

Efficient aerobic succinate production from glucose in minimal medium with *Corynebacterium glutamicum*

Boris Litsanov, Armin Kabus,[†] Melanie Brocker and Michael Bott*

Institut für Bio- und Geowissenschaften, IBG-1:
Biotechnologie, Forschungszentrum Jülich, D-52425
Jülich, Germany.

Summary

Corynebacterium glutamicum, an established industrial amino acid producer, has been genetically modified for efficient succinate production from the renewable carbon source glucose under fully aerobic conditions in minimal medium. The initial deletion of the succinate dehydrogenase genes (*sdhCAB*) led to an accumulation of 4.7 g l⁻¹ (40 mM) succinate as well as high amounts of acetate (125 mM) as by-product. By deleting genes for all known acetate-producing pathways (*pta-ackA*, *pqo* and *cat*) acetate production could be strongly reduced by 83% and succinate production increased up to 7.8 g l⁻¹ (66 mM). Whereas overexpression of the glyoxylate shunt genes (*aceA* and *aceB*) or overproduction of the anaplerotic enzyme pyruvate carboxylase (PCx) had only minor effects on succinate production, simultaneous overproduction of pyruvate carboxylase and PEP carboxylase resulted in a strain that produced 9.7 g l⁻¹ (82 mM) succinate with a specific productivity of 1.60 mmol g (cdw)⁻¹ h⁻¹. This value represents the highest productivity among currently described aerobic bacterial succinate producers. Optimization of the production conditions by decoupling succinate production from cell growth using the most advanced producer strain (*C. glutamicum* Δ *pqo* Δ *pta-ackA* Δ *sdhCAB* Δ *cat*/pAN6-*pyc*^{P458S}*ppc*) led to an additional increase of the product yield to 0.45 mol succinate mol⁻¹ glucose and a titre of 10.6 g l⁻¹ (90 mM) succinate.

Introduction

The four-carbon dicarboxylate succinate is used in the pharmaceutical, agricultural and food industry (Zeikus

et al., 1999). The present market for succinate is rather small but as succinate chemistry is similar to that of maleic anhydride, it could serve as a replacement for the synthesis of bulk chemicals such as butanediol, tetrahydrofuran and γ -butyrolactone, which are currently produced in a total annual amount of more than 500 000 t (McKinlay *et al.*, 2007).

Presently, succinate is predominantly produced chemically on the basis of petroleum, but microbial production of succinate from renewable carbon sources is a promising environmentally friendly approach for obtaining this building block. The US Department of Energy identified biotechnologically derived succinate as one of the top 12 value-added chemicals that could be produced in large quantities from biomass (Werpy and Peterson, 2004). Although the production of bio-based succinate as bulk chemical is still cost-intensive compared with petrochemically based alternatives, increasing oil and gas prices as well as the public's and the industry's growing interest for 'green' products raise the demand for the development of a competitive microbial production process (Hermann and Patel, 2007). These facts draw the attention to organisms which can produce succinate at high rates with little or no by-products and to efficiently used substrates to achieve an inexpensive fermentation and purification process.

Succinate can be formed by a wide variety of bacteria in the course of anaerobic fermentative metabolism, such as, for example, *Pasteurellaceae* species, *Escherichia coli* and *Corynebacterium glutamicum*. Whereas *Pasteurellaceae* species such as *Mannheimia succiniciproducens* and *Basfia succiniciproducens* or *Anaerobiospirillum succiniciproducens*, a member of the *Succinivibrionaceae*, produce succinate as the major end-product of fermentation, non-modified *E. coli* strains accumulate succinate only as a minor product (Samuelov *et al.*, 1991; Guettler *et al.*, 1999; Lee *et al.*, 2002; Inui *et al.*, 2004b; Jantama *et al.*, 2008; Scholten and Dagele, 2008). Under anaerobic conditions in the presence of bicarbonate, *C. glutamicum* does not grow, but efficiently converts glucose to succinate, lactate and acetate (Inui *et al.*, 2004b). Based on the above mentioned bacterial species a number of processes with metabolically engineered strains and/or optimized production conditions have been developed (Samuelov *et al.*, 1999; Okino *et al.*, 2008; Oh *et al.*, 2009; Scholten *et al.*, 2009; Zhang *et al.*, 2009). Although anaerobic succinate production

Received 27 May, 2011; revised 29 July, 2011; accepted 9 September, 2011. *For correspondence. E-mail m.bott@fz-juelich.de; Tel. (+49) 2461 613294; Fax (+49) 2461 612710. [†]Present address: Momentive Performance Materials GmbH, D-51368 Leverkusen, Germany.

allows high yields and titres, it also poses some problems: (i) the substrate spectrum which allows an optimal redox balance is limited. For example, glucose as substrate requires the formation of by-products in order to provide sufficient reducing equivalents for the maximal possible reduction of oxaloacetate to succinate (San *et al.*, 2002). In contrast, conversion of glycerol and carbon dioxide to succinate offers a closed redox balance and does not necessitate by-product formation. (ii) Long production processes combined with slow anaerobic growth, which are typical for anaerobic fermentations, can result in overall low volumetric productivity (McKinlay *et al.*, 2007). (iii) As most of the best anaerobic producers do not grow in minimal media (Lee *et al.*, 2001; Oh *et al.*, 2009; Scholten *et al.*, 2009), complex media supplements are needed which do not only raise the costs for the educts, but potentially also complicate product purification (McKinlay *et al.*, 2007). All these problems could be circumvented by using an aerobic succinate producer.

So far only a few aerobic succinate producers have been described. For example an extensively engineered *E. coli* strain has been developed that produces succinate from glucose under fully aerobic conditions and reaches the theoretical maximal yield of 1 mol mol⁻¹, but with low specific productivity of 0.62 mmol g cell dry weight (cdw)⁻¹ h⁻¹. However, probably due to its genetic modifications, the growth-coupled production process with this strain was performed in LB medium which contains trypton and yeast extract (Lin *et al.*, 2005a). The yeast *Yarrowia lipolytica* uses glycerol as substrate and accumulates succinate in non-buffered medium reaching a final pH of 3.2. By omitting pH neutralization, fermentation and purification costs could be decreased. However, the volumetric productivity is low (0.87 mM h⁻¹) and like for the *E. coli* production process described above, complex media are necessary for growth and product formation with *Y. lipolytica* (Yuzbashev *et al.*, 2010). A genetically engineered *Saccharomyces cerevisiae* strain for aerobic succinate production is also tolerant to low pH values and does not require pH neutralization, but accumulates only low succinate concentrations (30 mM) with a yield of 0.11 mol mol⁻¹ and a volumetric productivity of 0.17 mM h⁻¹ (Raab *et al.*, 2010).

