than 500 mM (i.e., 45 g/l). With these characteristics, this strain not only is a useful platform for pyruvate production but can probably also be exploited for the production of pyruvate-derived products, such as the dicarboxylic and tricarboxylic acids of the citric acid cycle.

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Bio-based production of organic acids with *Corynebacterium glutamicum*

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Running title
Organic acid production with *Corynebacterium glutamicum*

Keywords
Corynebacterium glutamicum, pyruvate, 2-ketoisovalerate, 2-ketoglutarate, D-, L-lactate, succinate, industrial biotechnology, metabolic engineering

Summary

The shortage of oil resources, the steadily rising oil prices and the impact of its use on the environment evokes an increasing political, industrial and technical interest for development of safe and efficient processes for the production of chemicals from renewable biomass. Thus, microbial fermentation of renewable feedstocks found its way in white biotechnology, replacing more and more traditional crude oil-based chemical processes. Rational strain design of appropriate microorganisms has become possible due to steadily increasing knowledge on metabolism and pathway regulation of industrially relevant organisms and, aside from process engineering and optimization, has an outstanding impact on improving the performance of such hosts. *Corynebacterium glutamicum* is well known as workhorse for the industrial production of numerous amino acids. However, recent studies also explored the usefulness of this organism for the production of several organic acids and great efforts have been made for improvement of the performance. This review summarizes the current knowledge and recent achievements on metabolic engineering approaches to tailor *C. glutamicum* for the bio-based production of organic acids. We focus here on the fermentative production of pyruvate, L- and D-lactate, 2-ketoisovalerate, 2-ketoglutarate, and succinate. These organic acids represent a class of compounds with manifold application ranges, e.g. in pharmaceutical and cosmetics industry, as food additives, and economically most interesting, as precursors for a variety of bulk chemicals and commercially important polymers.

Introduction

The depletion of earth`s fossil energy resources, accompanied by the strong impact of their use on the environment, particularly in form of higher CO_2 emissions, raises the demand for sustainable, safe and efficient substitution of hitherto crude oil derived chemicals and chemical building blocks from renewable resources. Besides chemical manufacturing of renewable feedstocks to valuable compounds, biotechnological processes afford more and more opportunities to produce fuels, building blocks, and solvents in a cost effective way from biomass (Bozell and Petersen, 2010). Chemical buildings blocks, such as some organic acids, serve as precursors for a variety of bulk chemicals and commercially important polymers (Werpy and Petersen, 2004). The cost effective bio-based production of these chemicals is a most relevant goal for the future and has to meet economic and environmental requirements. Therefore, the microbial production systems have to

perform excellent with regard to yield, productivity, product purity and flexibility to substrate consumption.

Corynebacterium glutamicum is a Gram-positive facultative anaerobic organism that grows on a variety of sugars, organic acids, and alcohols as single or combined carbon and energy sources (Eggeling and Bott, 2005; Liebl, 2006; Nishimura et al., 2007; Takeno et al., 2007). The organism is generally regarded as safe (GRAS status) and is traditionally employed for large scale production of amino acids, such as L-glutamate (> 2 million t/a) and L-lysine (> 1.4 million t/a) (Eggeling and Bott, 2005; Takors et al., 2007; Ajinomoto Co., 2010; 2011). In order to improve the production performance by metabolic engineering approaches, the central carbon metabolism, the physiology and the regulation of main and specific pathways of *C. glutamicum* were analyzed in detail and genetic tools as well as systems biology approaches on the 'omics' level have been developed and employed (overviews in Sauer and Eikmanns, 2005; Wendisch et al., 2006a; Eggeling and Bott, 2005; Takors et al., 2007; Bott, 2007; Burkowski 2008; Kirchner and Tauch, 2003; Brinkrolf et al., 2010; Teramoto et al., 2011; Becker and Wittmann, 2011; Vertes et al., 2012). Since *C. glutamicum* is regarded as a robust and easily manageable production host, recent studies also focused on the suitability of this organism for the production of other commodity chemicals, such as the biofuels isobutanol and ethanol (Blombach et al., 2011; Blombach and Eikmanns, 2011; Smith et al., 2010; Inui et al., 2004b), the diamines cadaverine and putrescine (Mimitsuka et al., 2007; Kind et al., 2010a; 2010b; Kind and Wittmann, 2011; Schneider and Wendisch, 2010; 2011), the sugar alcohol xylitol (Sasaki et al., 2010), and also several organic acids (reviewed in this article).

Six years ago, Wendisch et al. (2006b) reviewed the metabolic engineering of *C. glutamicum* and *Escherichia coli* for the biotechnological production of organic acids and amino acids. At that time, *E. coli* was the superior platform organism for the production of organic acids and it was hardly known that *C. glutamicum* forms lactate and succinate under oxygen-deprivation conditions (Dominguez et al., 1993; Inui et al., 2004a; Okino et al., 2005). However, it was foreseeable that genetically modified *C. glutamicum* strains will become promising biocatalysts for the production of at least some organic acids. As outlined in the following, great efforts have been made in the last seven years to successfully implement and/or to improve the production of several organic acids with *C. glutamicum*. This review focuses on the metabolic/genetic engineering approaches to tailor *C. glutamicum* for the fermentative production of pyruvate, D- and L-lactate, 2-ketoisovalerate, 2-ketoglutarate, and succinate from renewable carbon sources. Figure 1 gives an overview on pathways and enzymes of the central metabolism of *C. glutamicum*,

including the pathways for the degradation of selected substrates and those for the synthesis of organic acids produced with this organism.

Production of pyruvate

Pyruvate is broadly used as ingredient or additive in food, cosmetics and pharmaceuticals, but also for the synthesis of various chemicals and polymers (Li et al., 2001; Zhu et al., 2008). Chemical production of pyruvate is realized by dehydration and decarboxylation of tartaric acid, but in a cost-ineffective way (Li et al., 2001; Howard and Fraser, 1932). Different approaches were made for pyruvate production with eukaryotic microorganisms like multi-auxotrophic yeasts (reviewed in Li et al., 2001), however, prokaryotic microorganisms, such as *E. coli* and *C. glutamicum,* also were successfully engineered to produce pyruvate.

Pyruvate is a central intermediate in the carbon and energy metabolism (see Fig. 1) in all organisms and thus, for construction of an efficient pyruvate-producing *C. glutamicum* strain, the major pyruvate-drawing reactions had to be down-regulated or even eliminated. In the course of the molecular analysis of the pyruvate dehydrogenase complex (PDHC), Schreiner et al. (2005) inactivated this complex in *C. glutamicum* by deletion of the *aceE* gene, encoding the E1p subunit of the PDHC. The resulting strain *C. glutamicum* Δ*aceE* required acetate or ethanol as an additional carbon source for growth on glucose (Schreiner et al., 2005; Blombach et al., 2009). In an approach to engineer *C. glutamicum* for L-valine production, Blombach et al. (2007) observed that *C. glutamicum* Δ*aceE* showed a relatively high intracellular concentration of pyruvate and, when acetate was exhausted from the medium and growth stopped, secreted significant amounts of L-alanine (30 mM), L-valine (30 mM), and pyruvate (30 mM) from glucose. In subsequent studies, the PDHC-deficient strain turned out to be an excellent starting point to engineer *C. glutamicum* for the efficient production of L-valine (Blombach et al., 2007; 2008; 2009; Krause et al., 2009), isobutanol (Blombach et al., 2011), and also of 2-ketoisovalerate (Krause et al., 2010; see below), succinate (see below) and pyruvate. The additional inactivation of the pyruvate:quinone oxidoreductase (PQO) and NADH-dependent L-lactate dehydrogenase (L-LDH) significantly improved pyruvate formation (Wieschalka et al., 2012). In shake-flask experiments, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* accumulated in a growth-decoupled manner about 50 mM pyruvate with a substrate specific product yield ($Y_{P/S}$) of 0.48 mol per mol of glucose, aside from L-alanine (29 mM) and L-valine (21 mM) as by-products (Wieschalka et al., 2012). To abolish overflow metabolism towards L-valine, the native acetohydroxyacid synthase (AHAS) was substituted by a leaky variant (ΔC-T IlvN)