In view of these facts, we started to explore the potential of *C. glutamicum* for aerobic succinate production. *Corynebacterium glutamicum* is a facultatively anaerobic, Gram-positive soil bacterium with GRAS status (generally regarded as safe), which is used for the large-scale production of more than 2 million tons of L-glutamate and 1.1 million tons of L-lysine annually. In addition, *C. glutamicum* strains were developed for the production of several other industrially relevant products such as putrescine (Schneider and Wendisch, 2010), isobutanol (Blombach *et al.*, 2011) or ethanol (Inui *et al.*, 2004a). The

genome of *C. glutamicum* is known (Ikeda and Nakagawa, 2003; Kalinowski *et al.*, 2003; Yukawa *et al.*, 2007) and numerous genetic tools are available allowing genetic engineering (Kirchner and Tauch, 2003). Moreover, extensive knowledge on the central metabolism of *C. glutamicum* is available due to 60 years of research on amino acid production (Eggeling and Bott, 2005; Burkovski, 2008). Novel results relevant for succinate production represent the identification of two genes coding for succinate importers, *dccT* (Youn *et al.*, 2008) and *dctA* (Youn *et al.*, 2009), and of a gene coding for a succinate exporter, *sucE* (Huhn *et al.*, 2011). Based on the extensive knowledge available and the fact that anaerobic succinate production with this species has been demonstrated, a *C. glutamicum* strain for efficient aerobic succinate production from glucose was constructed in this work by metabolic engineering, using similar strategies as described for *E. coli* (Lin *et al.*, 2005a,b; Wendisch *et al.*, 2006). The key mutation required for succinate production was the deletion of the succinate dehydrogenase genes. Subsequent deletion of all known acetate-producing pathways as well as overproduction of phosphoenolpyruvate carboxylase and pyruvate carboxylase led to significantly increased succinate production (Fig. 1). Finally, the production process was optimized by limiting biomass formation, thereby providing more carbon for succinate production. In summary, our results demonstrate for the first time the potential of *C. glutamicum* for aerobic succinate production in minimal medium.

Results

Inactivation of the succinate dehydrogenase complex for aerobic succinate production

During aerobic metabolism *C. glutamicum* uses the TCA cycle for generation of reducing equivalents and precursors for amino acids (Bott, 2007). Under these conditions succinate is an intermediate of the oxidative TCA cycle and does not accumulate in the supernatant. In order to allow the accumulation of succinate as an end-product of aerobic metabolism, the succinate dehydrogenase complex encoded by the *sdhCAB* genes must be inactivated. For this purpose, a *C. glutamicum* Δ *sdhCAB* deletion mutant was constructed which is completely unable to oxidize succinate to fumarate (Fig. 1). The growth behaviour and product formation of this strain were compared with those of the wild type. For this purpose, independent batch cultivations in modified CGXII medium with 4% (w/v) glucose as carbon source were performed in the Multifors bioreactors. To prevent acidification of the medium caused by organic acid production the pH was kept at 7.0. Oxygen limitation was avoided by keeping pO₂ at > 30% saturation. During cultivation growth, glucose consumption and organic acid production were measured

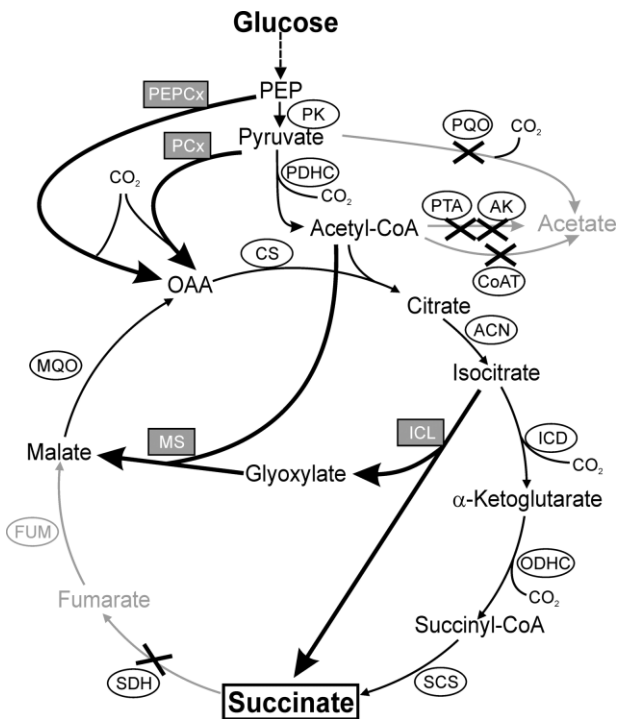


Fig. 1. Scheme of the central metabolism of *C. glutamicum* showing the genetic modifications used in this work to construct a strain for aerobic succinate production. Enzymes whose genes were deleted are indicated by 'X'. The reactions affected by these deletions and their products are displayed in grey. Enzymes whose genes were overexpressed are highlighted in grey boxes and the arrows for the corresponding reactions are thickened. Abbreviations: ACN, aconitase; AK, acetate kinase; CoAT, acetyl-CoA:CoA transferase; CS, citrate synthase; FUM, fumarase; ICD, isocitrate dehydrogenase; ICL, isocitrate lyase; MQO, malate:menaquinone oxidoreductase; MS, malate synthase; OAA, oxaloacetate; ODHC, 2-oxoglutarate dehydrogenase complex; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PEPCx, PEP carboxylase; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; PTA, phosphotransacetylase; PQA, pyruvate:menaquinone oxidoreductase; SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase.

(Fig. 2). *Corynebacterium glutamicum* Δ *sdhCAB* exhibited a 9% reduced growth rate and 28% decreased biomass formation compared with the wild type (Table 1). The wild-type cells consumed glucose with an uptake rate of $4.30 \text{ mmol g (cdw)}^{-1} \text{ h}^{-1}$ and did not produce measurable quantities of succinate or other organic acids in the culture broth. *Corynebacterium glutamicum* Δ *sdhCAB* showed growth-coupled accumulation of succinate from the beginning of the cultivation up to the end of the exponential growth phase with a specific production rate of $0.75 \text{ mmol g (cdw)}^{-1} \text{ h}^{-1}$. At the end of the cultivation after 22.5 h the succinate concentration in the supernatant reached 40 mM. The succinate yield ($0.18 \text{ mol succinate mol}^{-1} \text{ glucose}$) represented 18% of the theoretically maximal yield ($1 \text{ mol succinate mol}^{-1} \text{ glucose}$). The major product formed during cultivation of *C. glutamicum* Δ *sdhCAB* was

acetate, which accumulated in parallel to succinate and reached a concentration of 125 mM ($0.56 \text{ mol mol}^{-1} \text{ glucose}$) after 22.5 h.

Reduction of acetate formation

To reduce acetate formation, the genes for the known acetate-forming pathways in *C. glutamicum*, namely *pqo*, *cat* and *pta-ackA* coding for pyruvate:menaquinone oxidoreductase (PQO), acetyl-CoA:CoA transferase (CoAT), phosphotransacetylase (PTA) and acetate kinase (AK), respectively, were deleted in the Δ *sdhCAB* mutant. Because of organic acid production and the resulting growth defect of *C. glutamicum* Δ *sdhCAB*, the deletion of *pqo* and *pta-ackA* was performed first in *C. glutamicum* wild type and then the deletion of the *sdhCAB* operon was introduced. In the course of strain construction the identification of acetyl-CoA:CoA transferase (CoAT) and its gene *cat* in *C. glutamicum* (Veit *et al.*, 2009) revealed another target for reducing acetate production. The *cat* gene was deleted in the strain *C. glutamicum* Δ *pqo* Δ *pta-ackA* Δ *sdhCAB* resulting in strain *C. glutamicum* BL-1 (Δ *pqo* Δ *pta-ackA* Δ *sdhCAB* Δ *cat*) (Fig. 1). Subsequently, growth and organic acid production of strain BL-1 was analysed under the same conditions as described above. As shown in Fig. 2, it exhibited comparable growth rates ($\mu = 0.33 \text{ h}^{-1}$), biomass formation [$13.0 \text{ g (cdw)}^{-1}$] and glucose uptake rates [$5.54 \text{ mmol g (cdw)}^{-1} \text{ h}^{-1}$] as the strain *C. glutamicum* Δ *sdhCAB* (Table 1). The succinate concentration in the supernatant increased exponentially from the beginning of the cultivation reaching a maximal concentration of 66 mM after 22.5 h. This value represented a 65% increase of the final succinate titre and a 55% increase in yield ($0.28 \text{ mol succinate mol}^{-1} \text{ glucose}$) compared with *C. glutamicum* Δ *sdhCAB*. The specific succinate production rate [$1.39 \text{ mmol g (cdw)}^{-1} \text{ h}^{-1}$] was increased about 85% compared with that of the Δ *sdhCAB* strain. The deletion of *pqo*, *pta-ackA* and *cat* led to an 82% reduction in acetate formation compared with the Δ *sdhCAB* strain; however, with 22 mM formed after 22.5 h acetate was still the major by-product. The reasons for the residual acetate formation will be discussed below.