leading to an almost threefold increased $Y_{P/S}$ of 1.36 mol pyruvate per mol of glucose, and a strong increase of pyruvate production (up to 193 mM), while L-valine and L-alanine formation were reduced to 1 mM and 9 mM, respectively (Wieschalka et al., 2012). Additional deletion of the genes encoding alanine aminotransferase (AlaT) and valine-pyruvate aminotransferase (AvtA) resulted in cumulative reduction of L-alanine as undesired by-product by 50%. With the final strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC-T *ilvN* Δ*alaT* Δ*avtA* (designated as *C. glutamicum* ELB-P; see Fig. 2) up to 200 mM pyruvate were formed in shake-flask experiments, with a $Y_{P/S}$ of 1.49 mol per mol of glucose. The yields of the by-products L-alanine and L-valine were evanescent low with 0.03 and 0.01 mol per mol of glucose, respectively (Wieschalka et al., 2012). To study the relevance for industrial applications, fed-batch fermentations were performed with *C. glutamicum* ELB-P. When *C. glutamicum* ELB-P was cultivated with a constant pO_2 of about 30% a two-fold lower glucose consumption rate (0.28 mmol $g_{(CDW)}^{-1}$ h^{-1}) and a significantly lower $Y_{P/S}$ (0.8 mol pyruvate per mol of glucose) were observed when compared to shake flask experiments (0.58 mmol $g_{(CDW)}^{-1}$ h^{-1} and 1.49 mol pyruvate per mol of glucose, respectively). Implementation of low oxygen tension from the middle until the end of growth phase restored the production performance and led to the formation of more than 500 mM (45 g/l) pyruvate with a $Y_{P/S}$ of 0.97 mol pyruvate per mol of glucose in the production phase (Wieschalka et al., 2012). In comparison, the best pyruvate-producing *E. coli* strains (*E. coli* YYC202 and ALS1059) produced under optimized process conditions about 720 mM (63 g/l) and 1 M (90 g/l) pyruvate, with $Ys_{P/S}$ of 1.74 and 1.39 mol pyruvate per mol of glucose, respectively (Zelic et al., 2003; Zhu et al., 2008). Since the yield of *C. glutamicum* ELB-P in shake flask experiments is in the same range as in these *E. coli* strains, further process optimization might disclose the whole potential of *C. glutamicum* ELB-P for a further improved pyruvate production process.

Production of lactate

Lactate is widely used as both D- and L-isomers for pharmaceutical, cosmetic, leather and textile, chemical, biomedical and food industries, as well as for green solvent and biodegradable fiber and polymer production (Hofvendahl and Hahn-Hägerdal, 2000; Okano et al., 2010; Bozell and Petersen, 2010). Especially the latter, in form of D- and L-polylactic acid is a fully biodegradable substitute for polyethylene terephthalates and therefore, of great economical interest (Dodds and Gross, 2007; Lorenz and Zinke, 2005). In the past and still today, wildtype and recombinant lactic acid bacteria have been mainly employed for the production of both D- and L-lactate (reviewed in Okano et al., 2010). However, these bacteria have a demand for

complex media, which makes the cultivation of the organisms and the purification of the product relatively cost-intensive. Therefore, other less fastidious organisms, such as metabolically engineered *E. coli, Saccharomyces cerevisiae* and *C. glutamicum* have also been developed for efficient L- and D-lactic acid production (Okano et al., 2010).

C. glutamicum is facultatively anaerobic and grows aerobically and anaerobically in the presence of oxygen and nitrate, respectively (Nishimura et al., 2007; Takeno et al., 2007). The lack of oxygen or nitrate as external electron acceptors results in growth arrested cells, which still have the capability to ferment C6 sugars to L-lactate and succinate as major products. Dominguez et al. (1993) firstly reported that *C. glutamicum* forms lactate, succinate and acetate at small amounts when oxygen is limited during aerobic growth. Inui et al. (2004a) further studied this phenomenon in an attempt to utilize corynebacterial properties for the industrial production of lactate and succinate. These authors reported of organic acid production with *C. glutamicum* strain R and described that the bacteria showed no growth under oxygen-deprivation conditions, but produced significant amounts of L-lactate (~220 mM) and succinate (~20 mM) from about 130 mM glucose. Addition of bicarbonate to the medium led to an increase of the NAD^+/NADH ratio and, probably as consequence of a derepression of the glyceraldehyde-3-phosphate dehydrogenase gene *gapA*, to an increased glucose consumption (Inui et al., 2004a). Furthermore, the addition of bicarbonate led to an altered product spectrum, i.e., the formation of succinate and lactate increased by a factor of two to four and significant concentrations (about 10 mM) of acetate were formed (Inui et al., 2004a; Okino et al., 2005). In a high cell density [30 g $_{\text{dry cell weight (DCW)}}$/l] fed-batch system, *C. glutamicum* R already produced 574 mM L-lactate (i.e., 53 g/l), with only small amounts (< 10 mM) of succinate and acetate as side-products (Okino et al., 2005). Addition of 400 mM bicarbonate raised the L-lactate concentration to more than 1 M (97.5 g/l), but also the concentrations of the by-products succinate (192 mM) and acetate (50 mM) (Okino et al., 2005). Even without genetic modification of *C. glutamicum*, the resulting L-lactate titer from glucose and the $Y_{P/S}$ of 1.79 mol L-lactate per mol of glucose (i.e., 0.90 g/g) are highly competitive as e.g., the best known metabolically engineered L-lactate-producing *E. coli* strain SZ85 (*pflB, frdBC, adhE, ackA, ldhA::ldhL*, overexpressed *ldhL* gene from *Pediococcus acidilactici*) accumulated 505 mM L-lactate (i.e., 46 g/l) with a $Y_{P/S}$ of 1.9 mol per mol of glucose (i.e., 0.95 g/g; Zhou et al., 2003b) (see Table 1).

For D-lactate production with *C. glutamicum*, a L-LDH-deficient mutant was constructed, expressing the D-lactate dehydrogenase (D-LDH) from *Lactobacillus delbrueckii* (Okino et al., 2008b). Under oxygen-deprivation conditions, this mutant

(*C. glutamicum* R Δ*ldhA*/pCRB204) produced in a high cell density system (60 $g_{(DCW)}$ l^{-1}) about 1.34 M D-lactate (i.e. 120 g/l) within 30 h with a $Y_{P/S}$ of 1.73 mol per mol of glucose. But also significant amounts of succinate (146 mM) and actetate (52 mM) were formed, underlining product purity as major problem (Okino et al., 2008b). However, *C. glutamicum* R Δ*ldhA*/pCRB204 produced more D-lactate than *E. coli* JP203 (*pta, ppc*) (Chang et al., 1999) and SZ63 (W3110; *pflB, frdBC,adhE, ackA*) (Zhou et al., 2003a) (Tab. 1), the best known genetically defined D-lactate producing *E. coli* strains, harboring the native D-LDH of *E. coli*. With about 690 mM (62 g/l) and 530 mM (48 g/l), these strains formed approximately half of the titer obtained with *C. glutamicum* R Δ*ldhA*/pCRB204, however, with comparable $Ys_{P/S}$ of between 1.76 and 1.92 mol D-lactate per mol of glucose (0.90 to 0.99 g D-lactate per g of glucose; Chang et al., 1999; Zhou et al., 2003a).

It has to be noted that all described *C. glutamicum* and *E. coli* processes have to compete with those using recombinant yeast strains (*Saccharomyces* and *Kluyveromyces*) that produce L-lactic acid with titers of up to 1.3 M and $Ys_{P/S}$ of up to 1.6 mol L-lactate per mol of glucose (Saitoh et al., 2005; Okano et al., 2010).

Production of 2-ketoisovalerate and 2-ketoglutarate

In nature, 2-ketoisovalerate (3-methyl-2-oxobutanoic acid) is a precursor for L-valine, L-leucine, and pantothenate synthesis in bacteria and plants. In these organisms, it is formed from two molecules of pyruvate via the reactions catalyzed by AHAS, acetohydroxyacid isomeroreductase (AHAIR), and dihydroxyacid dehydratase (DHAD) (see Fig. 1). 2-Ketoisovalerate is used as substitute for L-valine or L-leucine in chronic kidney disease patients (Aparicio et al., 2009; Aparicio et al., 2012; Feiten et al., 2005; Teschan et al., 1998) and also has been used in therapy for uremic hyperphosphatemia (Schaefer et al., 1994). To our knowledge, 2-ketoisovalerate for these purposes is mainly synthesized chemically by different methods (Cooper et al., 1983) and only very recently, directed fermentative production of 2-ketoisovalerate with microorganisms has been reported for the first time (Krause et al., 2010; see below).

Since 2-ketoisovalerate stems from two molecules of pyruvate (see Fig. 1) and a PDHC-deficient *C. glutamicum* secreted significant amounts of pyruvate and L-valine (see above), *C. glutamicum* Δ*aceE* was an excellent basis to engineer *C. glutamicum* for the production of this 2-ketoacid. To avoid transamination of 2-ketoisovalerate to L-valine, the *ilvE* gene encoding transaminase B was deleted, leading to an auxotrophy for branched chain amino acids. Aerobically, *C. glutamicum* Δ*aceE* Δ*ilvE* formed about 76 mM pyruvate, 25 mM L-alanine, and 40 mM 2-ketoisovalerate in a growth-decoupled manner from glucose (Krause et al., 2010). Overexpression of the

AHAS, AHAIR, and DHAD genes shifted the product spectrum towards 2-ketoisovalerate and the resulting strain *C. glutamicum* Δ*aceE* Δ*ilvE* (pJC4ilvBNCD) produced in fed-batch fermentations about 85 mM 2-ketoisovalerate with a volumetric productivity of 1.9 mM h^{-1} and a $Y_{P/S}$ of about 0.38 mol per mol of glucose. Although the PQO has been found to be dispensable for growth and a deletion was only slightly beneficial on L-valine production (Schreiner et al., 2006, Blombach et al., 2008), PQO inactivation turned out to be highly beneficial for 2-ketoisovalerate production. Compared to the parental strain, *C. glutamicum* Δ*aceE* Δ*ilvE* Δ*pqo* (pJC4ilvBNCD) showed in fed-batch fermentations more than two times higher final titers (up to 220 mM = 25.5 g/l) and volumetric productivities of 4.6 mM h^{-1} (Krause et al., 2010; Tab. 1).

It is noteworthy to mention that the 2-ketoisovalerate-producer *C. glutamicum* Δ*aceE* Δ*ilvE* Δ*pqo* (pJC4ilvBNCD) was used as a basis for the generation of a series of *C. glutamicum* strains producing isobutanol via the so-called “Ehrlich pathway” (Blombach et al., 2011; Blombach and Eikmanns, 2011). The most promising strain of this series, *C. glutamicum* Iso7, carries additional deletions of the L-LDH and malate dehydrogenase genes (Δ*ldhA* and Δ*mdh,* respectively) and overexpresses additionally the *E. coli* transhydrogenase genes *pntAB*, the *Lactococcus lactis* ketoacid decarboxylase gene *kivD,* and the homologous alcohol dehydrogenase gene *adhA* (Blombach et al., 2011).

2-Ketoglutarate is an intermediate of the tricarboxylic acid (TCA) cycle and the precursor for the synthesis of glutamate and the glutamate family of amino acids. 2-Ketoglutarate is used in dairy industry (Banks et al., 2001; Gutiérrez-Mendéz et al., 2008) and also is suitable to treat chronic renal insufficiency in hemodialysis patients (Riedel et al., 1996). An enzymatic process to synthesize 2-ketoglutarate from glutamate via the coupled reactions of glutamate dehydrogenase and NADH oxidase has been established (Ödmann et al., 2004), however, this bioconversion seems not very efficient. Therefore, Jo et al. (2012) very recently used a glutamate-overproducing mutant of *C. glutamicum* for the construction of a 2-ketoglutarate-producer. Inactivation of the genes encoding glutamate dehydrogenase, glutamate synthase and isocitrate lyase (*gdh, gltB*, and *aceA*, respectively) led to abolition of glutamate formation and concomitantly to 2-ketoglutarate accumulation to concentrations of up to 325 mM (47.5 g/l) after 120 h of cultivation in medium containing glucose, molasses, glutamate, and soybean hydrolysate (Jo et al., 2012). To our knowledge, there were no other approaches to produce 2-ketoglutarate by fermentation with any other bacterium. However, Zhou et al. (2012) recently reported efficient 2-ketoglutarate production (up to about 380 mM) with a recombinant (“non-

conventional") yeast strain of *Yarrowia lipolytica* with enhanced acetyl-CoA availability.

Production of succinate

The C4 dicarboxylate succinate has been denoted as "a LEGO® of chemical industry" (Sauer et al., 2008) and as such, can be used as precursor for known petrochemical bulk products, such as 1,4-butanediol, tetrahydrofuran, γ-butyrolactone, adipic acid, maleic anhydride, various n-pyrrolidinones, and linear aliphatic esters (Bozell and Petersen, 2010; Sauer et al., 2008; Zeikus et al., 1999). Moreover, succinate (or succinic acid) is directly used as surfactant, ion chelator, and as an additive in pharmaceutical, and food industry (McKinlay et al. 2007). The market potential for succinic acid and its direct derivatives has been estimated to be 245,000 tons per year, that for succinic acid-derived polymers about 25,000,000 tons per year, and with the transition to cost-efficient bio-based production of succinate or succinic acid, the market is predicted to steadily increase (Werpy and Petersen 2004; Bozell and Petersen, 2010).

Aside from L-lactate and acetate, succinate is a natural fermentative endproduct of the wildtype of *C. glutamicum,* when incubated with glucose under oxygen deprivation (Dominguez et al., 1993; Inui et al. 2004a). Under these conditions, succinate is formed via glycolysis, carboxylation of phosphoenolpyruvate (PEP) or pyruvate to oxaloacetate (OAA) by PEP carboxylase (PEPCx) and/or pyruvate carboxylase (PCx), and subsequent conversion of OAA by malate dehydrogenase (Mdh), fumarase (Fum), and succinate dehydrogenase (SDH) (Inui et al. 2004a; see Fig. 1).

A two-stage succinate production process with *C. glutamicum* strain R was developed by Okino et al. (2008a), using a derivative devoid of LDH activity and overexpressing the native PCx gene (*pyc)*, *C. glutamicum* R Δ*ldhA* pCRA717. In a first step, cells of this strain were grown under fully aerobic conditions. Then, the cells were harvested, washed and transferred to closed bottles, to give a high cell density of about 50 $g_{(DCW)}$ l^{-1}. With repeated intermittent addition of glucose and sodium bicarbonate, a final titer of 1.24 M succinate (146 g/l) was obtained within 46 h, with a $Y_{P/S}$ of 1.4 mol per mol of glucose (Okino et al. 2008a). The cells did not form any lactate, however they produced significant amounts of acetate (0.3 M = 16 g/l) as by-product.

Recently, also Litsanov et al. (2012b) engineered *C. glutamicum* ATCC 13032 for high yield succinate production by further extending the experimental approach by Okino et al. (see above). Deletion of the LDH gene, chromosomal integration of an allele for a deregulated PCx (*pyc*^{P458S}) and deletion of the genes encoding enzymes

responsible for acetate synthesis (Δ*cat*, Δ*pqo*, Δ*pta-ack*) resulted in *C. glutamicum* BOL-2, that produces up to 116 mM succinate with a $Y_{P/S}$ of 1.03 mol per mol of glucose, and pyruvate (23 mM) as well as 2-ketoglutarate (12 mM) as major by-products (Litsanov et al., 2012b). To increase NADH and CO_2 availability and to increase the glycolytic flux, the authors then integrated the formate dehydrogenase gene *fdh* from *Mycobacterium vaccae* into the genome of *C. glutamicum* BOL-2 and additionally overexpressed the homologous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (*gapA*) from plasmid. In a fed-batch fermentation with glucose, formate and bicarbonate as substrates, the ultimate strain *C. glutamicum* BOL-3/pAN6-*gap* (see Fig. 3) produced 1.13 M succinate (134 g/l) with a $Y_{P/S}$ of 1.67 mol per mol of glucose (Litsanov et al. 2012b). Aside from succinate, 2-ketoglutarate (35 mM), malate (33 mM), acetate (20 mM), fumarate (13 mM), and pyruvate (6 mM) were formed as by-products.