Role of anaplerotic reactions and of the glyoxylate shunt for aerobic succinate production by *C. glutamicum* BL-1

The production of succinate from glucose under aerobic conditions is strongly dependent on anaplerotic reactions forming oxaloacetate as substrate for citrate synthase and thus for initiation of the oxidative TCA cycle. Therefore three strategies to improve the availability of oxaloacetate were tested (Fig. 1). (i) Overproduction of pyruvate carboxylase (PCx): previous work showed that the ATP-dependent carboxylation of pyruvate to oxaloacetate

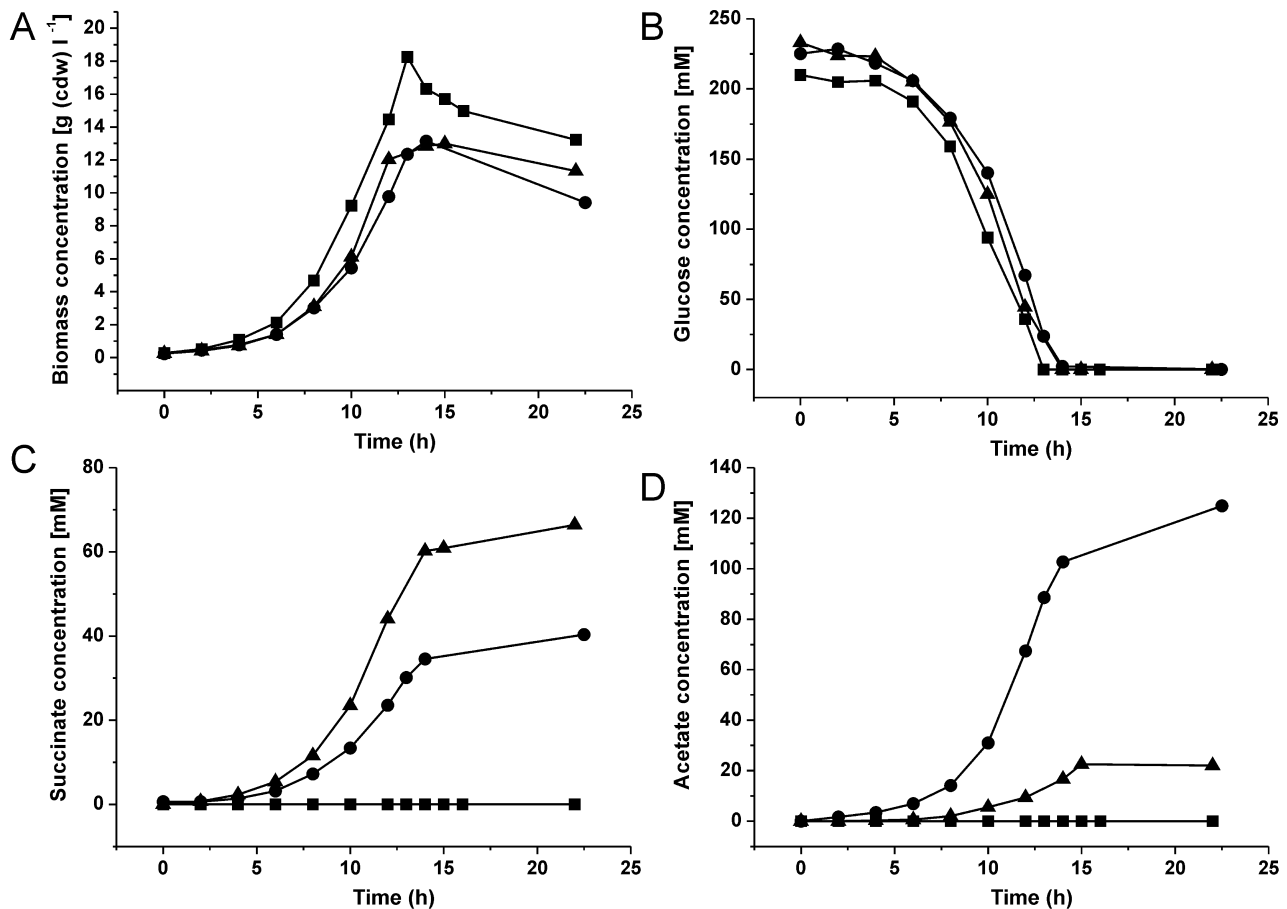


Fig. 2. Comparison of biomass formation (A), glucose consumption (B), succinate production (C) and acetate production (D) of *C. glutamicum* wild type (squares, $n = 3$), *C. glutamicum* Δsdh (circles, $n = 4$) and *C. glutamicum* BL-1 (triangles, $n = 2$) in aerobic batch cultivations in modified CGXII medium containing 4% (w/v) glucose under constantly controlled conditions of pH 7.0 and $pO_2 > 30\%$ using a Multifors bioreactor system. The results displayed are mean values from n independent experiments.

catalysed by PCx is responsible for 90% of the oxaloacetate replenishment during aerobic growth of *C. glutamicum* on glucose (Petersen *et al.*, 2000). The amino acid exchange P458S in PCx was reported to be beneficial for lysine production by *C. glutamicum* (Ikeda

et al., 2006), although the molecular effect on PCx is not yet clear. To test the influence of a higher PCx activity on succinate production, the expression plasmid pAN6-*pyc*^{P458S} was constructed. (ii) Simultaneous over-production of pyruvate carboxylase (PCx) and phos-

Table 1. Growth and organic acid production parameters of different *C. glutamicum* succinate producer strains during aerobic cultivation in modified CGXII medium containing 4% (w/v) glucose, 25 mg l⁻¹ kanamycin (plasmid-containing strains only) and 0.5 mM IPTG (plasmid-containing strains only) at pH 7.0 and $pO_2 > 30\%$ saturation.

<i>C. glutamicum</i> strain	Growth rate (h ⁻¹)	Biomass [g (cdw) l ⁻¹]	Glucose consumed (mM)	Glucose uptake rate [mmol g (cdw) ⁻¹ h ⁻¹]	Final acetate titre (mM)	Final succinate titre (mM)	Succinate production rate [mmol g ⁻¹ (cdw) h ⁻¹]	Yield succinate/glucose (mol mol ⁻¹)
Wild type	0.34	18.1	210	4.30	n.d.	n.d.	n.d.	n.d.
Δsdh	0.31	13.1	225	5.29	125	40	0.75	0.18
BL-1	0.33	13.0	233	5.54	22	66	1.39	0.28
BL-1 (pAN6- <i>pyc</i> ^{P458S})	0.30	12.2	242	5.49	20	68	1.44	0.28
BL-1 (pAN6- <i>pyc</i> ^{P458S} <i>ppc</i>)	0.29	12.2	230	5.15	20	82	1.60	0.36
BL-1 (pAN6- <i>aceAaceB</i>)	0.27	10.5	235	5.19	22	72	1.38	0.30

All parameters describing rates were calculated for the exponential growth phase of the cultures. The data presented are mean values from at least two independent batch cultivations.

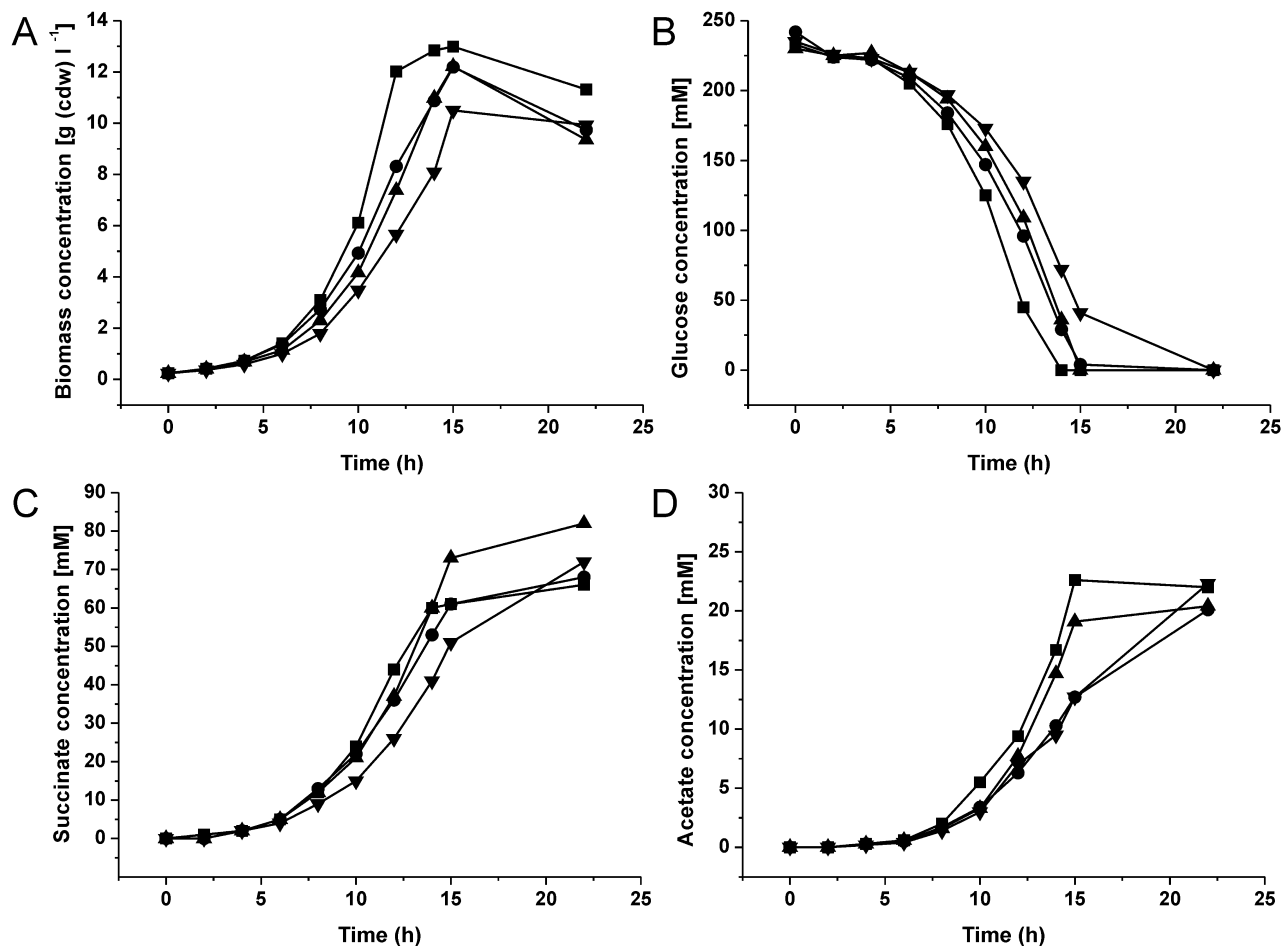


Fig. 3. Influence of overexpression of genes for anaplerotic enzymes in *C. glutamicum* strain BL-1 on biomass formation (A), glucose consumption (B), succinate production (C) and acetate production (D). The following strains were tested: BL-1 (squares), BL-1 with pAN6-*pcx*^{P458S} (pyruvate carboxylase, circles), BL-1 with pAN6-*pcx*^{P458S}/*ppc* (pyruvate carboxylase and PEP carboxylase, triangles pointing upwards) and BL-1 with pAN6-*aceAaceB* (isocitrate lyase and malate synthase, triangles pointing downwards). The strains were cultivated in modified CGXII medium containing 4% (w/v) glucose, 25 mg l⁻¹ kanamycin and 0.5 mM IPTG under constantly control conditions of pH 7.0 and pO₂ > 30% using a Multifors bioreactor system. Mean values from two independent cultivations are shown.

phenolpyruvate carboxylase (PEPCx): PEPCx, which catalyses the carboxylation of PEP to oxaloacetate (Mori and Shiio, 1985; Eikmanns *et al.*, 1989), was described to be responsible for only 10% of the oxaloacetate replenishment in *C. glutamicum* during growth on glucose (Petersen *et al.*, 2000). Nevertheless, the combined overproduction of PEPCx (encoded by the *ppc* gene) with PCx^{P458S} was expected to further stimulate the synthesis from oxaloacetate and therefore the expression plasmid pAN6-*pcx*^{P458S}/*ppc* was constructed. (iii) Overproduction of the glyoxylate shunt enzymes isocitrate lyase (ICL) and malate synthase (MS): an enhanced carbon flux through the glyoxylate shunt by overproduction of ICL and MS could be a very promising option for anaplerotic oxaloacetate formation. By bypassing isocitrate dehydrogenase and the 2-oxoglutarate dehydrogenase complex (ODHC) in the TCA cycle, less CO₂ is produced and the glyoxylate

shunt itself could be used as alternative succinate production pathway besides the oxidative part of the TCA cycle (Fig. 1). This strategy was also used for the design of the currently best aerobic succinate producer of *E. coli* (Lin *et al.*, 2005b). For overproduction of ICL and MS the expression plasmid pAN6-*aceAaceB* was constructed which contains the genes *aceA* and *aceB* coding for ICL and MS respectively (Reinscheid *et al.*, 1994a,b).