In a further approach, the pyruvate-producing strain *C. glutamicum* ELB-P (see above and Fig. 2) was employed for succinate production (Wieschalka and Eikmanns, unpublished). Due to the inactivation of the PDHC, PQO, and LDH, this strain does not form significant amounts of acetate or lactate as by-products under any aerobic and anaerobic condition tested (Wieschalka et al., 2012). In contrast to the two-stage-processes described above (i.e., aerobic growth in complex or minimal media and, after harvest of the cells and resuspension in new medium, transfer to sealed bottles or fermenters, respectively; Okino et al., 2008; Litsanov et al., 2012b), a one-stage fed-batch fermentation process with *C. glutamicum* ELB-P was established, combining biomass formation and succinate production in a single bioreactor. This process includes three phases: (i) an aerobic growth phase on glucose plus acetate, (ii) a self-induced microaerobic phase at the end of the exponential growth by minimal aeration, and (iii) an anaerobic production phase, realized by gassing the fermenter with CO_2 (Fig. 4). This optimized process led to growth-decoupled succinate production of more than 330 mM (i.e., 39 g/l) with a $Y_{P/S}$ of 1.02 mol succinate per mol of glucose. The final $Y_{P/S}$ obtained, together with the formation of pyruvate (about 30 mM) as by-product, however, still indicates a limitation, which might be overcome by increasing the carbon flux from PEP/pyruvate to OAA or by integration of the *fdh* gene and the use of formate as an additional substrate for reduction equivalents, as described above by Litsanov et al. (2012b).

The experimental setup of a one-stage process (consecutive aerobic growth and anaerobic production in a single bioreactor) as done with *C. glutamicum* ELB-P, see above) represents an industrially feasible process. However, a recent study on isobutanol production with *C. glutamicum* disclosed the differences in the production performance between two-stage fermentations (aerobic growth in complex or minimal

media and anaerobic production in different containments, see above) and one-stage fermentations in a single bioreactor: The isobutanol $Y_{P/S}$ in the one-stage fermentation was significantly lower (0.48 mol vs. 0.77 mol of isobutanol per mol of glucose), indicating that the transition from the aerobic environment (growth phase) to the anaerobic environment (production phase) has a strong impact on the overall production behavior (Blombach et al., 2011; Blombach and Eikmanns, 2011). A strong negative impact was also seen on pyruvate production with *C. glutamicum* ELB-P cells grown up at constant high pO_2 (> 30 % saturation) in a fermenter instead of grown up in shake flasks, when the cells face increasing oxygen-limitation in the late exponential growth phase (see above; Wieschalka et al., 2012). Similarly, Martínez et al. (2010) recently observed that introducing a microaerobic phase at the end of the aerobic growth phase of an *E. coli* succinate-producer led to an adjustment of the enzymatic machinery and to improved succinate production under anaerobic conditions. To our knowledge, the physiological changes of *C. glutamicum* during a (slow or fast) shift from aerobic to anaerobic conditions have so far not been investigated. However, it can be foreseen that the insight into the metabolic adaptation of the cells to such alternating culture conditions will help to further optimize organic acid production by novel metabolic engineering approaches and also by applying optimally adapted process conditions.

The $Ys_{P/S}$ of the most efficient *E. coli* strains producing succinate under anaerobic conditions, *E. coli* SBS550MG/pHL413 and *E. coli* KJ134, were 1.60 mol and 1.53 mol succinate per mol of glucose, respectively (Sánchez et al. 2005; Jantama et al., 2008; Tab. 1). Thus, both recombinant *E. coli* strains and in particular, *C. glutamicum* BOL-3/pAN6-*gap* (Tab. 1) showed higher $Ys_{P/S}$ than all known natural succinate-producing bacteria, such as *Anaerospirillium succiniproducens* (1.37 mol/mol of glucose; Glassner and Datta, 1992) or *Mannheimia succiniproducens* (1.16 mol /mol glucose; Lee et al., 2006). A further advantage of employing the recombinant *C. glutamicum* or *E. coli* strains is the potential use of mineral media, keeping production and purification costs lower than with *Mannheimia* or *Anaerospirillum*, which both require complex media. *C. glutamicum* BOL-3/pAN6-*gap* and *C. glutamicum* R Δ*ldhA* pCRA717 produced about threefold higher succinate titers than *E. coli* SBS550MG/pHL413 (Tab. 1) and thus, *Corynebacterium* seems to be the superior organism for succinate production.

Very recently, Litsanov et al. (2012a) reported also on aerobic succinate production with *C. glutamicum* for the first time. Deletion of the SDH genes initiated aerobic succinate production in *C. glutamicum* via glycolysis, PEP and/or pyruvate carboxylation, the oxidative branch of the TCA cycle, and the glyoxylate shunt. Acetate formation was mostly prohibited by shut-down of the known pathways for

acetate synthesis, resulting in *C. glutamicum* BL-1 (genotype: Δ*sdhCAB*, Δ*cat*, Δ*pqo*, Δ*pta-ack*; Litsanov et al., 2012a). To reduce carbon-loss into cell mass, nitrogen-limited growth conditions were established, forcing the cells into a resting state after a certain period. With additional, plasmid-bound overproduction of both PEPCx and the PCx^{P458S}-variant, final succinate titers and $Ys_{P/S}$ of up to 90 mM and 0.45 mol succinate per mol of glucose, respectively, were observed (Litsanov et al., 2012a; Tab. 1). Concerning the specific productivity of 1.6 mmol $g_{(CDW)}^{-1}$ h^{-1}, *C. glutamicum* BL-1/pAN6-*pyc*^{P458S}*ppc* showed the highest value described so far for aerobic succinate production from glucose with bacteria.

In comparison to other bacterial succinate producers, *C. glutamicum* BL-1/pAN6-*pyc*^{P458S}*ppc* is exceedingly competitive in aerobic succinate production (Tab. 1). Lin et al. (2005) described various *E. coli* strains approaching the maximal theoretical $Y_{P/S}$ of about 1 mol succinate per mol of glucose under aerobic conditions. *C. glutamicum* BL-1/pAN6-*pyc*^{P458S}*ppc* did not reach this high $Y_{P/S}$, but the recombinant *C. glutamicum* strains produced significantly higher final succinate titers in minimal instead of complex media (Tab. 1).

Production of organic acids with *C. glutamicum* from alternative substrates

Economical relevant and sustainable production of organic acids with microorganisms in an industrial scale is dependent on the use of low-cost carbon sources, in particular from renewable resources. So far, we focused on the fermentative organic acid production from pre-treated and purified carbon sources, such as glucose and glucose plus formate, since the most promising attempts to produce organic acids with *C. glutamicum* were made with these substrates. To simplify feedstock purchase and to improve the economic efficiency, utilization of alternative, crude materials is of great interest. However, *C. glutamicum* naturally cannot utilize certain industrially relevant substrates, such as glycerol, starch (from corn, wheat, rice, or potato), whey, straw, or hemi- and lignocellulose. Especially lignocellulose, consisting largely of cellulose, hemicellulose, and lignin, is a widely abundant and potentially attractive source of renewable feedstock. Hemicellulose, consisting mainly of glucose but also to a significant portion of C_5 sugars (xylose and arabinose) (Wiselogel et al., 1996; Aristidou and Penttilä, 2000), can be depolymerized by chemical or enzymatic processes, and the resulting sugar mixtures are also of interest as alternative feedstock for *C. glutamicum*. Whereas some organisms (e.g., *E. coli*) are naturally able to consume the majority of sugars in the mixtures resulting from saccharification from hemicellulose, *C. glutamicum* needs metabolic engineering to expand the spectrum of sugars that can be utilized. Thus, the extension of the substrate spectrum of *C. glutamicum* to cheap, easily accessible

and renewable monomeric and polymeric carbon sources is desired and therefore, an ongoing field of intensive research (Wendisch et al., 2006; Rumbold et al., 2010; Okano et al., 2010; Blombach and Seibold, 2010; Becker and Wittmann, 2011).