For the succinate production experiments, strain BL-1 was transformed with each of the three above mentioned plasmids resulting in the *C. glutamicum* strains BL-1/pAN6-*pcx*^{P458S}, BL-1/pAN6-*pcx*^{P458S}/*ppc* and BL-1/pAN6-*aceAaceB*. These strains were analysed and compared in independent batch cultivations with modified CGXII medium containing 4% (w/v) glucose, 25 mg l⁻¹ kanamycin and 0.5 mM IPTG at pH 7.0 and pO₂ > 30% saturation (Fig. 3). Surprisingly, overproduction of PCx had no

influence on the glucose uptake rate and on succinate production compared with the parental strain BL-1, as no significant increase of the succinate titre (68 mM), the succinate production rate [$1.44 \text{ mmol g (cdw)}^{-1} \text{ h}^{-1}$] or the succinate yield ($0.28 \text{ mol mol}^{-1}$ glucose) was detected (Table 1). Overproduction of PCx led to a decreased growth rate (0.30 h^{-1}) and biomass formation [$12.2 \text{ g (cdw)} \text{ l}^{-1}$]. Acetate accumulation was decelerated, but the final acetate concentration (20 mM) reached the level of the BL-1 strain. The combined overexpression of *pyc*^{P458S} and *ppc* in the strain BL-1/pAN6-*pyc*^{P458S}*ppc* led to an increased succinate production rate [$1.60 \text{ mmol g (cdw)}^{-1} \text{ h}^{-1}$] and the final succinate titre was raised about 25% to 82 mM compared with the parental strain (Table 1), which is also reflected in a 28% higher succinate yield ($0.36 \text{ mol mol}^{-1}$ glucose). Interestingly, although the succinate production rate was increased by 15%, the glucose consumption rate was reduced by 7% to $5.15 \text{ mmol g (cdw)}^{-1} \text{ h}^{-1}$. Overexpression of *pyc*^{P458S} and *ppc* had no effect on acetate accumulation (Table 1). The functional overexpression of *aceA* and *aceB* in BL-1/pAN6-*aceAaceB* was confirmed by *in vitro* enzymatic activity measurements. The specific activity of ICL (encoded by *aceA*) was 0.79 U mg^{-1} protein, the specific activity of MS (*aceB*) was 0.51 U mg^{-1} protein. The overexpression of *aceA* and *aceB* in strain BL-1/pAN6-*aceAaceB* had a negative influence on the growth rate (0.27 h^{-1}) and on biomass formation [$10.5 \text{ g (cdw)} \text{ l}^{-1}$] compared with the Δ *sdhCAB* mutant (Table 1). The glucose uptake rate was reduced compared with the BL-1 strain [5.19 versus $5.54 \text{ mmol g (cdw)}^{-1} \text{ h}^{-1}$], whereas the succinate production rate was the same as for the BL-1 strain [1.38 versus $1.39 \text{ mmol g (cdw)}^{-1} \text{ h}^{-1}$]. The final succinate titre (72 versus 66 mM) as well as the succinate yield (0.30 versus $0.28 \text{ mol mol}^{-1}$) were slightly increased compared with the BL-1 strain. Overproduction of ICL and MS had no influence on the final acetate titre in the supernatant although its accumulation was delayed probably due to the slower growth.

Carbon balance of selected succinate producers

The reduction of acetate formation combined with an improved oxaloacetate provision in a Δ *sdhCAB* deletion mutant resulted in the currently best aerobic succinate producer *C. glutamicum* BL-1/pAN6-*pyc*^{P458S}*ppc*. However, the succinate yield ($0.36 \text{ mol mol}^{-1}$ glucose) of this strain was still far below the theoretical maximum of $1 \text{ mol succinate mol}^{-1}$ glucose. In order to identify which carbon pools (biomass, by-products) could be diminished to increase succinate synthesis, the carbon balances of the wild type and the three succinate producer strains Δ *sdhCAB*, BL-1 and BL-1/pAN6-*pyc*^{P458S}*ppc* were calculated and analysed (Fig. 4).

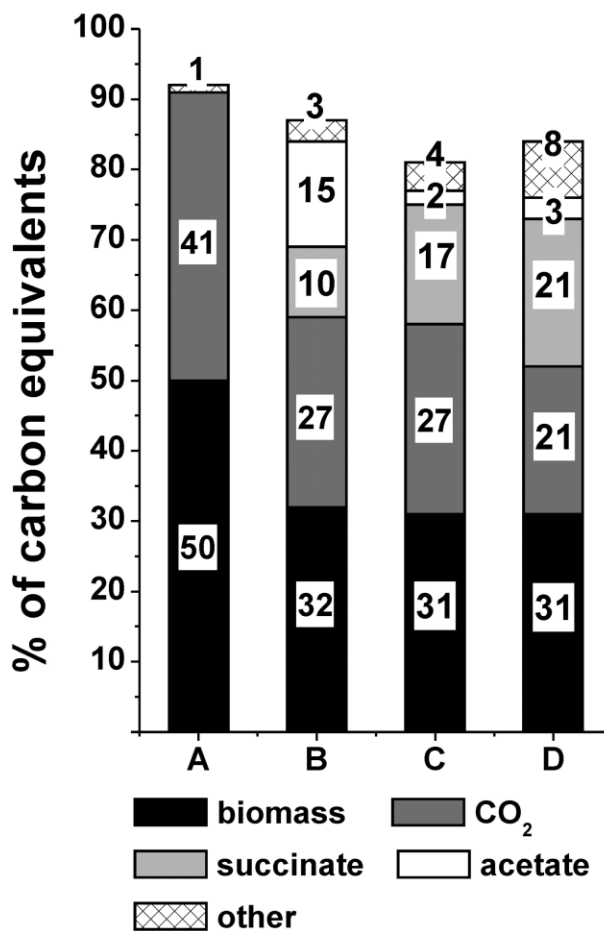


Fig. 4. Comparison of the carbon balances of *C. glutamicum* aerobic succinate producer strains calculated as percentage of product (mmol carbon) of the consumed glucose (mmol carbon) at the end of glucose consumption phase presented as mean values from the performed experiments. For cultivation conditions see legend of Fig. 3. (A) *Corynebacterium glutamicum* wild type after 12 h; (B) *C. glutamicum* Δ *sdh* after 14 h; (C) *C. glutamicum* BL-1 after 14 h; (D) *C. glutamicum* BL-1 with pAN6-*pyc*^{P458S}*ppc* after 14 h.

In *C. glutamicum* wild type, 50% of the carbon consumed ended up in biomass, 41% in carbon dioxide and < 1% in lactate, completing the carbon balance to nearly 92%. As no additional products such as organic acids or amino acids were detected in the supernatant of the wild type by our analysis, the missing 8% were assigned as 'unidentified products' and ignored in the further comparison of the strains. *Corynebacterium glutamicum* Δ *sdhCAB* as first-generation aerobic succinate producer used 32% of the consumed glucose for biomass formation, 27% was released as CO₂, 15% was converted to acetate and 10% to succinate. The sum of the minor products, i.e. lactate, pyruvate, α -ketoglutarate, lysine, alanine and valine, was very low and represented only 3% of the carbon consumed. The carbon recovery of the analysis for the Δ *sdhCAB* strain reached 88%. In the

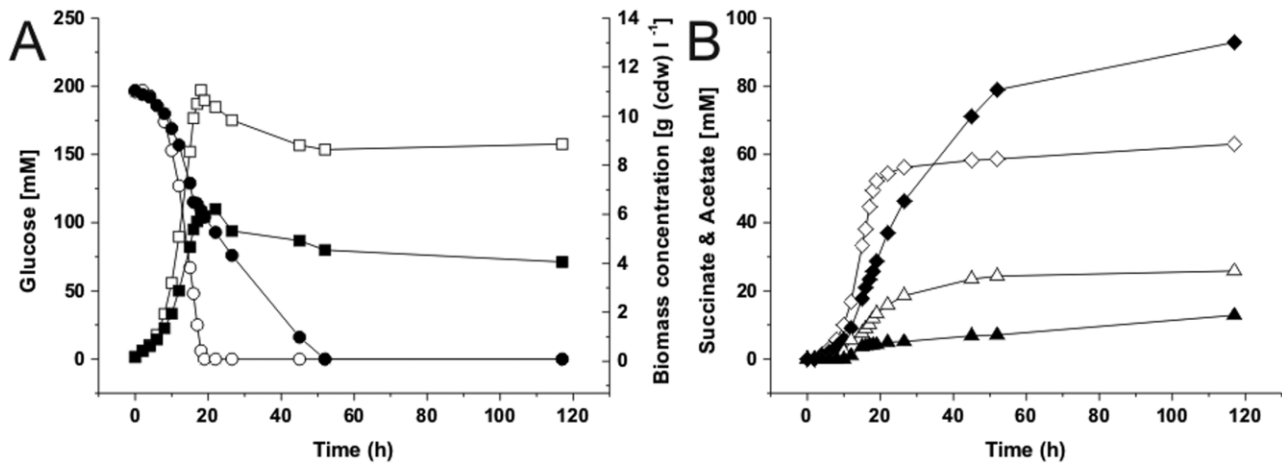


Fig. 5. Growth (squares), glucose consumption (circles), succinate production (diamonds) and acetate production (triangles) of *C. glutamicum* BL-1 with pAN6-*pyc*^{P458S}*ppc* under conditions of nitrogen excess (open symbols) and nitrogen limitation (solid symbols) during batch cultivation in modified CGXII medium containing 4% (w/v) glucose, 25 mg l⁻¹ kanamycin and 0.5 mM IPTG under constantly control conditions of pH 7.0 and pO₂ > 30% in a Multifors bioreactor system. Under nitrogen excess, the medium contained 151 mM (NH₄)₂SO₄ and no urea; under nitrogen limitation, the medium contained no urea and 20 mM (NH₄)₂SO₄. Mean values from at least two independent cultivations are shown.

improved succinate producer strain BL-1, the percentage of carbon going into biomass (32%) and CO₂ (27%) did not change compared with the Δ *sdhCAB* strain, but as a result of metabolic engineering the carbon equivalents converted to acetate decreased to 2%. The minor products (4%) showed a small increase compared with the parental strain Δ *sdhCAB*. The calculated carbon balance for the BL-1 strain was completed to 82%. The carbon balance of the aerobic succinate producer strain BL-1/pAN6-*pyc*^{P458S}*ppc* was similar to that of BL-1 with respect to carbon converted into biomass (31%), but only 21% of the carbon was released as CO₂. The carbon incorporated into acetate (3%) remained similar to strain BL-1, but the minor products rose to 8% of the total carbon. The most important feature of this strain was the increase in succinate by 4% compared with its parent BL-1 due to the enhanced carboxylation, reaching a percentage of 21%. The carbon balance of strain BL-1/pAN6-*pyc*^{P458S}*ppc* was completed to 84%.

Growth-decoupled succinate production

The carbon balance analysis described above demonstrated that the major portion of the utilized carbon source was converted into biomass and CO₂. As the theoretically maximal yield of 1 mol succinate mol⁻¹ glucose can only be obtained with non-growing cells, the possibility was explored to redirect the carbon flux at a certain stage during cultivation from growth to product synthesis, thus increasing the yield. The strategy chosen to achieve this goal was to restrict growth of the culture after reaching half of the biomass formed in the previous experiments. For this purpose, the nitrogen supply was limited by

omitting urea from the medium and reducing the (NH₄)₂SO₄ concentration from 20 g l⁻¹ to 2.71 g l⁻¹. Nitrogen is essential for growth, but not necessary for succinate formation. The production experiments were performed with the best aerobic succinate producer strain BL-1/pAN6-*pyc*^{P458S}*ppc* in batch cultivations with modified CGXII medium at pH 7.0 and pO₂ > 30% saturation. Growth, glucose utilization and product formation of the nitrogen-limited (N-limited) cultures as well as of control cultures containing 20 g l⁻¹ (NH₄)₂SO₄, which were run in parallel, were analysed and compared (Fig. 5).

The cultivation process of the N-limited cultures could be divided into two phases, a growth-coupled and a growth-decoupled production phase. The transition point was reached when the N-limited culture had reached its maximal biomass concentration after 22 h. In contrast, the cultivation process under N-excess conditions comprised only a growth-coupled production phase (see above). The maximal biomass concentration reached under N limitation [6.05 g (cdw) l⁻¹] was reduced by 45% compared with the control culture [11.06 g (cdw) l⁻¹]. When reaching the maximal biomass concentration, the N-limited cultures had consumed 52% of the initial glucose concentration and accumulated 40% (36 mM) of its final succinate titre. Thus, 48% (96 mM) of the initial glucose concentration was still available for product synthesis. In contrast, the non-limited culture had consumed glucose almost completely when it reached its maximal biomass after 18 h and the accumulated succinate represented 78% (49 mM) of the final titre. At the end of the cultivation (117 h), the N-limited cultures of strain BL-1/pAN6-*pyc*^{P458S}*ppc* had accumulated 90 mM succinate, corresponding to a 43% increase of the succinate titre

compared with the non-limited control cultures (63 mM). Further consequences of N limitation were the reduction of acetate formation by 50% (15 mM) and the formation of additional by-products, pyruvate (25 mM) and α -ketoglutarate (18 mM). The succinate yield of the N-limited cultures reached 0.45 mol mol⁻¹ glucose and thus was 41% higher than the yield of the N-excess cultures. Interestingly, under N limitation CO₂ production was lowered. At the time of glucose depletion, the N-limited culture had formed 113 mM CO₂, where the nitrogen excess culture had formed 219 mM CO₂.

Discussion

In this work, *C. glutamicum* strains for aerobic succinate production from glucose were constructed and analysed. The initial deletion of the *sdhCAB* genes encoding succinate dehydrogenase led to a strain that produced 40 mM succinate, a titre which was quite high compared with the titre reached by strains of *E. coli* (< 2 mM) and *S. cerevisiae* (24 mM) with equivalent deletions (Lin *et al.*, 2005b; Raab *et al.*, 2010). This fact encouraged further metabolic engineering of *C. glutamicum* towards efficient succinate production. As the major by-product of *C. glutamicum* Δ *sdhCAB* was found to be acetate, the next step was to inactivate the known acetate-forming pathways, i.e. the conversion of acetyl-CoA to acetyl phosphate and then to acetate by phosphotransacetylase and acetate kinase (Reinscheid *et al.*, 1999), the conversion of pyruvate to acetate and CO₂ with concomitant reduction of menaquinone to menaquinol by pyruvate:menaquinone oxidoreductase (Schreiner *et al.*, 2006) and the conversion of acetyl-CoA to acetate by an acetyl-CoA:CoA transferase, which requires a second substrate that is converted to its CoA derivative in this reaction (Yasuda *et al.*, 2007; Veit *et al.*, 2009). By deletion of the corresponding genes *ackA*, *pta*, *pqo* and *cat* (Fig. 1), acetate formation could be strongly reduced, but not completely abolished. The new strain BL-1 formed 65% more succinate and its productivity was nearly doubled compared with the Δ *sdhCAB* strain. The gain of product was on the cost of acetate reduction, as seen by the comparison of the carbon balances (Fig. 4). It is yet unclear which reactions and enzymes are responsible for the remaining production of acetate in strain BL-1. It is probably due to the hydrolysis and/or conversion of acetyl-CoA to acetate. Several genes of *C. glutamicum* were annotated as putative acetyltransferases or hydrolases and might be involved in acetate formation. The genes *cg0358* and *cg2472* are annotated as putative ac(et)yltransferases/hydrolases, *cg2860* as acyl-CoA thioester hydrolase, and *cg0592* as butyryl-CoA:acetate CoA transferase (Kalinowski *et al.*, 2003). Analysis of the corresponding protein sequences in the PFAM database

for domain structures (PFAM version 24.0, <http://pfam.sanger.ac.uk/>) revealed that *Cg0358*, *Cg2472* and *Cg2860* contain domains of the thioesterase superfamily and in the case of *Cg2472* even catalytic residues could be identified (Ollis *et al.*, 1992; Benning *et al.*, 1998). Therefore these enzymes might be able to catalyse acetyl-CoA hydrolysis. For *Cg0592* no significant domain match was found within PFAM. This protein is about one-third of the length of *cat* gene product and has very high sequence similarity to the C-terminal part of the *Cat* protein, but apparently lacks the N-terminal catalytic domain. Deletion of one or more of these putative acetate-producing enzymes will be a target for further strain development to reduce acetate formation and increase succinate production.