Several attempts have been made to broaden the natural substrate spectrum of *C. glutamicum* towards starch (Seibold et al., 2006; Tateno et al., 2007), whey (Barret et al., 2004), rice straw and wheat bran hydrolysates (Gopinath et al., 2011), glucosides and D-cellobiose (Kotrba et al., 2003), glycerol (Rittmann et al., 2008), or pentose sugars for growth and for the production of amino acids or other value-added products (Blombach and Seibold, 2010; Gopinath et al., 2012; Jojima et al., 2010; Schneider et al., 2011; Buschke et al., 2011). The first approaches to extend the substrate spectrum especially for organic acid production were performed by Kawagutschi et al. (2006; 2008) and Sasaki et al. (2008; 2009). Plasmid-bound introduction of the xylose isomerase and xylulokinase genes (*xylA* and *xylB*, respectively) from *E. coli* into *C. glutamicum* R enabled both aerobic growth on xylose as sole carbon source and production of L-lactate and succinate with resting cells under oxygen deprivation conditions (Kawagutchi et al., 2006). Although the sugar consumption rate and the specific productivity of the recombinant *C. glutamicum* CRX2 was lower with xylose than with glucose, the $Y_{P/S}$ for succinate was even higher on xylose (0.42 mol/mol) than on glucose (0.23 mol/mol). In contrast, the $Y_{P/S}$ for L-lactate was lower with xylose as substrate (1.06 and 1.36 mol/mol, respectively; Kawagutchi et al., 2006). A similar behavior was shown for succinate and L-lactate production from arabinose with *C. glutamicum* CRA1, which expresses the *E. coli* genes *araA*, *araB*, and *araD* (encoding L-arabinose isomerase, L-ribulokinase, and L-ribulose-5-phosphate 4-epimerase, respectively) and therefore is able to metabolize this C5 sugar (Kawagutchi et al., 2008). In this case, with 200 mM arabinose as substrate, the $Ys_{P/S}$ for succinate and L-lactate were 0.67 mol/mol and 0.75 mol/mol, respectively (Kawagutchi et al. 2008).

Co-utilization of different C5 sugars with C6 sugars was investigated to study catabolite repression effects in *C. glutamicum* and to expand sugar utilization on conditioned hemi- and lignocellulosic biomass hydrolysates (Sasaki et al., 2008). These efforts resulted in a *C. glutamicum* strain harbouring *xylA* and *xylB* as well as $bglF^{V317A}$ and *bglA* (encoding PTS β-glucoside-specific enzyme IIBCA component and phospho-β-glucosidase, respectively). This strain produced from a mixture of D-cellobiose (10 g/l), glucose (40 g/l), and D-xylose (20 g/l) about 460 mM L-lactate, 110 mM succinate, and 30 mM acetate under anaerobic conditions, with a combined yield of 0.85 g acids per g of sugar (Sasaki et al., 2008). A combined strain, containing all named modifications for D-xylose, L-arabinose and D-cellobiose consumption, and additionally overexpressing the arabinose transporter gene *araE*

from *C. glutamicum* ATCC31831, was even able to consume glucose (35 g/l), D-xylose (17.5 g/l), L-arabinose (7 g/l), and cellobiose (7 g/l) simultaneously and completely under oxygen-deprived conditions within 14 h (Sasaki et al., 2009).

Recently, Sasaki et al. (2011) developed a *C. glutamicum* strain overexpressing the mannose 6-phosphate isomerase and fructose permease genes *manA* and *ptsF*, respectively. This strain consumed mannose and glucose simultaneously and produced about 400 mM L-lactate, 100 mM succinate and 30 mM acetate from a sugar mixture of 200 mM glucose and 100 mM mannose under oxygen deprivation conditions (Sasaki et al., 2011).

Litsanov et al. (2012c) very recently showed aerobic succinate production with glycerol as sole carbon source, by plasmid-bound transfer of the glycerol utilizing genes *glpFKD* from *E. coli* into *C. glutamicum* BL-1. Glycerol is a main by-product of biodiesel and bioethanol production (Yazdani and Gonzales, 2007) and using this carbon source for the production of value-added chemicals (such as succinate), the economic efficiency of these biofuel production processes can be increased (Wendisch et al., 2011). Plasmid pVWEx1-*glpFKD* has previously been shown to enable growth and amino acid production of *C. glutamicum* on glycerol as sole carbon source (Rittmann et al., 2008). Consequently, using the conditions established for *C. glutamicum* BL-1/pAN6-*pyc*^{P458S}*ppc* (see above), *C. glutamicum* BL-1 (pVWEx1-*glpFKD)* aerobically produced up to 79 mM succinate (9.3 g/l) with a $Y_{P/S}$ of 0.21 mol per mol of glycerol (Litsanov et al. 2012c). The specific succinate productivity of *C. glutamicum* BL-1 pVWEx1-*glpFKD* on glycerol was as high as for *C. glutamicum* BL-1/pAN6-*pyc*^{P458S}*ppc* on glucose with 1.6 mmol $g_{(CDW)}^{-1}$ h^{-1}. However, the volumetric productivity of 3.59 mM h^{-1} is the highest productivity so far described for aerobic succinate production (Litsanov et al., 2012c; Tab. 1).

In summary, the above mentioned studies showed the feasibility to expand the substrate spectrum of *C. glutamicum* to the main C5 and C6 sugars found in agricultural residues, in hydrolyzed hemicellulose and lignocellulosic biomass, and to glycerol. For directed production of organic acids from hemicellulose feedstock, the modifications made for broadening the substrate spectrum and those made for optimal carbon flux to a desired organic acid must be combined. The successful aerobic production of succinate from glycerol instead of glucose by introduction of the glycerol utilizing genes from *E. coli* to *C. glutamicum* (Litsanov et al. 2012c; see above), is one such example and promises the feasibility of such approaches.

Summary and outlook

Driven by old and new knowledge and genome-based metabolic and genetic engineering strategies, *C. glutamicum* has become a major candidate as platform

organism for bio-based, industrial production of a variety of organic acids from renewable biomass. As outlined above and highlighted in Table 1, titers, $Ys_{P/S}$, and productivities of recently developed *C. glutamicum* producer strains are highly competitive, in several cases already superior in comparison to other bacterial, well established production systems. From current studies on transcriptome, proteome, metabolome, and intracellular fluxes, and from recent advances in evolutionary engineering tools it can be expected that a variety of further metabolic (or genetic) targets for strain development/improvement will be identified in *C. glutamicum*. Future approaches to optimize organic acid production certainly will not only aim at substrate flexibility (low cost and eco-efficient feedstocks), product extension (e.g., fumarate, malate, or itaconate), and/or the paths from a substrate or substrate mixtures to the desired products (i.e., substrate uptake, central metabolism, precursor supply, synthetic pathways, and export of the respective organic acid). They will also focus on maintenance of a well-balanced redox state within the cells, on optimal adaptation of the cells to alternating culture conditions (e.g., shift from aerobic to anaerobic conditions), on strain robustness, and on an increased acid-resistance of the producer strains. Tolerance to organic acid stress represents a highly relevant factor for process design and downstream processing of large-scale production processes, since organic acid recovery from low pH fermentation broth in general is more cost-efficient than from neutral broth. However, the achievements obtained in the last 6 years and the wealth of new knowledge about the physiology, the metabolism and its regulation, and the proven production capabilities of *C. glutamicum* bode well for the implementation of this organism as a platform for new and even more (cost) efficient processes for the production of a variety of organic acids and also of other specialty, fine, and bulk chemicals.