A second approach to improve succinate production was to enhance the anaplerotic reactions. Whereas overexpression of the *pyc* gene was without effect on succinate production, the combined overexpression of *pyc* and *ppc* improved succinate production. The succinate titre, yield and productivity were increased compared with the BL-1 strain by 24%, 28% and 14% respectively. The enhanced succinate production could therefore be assigned to PEPCx. This was surprising since previous flux analysis had shown that during aerobic growth of *C. glutamicum* on glucose 90% of the anaplerotic C3 carboxylation to oxaloacetate was performed by PCx and only 10% by PEPCx (Petersen *et al.*, 2000). In addition, PCx was reported to be the major bottleneck for the aerobic production of glutamate and lysine, both derivatives of TCA cycle intermediates (Peters-Wendisch *et al.*, 2001). Our data suggest that major (regulatory) differences exist with respect to the importance of PCx and PEPCx between succinate production strains and lysine or glutamate production strains. In the amino acid production strains, oxaloacetate and α -ketoglutarate are removed from the TCA cycle, but the cycle is not interrupted and thus there is no accumulation of TCA cycle intermediates. In the aerobic succinate producers, the cycle is blocked at the succinate dehydrogenase reaction, causing the accumulation of succinate and possibly also other TCA cycle intermediates. Moreover, the replenishment of oxaloacetate for the citrate synthase reaction completely depends on anaplerotic reactions. If the rate of oxaloacetate formation is lower than the rate of acetyl-CoA formation, the latter will accumulate, in particular in the absence of the most prominent acetate-forming enzymes. The possibly elevated concentrations of succinate, other TCA cycle intermediates and acetyl-CoA in *C. glutamicum* succinate producer strains might trigger regulatory effects on enzymes participating in the product synthesis pathway. In particular, it has been reported that PCx and PEPCx are regulated contrarily by acetyl-CoA: 110 μ M acetyl-CoA inhibits PCx activity, whereas PEPCx

is activated in the presence of 100 μM acetyl-CoA (Mori and Shiio, 1985; Peters-Wendisch *et al.*, 1998). This opposed regulation of the PEP/pyruvate carboxylation reactions through acetyl-CoA could explain the missing effect of *pyc* overexpression and the stimulating effect of *ppc* overexpression on succinate production. A beneficial effect of *ppc* overexpression in comparison with *pyc* overexpression was also reported for glutamate production by a ΔdtsR1 mutant in which fatty acid synthesis is disturbed, which might also result in higher acetyl-CoA levels within the cell (Yao *et al.*, 2009).

A highly active glyoxylate shunt could not only serve as OAA replenishment pathway, but also as an alternative succinate production route that avoids the loss of carbon as carbon dioxide in the isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase reactions. Therefore, overexpression of *aceA* and *aceB* was expected to improve aerobic succinate production. This approach was previously shown to be very efficient for construction of aerobic *E. coli* succinate producer strains (Lin *et al.*, 2005a). Strain BL-1 carrying pAN6-*aceAaceB* showed a 9% increased succinate titre and a 7% increased yield compared with the reference strain BL-1, but the growth rate was decreased by 18%. These results together with the enzyme activity measurements of the overproduced ICL and MS proved that the glyoxylate shunt was functional, but the improvements were rather small. This could be due to the inhibition of ICL from *C. glutamicum* by succinate (K_i of 1.48 mM) and also by intermediates of glycolysis (Reinscheid *et al.*, 1994a). Although the BL-1/pAN6-*aceAaceB* strain has high *in vitro* ICL activity (0.79 U mg^{-1} protein), the *in vivo* activity is probably very low due to elevated intracellular levels of succinate and perhaps also glycolytic intermediates. The reason for the reduced growth rate remains unclear.

The carbon balance analysis and comparison of the three aerobic succinate producers ΔsdhCAB , BL-1 and BL-1/pAN6-*pyc*^{P458S}*ppc* delivered three important conclusions. First, the increase in succinate production of the BL-1 strain compared with the ΔsdhCAB mutant did not match with the reduction in acetate accumulation, as only about half of the carbon equivalents becoming available in the BL-1 strain by the decreased acetate production (13%) were directed to succinate. This was probably due to an insufficient provision of oxaloacetate, as overexpression of *ppc* (together with *pyc*) in strain BL-1 shifted another 4% of carbon equivalents towards succinate. Second, deletion of the *sdhCAB* genes caused the accumulation of unidentified products, as the total carbon recovery of the ΔsdhCAB mutant decreased by 4% compared with the wild type. This effect was further increased in strains BL-1 and BL-1/pAN6-*pyc*^{P458S}*ppc*, in which 10% and 8% of the consumed carbon equivalents from glucose could not be assigned to products. Third,

despite the improvements made by metabolic engineering, the major proportion of the carbon source was still used for biomass and CO_2 formation. By limiting biomass formation through a reduced nitrogen supply, CO_2 production was strongly reduced and growth-decoupled succinate production from glucose was obtained. Under these conditions, the succinate yield and the succinate titre of strain BL-1/pAN6-*pyc*^{P458S}*ppc* could be increased by more than 40%.

In summary, our results show for the first time the potential of *C. glutamicum* strains for aerobic succinate production in a minimal medium. The best strain obtained in this work, BL-1/pAN6-*pyc*^{P458S}*ppc*, is able to accumulate succinate with a yield of 0.45 mol mol^{-1} glucose and reaches the highest specific productivity [1.60 mmol g^{-1} (cdw) h^{-1}] currently described for aerobic bacterial succinate producers. The use of minimal medium for succinate production with *C. glutamicum* offers an advantage to most alternative processes described for other microorganisms, which rely on complex medium additives (Lin *et al.*, 2005a; Yuzbashev *et al.*, 2010). Moreover, it can be envisaged that further improvements of our *C. glutamicum* strains and of the process, e.g. by using fed-batch cultivation conditions, will allow significant further increases in the titre and the yield of succinate.