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Figure legends

Fig. 1. Schematic presentation of the central carbon metabolism of *C. glutamicum* including pathways for the degradation of carbon sources (glucose, glycerol, D-cellobiose, L-arabinose, D-xylose, mannose, formate, acetate) used for the production of pyruvate, D,L-lactate, 2-ketoisovalerate and succinate. Ellipses represent enzymes and transport systems present in *C. glutamicum*. Rectangles represent heterologous enzymes. *Abbreviations*: Coding genes are given in brackets. 6PG, 6P-gluconate; 6PGDH (*gnd*), 6PG dehydrogenase; AHAIR (*ilvC*), acetohydroxyacid isomeroreductase; AHAS (*ilvBN*), acetohydroxyacid synthase; AK (*ack*), acetate kinase; AlaT (*alaT*), alanine aminotransferase; AraA (*araA* from *E. coli*), arabinose isomerase; AraB (*araB* from *E. coli*), ribulokinase; AraD (*araD* from *E. coli*), L-ribulose-5-phosphate 4-epimerase; AraE (*araE* from *E. coli*), L-arabinose transporter; AvtA (*avtA*), valine-pyruvate aminotransferase; BglA (*bglA1*, *bglA2*), phospho-β-glucosidases; BglF ($bglF^{V317A}$), mutated PTS permease enabling D-cellobiose import; CtfA (*cat*), CoA transferase A; DHAD (*ilvD*), dihydroxyacid dehydratase; DHAP, dihydroxyacetone-P; F1,6P, fructose-1,6P; F6P, fructose-6P; FDH (*fdh* from *Mycobacterium vaccae*), formate dehydrogenase; Fum (*fum*), fumarase; GAP, glyceraldehyde-3P; GAPDH (*gapA*), GAP dehydrogenase; GlpD (*glpD* from *E. coli*), glycerol-3P dehydrogenase; GlpF (*glpF* from *E. coli*), glycerol facilitator; GlpK (*glpK* from *E. coli*), glycerol kinase; G6P, glucose-6P; G6PDH (*zwf*, *opcA*), G6P dehydrogenase; ICD (*icd*), isocitrate dehydrogenase; ICL (*aceA*), isocitrate lyase; LDH (native *ldhA* or *ldhA* from *L. delbrueckii*), L- and D-lactate dehydrogenase, respectively; MalE (*malE*), malic enzyme; MctC (*mctC*) monocarboxylic acid transporter; Mdh (*mdh*), malate dehydrogenase; MQO (*mqo*), malate:quinone oxidoreductase; MS (*aceB*), malate synthase; ODHC (*odhA*, *aceF*, *lpd*), 2-oxoglutarate dehydrogenase complex; ODx (*odx*), oxaloacetate decarboxylase; PCx (*pyc*), pyruvate carboxylase; P, phosphate; PDHC (*aceE*, *aceF*, *lpd*), pyruvate dehydrogenase complex; PEP phosphoenolpyruvate; PEPCk (*pck*), PEP carboxykinase; PEPCx (*ppc*), PEP carboxylase; Pyk (*pyk*), pyruvate kinase; PMI (*manA*), phosphomannose isomerase; PQO (*pqo*), pyruvate:quinone oxidoreductase; PTA (*pta*), phosphotransacetylase; PTS (*ptsG*, *hpr*; *ptsI*), phosphotransferase system; Rpe (*rpe*), ribulose-5-phosphate epimerase; SDH (*sdhABC*), succinate dehydrogenase; TA (*ilvE*), transaminase B; XylA (*xylA* from *E. coli*), xylose isomerase; XylB (*xylB* from *E. coli*), xylulokinase.

Fig. 2. Schematic presentation of the central carbon metabolism of *C. glutamicum* ELB-P with the corresponding enzymes and modifications, leading to pyruvate production under aerobic conditions and reductive succinate production under anaerobic conditions. For most abbreviations see legend to Fig. 1. Further abbreviations: BCAAs, branched-chain amino acids; LdhA, NAD^+-dependent L-lactate dehydrogenase; SucE, succinate exporter; TCA, tricarboxylic acid. Down arrow at AHAS indicates decreased activity of the truncated AHAS derivative, crosses indicate inactivation of the enzyme by deletion of the respective gene.

Fig. 3. Schematic presentation of the central carbon metabolism of *C. glutamicum* BOL-3/pAN6-*gap* during anaerobic succinate production. For most abbreviations see legend to Fig. 1. Further abbreviations: BCAAs, branched-chain amino acids; LdhA, NAD^+-dependent L-lactate dehydrogenase; SucE, succinate exporter; TCA, tricarboxylic acid. Dark ellipses indicate homologous/heterologous enzymes, crosses indicate inactivation of the enzyme by deletion of the respective gene.

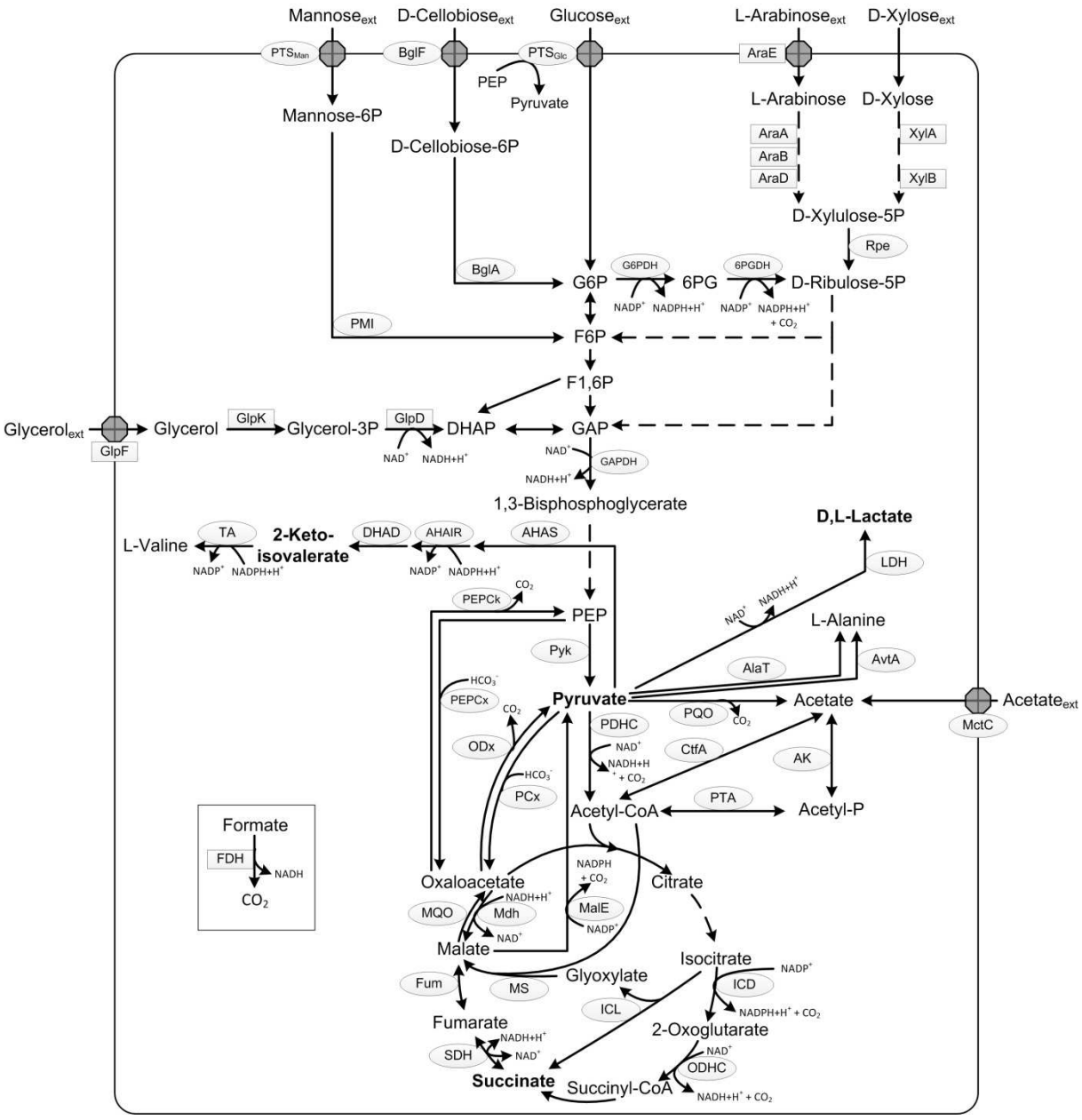
Fig. 4. Growth, glucose consumption, and product formation (A) and acetate consumption and the course of the pO2 (B) during a representative pH-controlled tri-phasic fed-batch cultivation of *C. glutamicum* ELB-P in a 400 ml bioreactor with minimal medium, initially containing 4% (*w*/*v*) glucose, 1% (*w*/*v*) acetate and 6 mM L-alanine. ▲ growth; □ glucose; ● pyruvate; ○ succinate; ▼ malate; ⬢ lactate: Roman numerals indicate (I) aerobic growth phase, (II) self-induced microaerobic phase, and (III) oxygen deprivation by CO_2 gassing. The pO_2 peak at the end of the microaerobic phase (marked with the asterisk in 4B) indicated the end of aerobic growth, as O_2 consumption stopped, leading to increasing DO in the medium. Immediately, aeration was replaced by CO_2 sparging and the production phase started. A batch of glucose at the beginning of phase III should ensure carbon availability for succinate production. At least five independent fermentations were performed, showing comparable results.

Table 1: Maximal titers, substrate specific yields ($Y_{P/S}$), productivities, by-products and the respective references of the so far most efficient processes for organic acid production with *C. glutamicum* and *E. coli* strains.

Strain	Medium	Maximal titer [mM]	$Y_{P/S}$ [$mol_{product}$ per $mol_{substrate}$]	Productivity[a] [mM h^{-1}]	By-products[b]	Reference
Pyruvate						
C. glutamicum ELB-P	minimal medium, **glucose**	512	1.49	5.6	-	Wieschalka et al., 2012
E. coli ALS1059	minimal medium, **glucose**, L-isoleucine, betaine	1022	1.39	23.9	-	Zhu et al., 2008
E. coli YYC202	minimal medium, **glucose**	720	1.74	37	-	Zelic et al., 2003
L-Lactate						
C. glutamicum R	minimal medium, **glucose**	574	1.42	71.8	-	Okino et al., 2005
C. glutamicum R	minimal medium, **glucose**, bicarbonate	1061	1.79	176.8	acetate, succinate	Okino et al., 2005
E. coli SZ85	minimal medium, **glucose**	505	1.90	7.2	-	Zhou et al., 2003b
D-Lactate						
C. glutamicum R Δ*ldhA* pCRB204	minimal medium, **glucose**	1340	1.73	44.5	acetate, succinate	Okino et al., 2008b
E. coli JP203	complex medium, **glucose**	691	1.80	11.6	-	Chang et al., 1999
E. coli SZ63	minimal medium, **glucose**	528	1.92	9.8	-	Zhou et al., 2003a
2-ketoisovalerate						

C. glutamicum Δ*aceE* Δ*pqo* Δ*ilvE* (pJC4ilvBNCD)	minimal medium, **glucose**, yeast extract	188	0.56	4.6	L-valine	Krause et al., 2010
2-ketoglutarate						
C. glutamicum R Δ*gdh* Δ*gltB* Δ*aceA*	complex medium, glucose, molasses, soybean hydrolysate	325	n.s.[c]	2.7	-	Jo et al., 2012
***Succinic acid* (anaerobic)**						
C. glutamicum R Δ*ldhA* pCRA717	minimal medium, **glucose**, bicarbonate	1240	1.40	27	acetate	Okino et al., 2008a
C. glutamicum ELB-P	minimal medium, **glucose**	330	1.02	5.6	pyruvate	own unpublished data
C. glutamicum BOL-3/pAN6-*gap*	saline, **glucose**, **formate**, bicarbonate	1134	1.67	21	2-oxoglutarate, acetate, fumarate, malate	Litsanov et al., 2012b
E. coli SBS550MG/pHL413	complex medium, **glucose**	330	1.61	10	acetate, formate	Sánchez et al., 2005
E. coli KJ134	minimal medium, **glucose**	606	1.53	6.4	acetate, pyruvate, malate	Jantama et al., 2008
***Succinic acid* (aerobic)**						
C. glutamicum BL-1/pAN6-*pyc*^{P458S}*ppc*	minimal medium, **glucose**	90	0.45	0.8	2-oxoglutarate, acetate, pyruvate	Litsanov et al., 2012a
C. glutamicum BL-1 pVWEx1-*glpFKD*	minimal medium, **glycerol**	79	0.21	3.6	acetate	Litsanov et al., 2012c
E. coli HL51276k(pKK313)	complex medium, **glucose**, bicarbonate	70	1.09	1.2	acetate, pyruvate	Lin et al., 2005
E. coli HL27659k(pKK313)	complex medium, **glucose**, bicarbonate	60	0.95	2.3	acetate	Lin et al., 2005

[a] during production phase, [b] significant concentrations above 10 mM, [c] n.s. = not specified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D-Xylose$_{ext}$
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BglF
PTS$_{Glc}$
AraE
PEP
Pyruvate
Mannose-6P
D-Cellobiose-6P
L-Arabinose
D-Xylose
AraA
AraB
AraD
XylA
XylB
D-Xylulose-5P
Rpe
BglA
G6PDH
6PGDH
G6P
6PG
D-Ribulose-5P
NADP$^+$
NADPH+H$^+$
+ CO$_2$
PMI
F6P
F1,6P
Glycerol$_{ext}$
GlpF
Glycerol
GlpK
Glycerol-3P
GlpD
DHAP
GAP
NAD$^+$
NADH+H$^+$
GAPDH
1,3-Bisphosphoglycerate
D,L-Lactate
LDH
L-Valine
TA
2-Keto-isovalerate
DHAD
AHAIR
AHAS
PEPCk
CO$_2$
PEP
L-Alanine
Pyk
AlaT
AvtA
HCO$_3^-$
PEPCx
Pyruvate
Acetate
Acetate$_{ext}$
PQO
PDHC
MctC
ODx
CtfA
AK
PCx
Acetyl-CoA
PTA
Acetyl-P
Formate
FDH
NADH
Oxaloacetate
Citrate
MQO
Mdh
MalE
Malate
Isocitrate
Fum
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ICD
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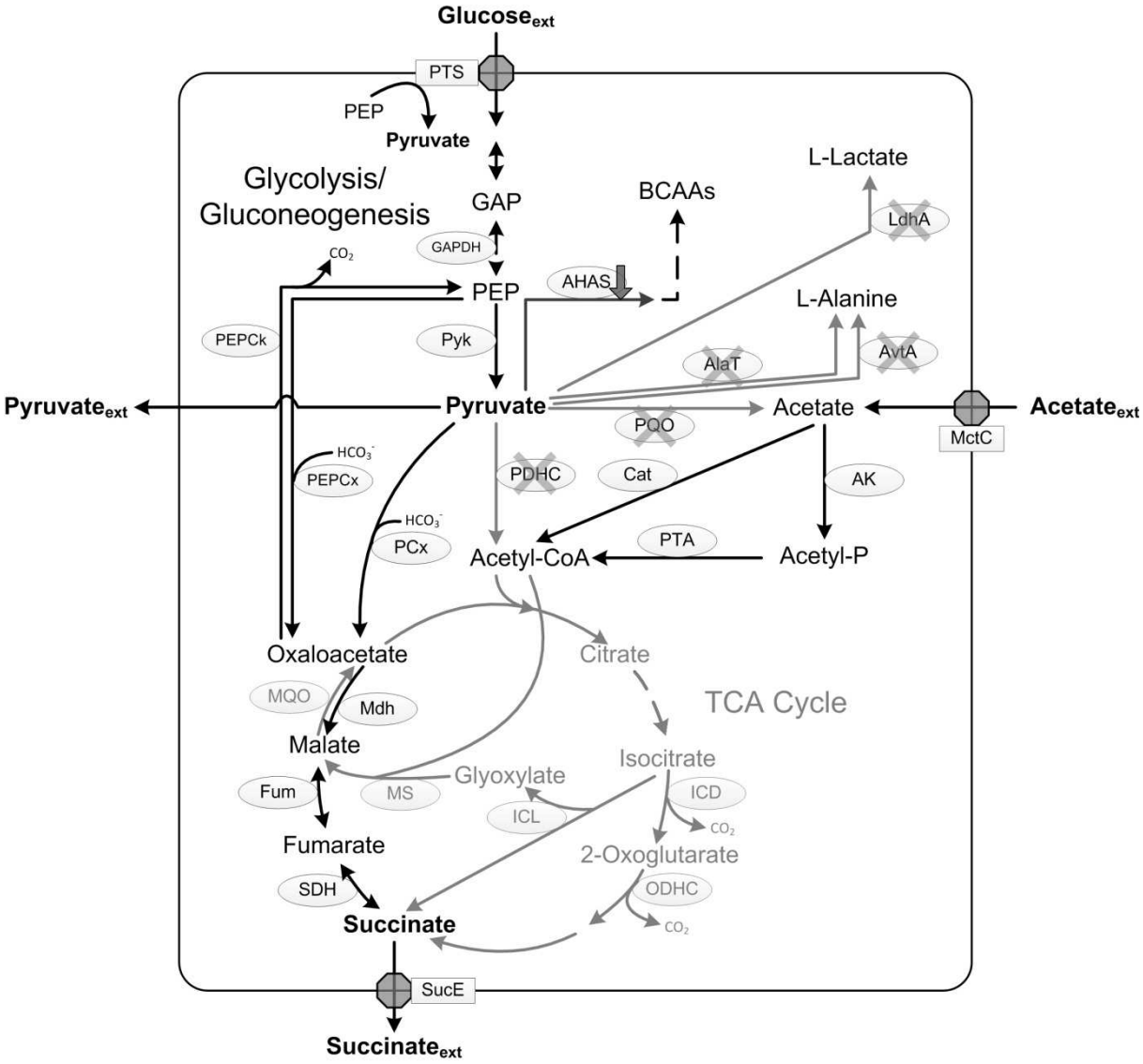
SDH
Succinate
Succinyl-CoA
ODHC

Fig. 1.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LdhA
L-Alanine
AvtA
AlaT
PEPCk
Pyk
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Acetate
Acetate$_{ext}$
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POO
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PEPCx
PDHC
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AK
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PTA
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Acetyl-P
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Citrate
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Mdh
Malate
Isocitrate
Glyoxylate
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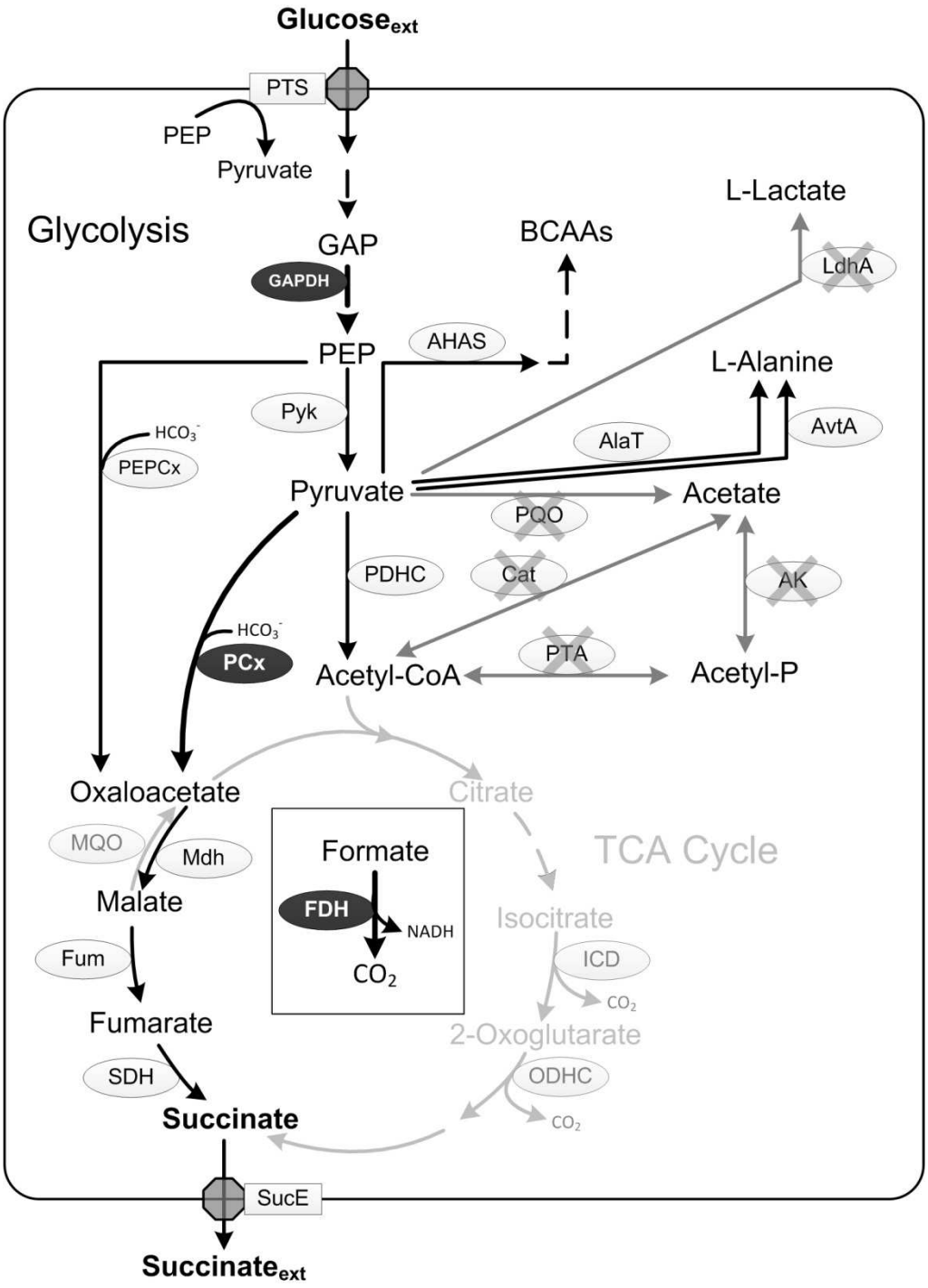
Fig. 2.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Pyk
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Pyruvate
Acetate
PQO
PDHC
Cat
AK
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PCx
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Succinate
SucE
Succinate$_{ext}$

Fig. 3.

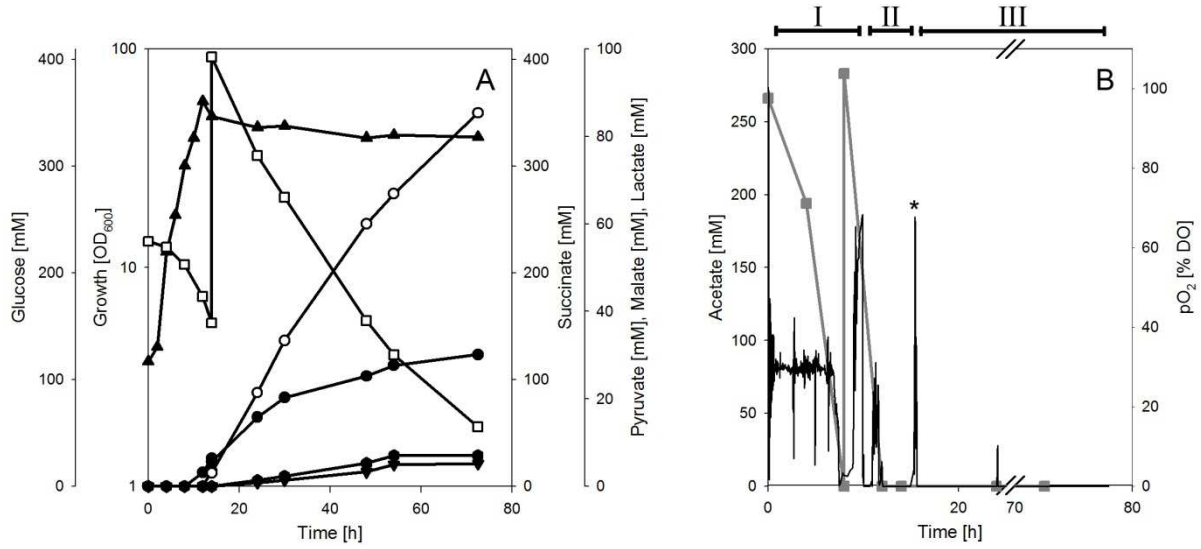
A
Glucose [mM]
Growth [OD600]
Succinate [mM]
Pyruvate [mM], Malate [mM], Lactate [mM]
Time [h]
B
I
II
III
Acetate [mM]
pO2 [% DO]
Time [h]
*

Fig. 4.

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Lebenslauf

Lebenslauf aus datenschutzrechtlichen Gründen entfernt.

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Alle aus der Literatur wörtlich oder inhaltlich übernommenen Zitate sind als solche kenntlich gemacht.

Weiterhin erkläre ich, dass diese Arbeit weder vollständig noch in Auszügen einer anderen Fakultät vorgelegt worden ist, mit dem Ziel einen akademischen Titel zu erwerben. Ich bewerbe mich hiermit erstmalig um den Doktorgrad der Naturwissenschaften der Universität Ulm.

Ulm, den 19.10.2012

Stefan Wieschalka