Experimental procedures

Bacterial strains, plasmids and culture conditions

All bacterial strains and plasmids used or constructed in the course of this work are listed in Table 2. *Corynebacterium glutamicum* strains were routinely cultivated at 30°C in Brain-Heart-Infusion medium (Difco Laboratories, Detroit, MI, USA) supplemented with 2% (w/v) glucose or in a modified CGXII medium containing $(\text{NH}_4)_2\text{SO}_4$ (20 g l^{-1}), urea (5 g l^{-1}), KH_2PO_4 (1 g l^{-1}), K_2HPO_4 (1 g l^{-1}), $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ (0.25 g l^{-1}), CaCl_2 (10 mg l^{-1}), $\text{FeSO}_4 \times 7 \text{H}_2\text{O}$ (10 mg l^{-1}), $\text{MnSO}_4 \times \text{H}_2\text{O}$ (0.1 mg l^{-1}), $\text{ZnSO}_4 \times 7 \text{H}_2\text{O}$ (1 mg l^{-1}), $\text{CuSO}_4 \times 5 \text{H}_2\text{O}$ (0.2 mg l^{-1}), $\text{NiCl}_2 \times 6 \text{H}_2\text{O}$ (20 $\mu\text{g l}^{-1}$), biotin (0.2 mg l^{-1}), 3-morpholinopropanesulfonic acid (MOPS) (42 g l^{-1}) and 4% (w/v) glucose as carbon and energy source. The pH of the medium was adjusted to pH 7 with KOH. For cultivations of the BL-1 strain of *C. glutamicum* and its derivatives the biotin concentration was doubled to 0.4 mg l^{-1} . *Escherichia coli* DH5 α was routinely grown in LB medium at 37°C. If appropriate, kanamycin (25 $\mu\text{g ml}^{-1}$ for *C. glutamicum* or 50 $\mu\text{g ml}^{-1}$ for *E. coli*) and 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) were added.

Recombinant DNA work

The enzymes for recombinant DNA work were obtained from Roche Diagnostics (Mannheim, Germany) or New England Biolabs (Frankfurt am Main, Germany). All oligonucleotides were synthesized by Eurofins MWG Operon (Table S1). Routine methods like PCR, restriction or ligation were carried

Table 2. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>C. glutamicum</i> ATCC 13032	Wild-type strain, biotin auxotroph	Abe <i>et al.</i> (1967)
<i>C. glutamicum</i> Δ <i>sdhCAB</i>	ATCC 13032 derivative with an in-frame deletion of the <i>sdhCAB</i> operon	This work
<i>C. glutamicum</i> BL-1	ATCC 13032 derivative with in-frame deletions of <i>pqo</i> , <i>pta-ackA</i> , <i>sdhCAB</i> and <i>cat</i>	This work
<i>C. glutamicum</i> DM1727	ATCC 13032 derivative with a Pro458Ser exchange in the pyruvate carboxylase gene (<i>pyc</i> ^{P458S})	B. Bathe, Evonik Degussa
<i>E. coli</i> DH5 α	F ⁻ ϕ 80 <i>dlac</i> Δ (<i>lacZ</i>)M15 Δ (<i>lacZYA-argF</i>) U169 <i>endA1 recA1 hsdR17</i> (<i>r</i> _K ⁻ , <i>m</i> _K ⁺) <i>deoR thi-1 phoA supE44</i> λ ⁻ <i>gyrA96 relA1</i>	Invitrogen
Plasmids		
pAN6	Kan ^R ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector for regulated gene expression (P _{<i>tac</i>} , <i>lac</i> ^q , pBL1 <i>oriV</i> _{C.G.} , pUC18 <i>oriV</i> _{E.C.}) derived from pEKEx2	Frunzke <i>et al.</i> (2008)
pAN6- <i>pyc</i> ^{P458S}	Kan ^R ; pAN6 derivative containing the <i>pyc</i> ^{P458S} gene from <i>C. glutamicum</i> DM1727 under control of the <i>tac</i> promoter	This work
pAN6- <i>pyc</i> ^{P458S} <i>ppc</i>	Kan ^R ; pAN6 derivative containing the <i>pyc</i> ^{P458S} gene from <i>C. glutamicum</i> DM1727 and the <i>ppc</i> gene from <i>C. glutamicum</i> ATCC13032 under control of the <i>tac</i> promoter	This work
pAN6- <i>aceAaceB</i>	Kan ^R ; pAN6 derivative containing the <i>aceA</i> and <i>aceB</i> genes from <i>C. glutamicum</i> ATCC13032 under control of the <i>tac</i> promoter	This work
pK19 <i>mobsacB</i>	Kan ^R ; vector for allelic exchange in <i>C. glutamicum</i> (pK18 <i>oriV</i> _{E.C.} <i>sacB lacZ</i> α)	Schäfer <i>et al.</i> (1994)
pK19 <i>mobsacB</i> - Δ <i>cat</i>	Kan ^R ; pK19 <i>mobsacB</i> derivative containing a 1.1 kb overlap-extension PCR product (Sall/XbaI) which covers the flanking regions of the <i>C. glutamicum cat</i> gene	Veit <i>et al.</i> (2009)
pK19 <i>mobsacB</i> - Δ <i>pqo</i>	Kan ^R ; pK19 <i>mobsacB</i> derivative containing a 1 kb overlap-extension PCR product (HindIII/EcoRI) which covers the flanking regions of the <i>C. glutamicum pqo</i> gene	This work
pK19 <i>mobsacB</i> - Δ <i>pta-ackA</i>	Kan ^R ; pK19 <i>mobsacB</i> derivative containing a 1.1 kb overlap-extension PCR product (HindIII/XbaI) which covers the flanking regions of the <i>C. glutamicum pta-ackA</i> genes	This work
pK19 <i>mobsacB</i> - Δ <i>sdhCAB</i>	Kan ^R ; pK19 <i>mobsacB</i> derivative containing a 1.1 kb overlap-extension PCR product (HindIII/XbaI) which covers the flanking regions of the <i>C. glutamicum sdhCAB</i> genes	This work

out according to standard protocols (Sambrook *et al.*, 2001). The generation of all PCR products was performed with KOD Hot Start polymerase (Novagen, Darmstadt, Germany). Plasmids were isolated from *E. coli* with the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). *Escherichia coli* was transformed by the RbCl method (Hanahan, 1985). Transformation of *C. glutamicum* was performed as described previously (van der Rest *et al.*, 1999). All plasmid constructs described below were controlled by DNA sequencing (LGC genomics, Berlin, Germany).

Construction of deletion mutants and plasmids

Corynebacterium glutamicum mutants with in-frame deletions of *sdhCAB* (Δ *sdhCAB*), *pta-ackA* (Δ *pta-ackA*), *pqo* (Δ *pqo*) and *cat* (Δ *cat*) were constructed via a two-step homologous recombination procedure as described previously (Niebisch and Bott, 2001). The regions up- and downstream (approximately 500 bp each) of the gene/operon to be deleted were amplified using pairs of oligonucleotides designated Δ -gene/operon-1 and Δ -gene/operon-2 and Δ -gene/operon-3 and Δ -gene/operon-4 (Table S1), respectively, and the two PCR products served as templates for an overlap-extension PCR using the oligonucleotides Δ -gene/operon-1 and Δ -gene/operon-4. The resulting PCR product of about 1 kb was digested with the restriction enzymes indicated in Table S1 and cloned into pK19*mobsacB* cut with the

same enzymes. The plasmid pK19*mobsacB*- Δ *cat* was kindly provided by Volker F. Wendisch (University of Bielefeld). The transfer of the resulting plasmids, designated pK19*mobsacB*- Δ gene/operon, into *C. glutamicum* and selection for the first and second recombination events were performed as described previously (Niebisch and Bott, 2001). Kanamycin-sensitive and saccharose-resistant clones were tested by colony PCR with an oligonucleotide pair designated Δ -gene/operon-out-fw and Δ -gene/operon-out-rv (Table S1). Clones which had the desired in-frame deletion of the gene/operon revealed a 1 kb fragment in which all nucleotides except for the first six codons (in case of Δ *cat* the first seven codons) and the last 12 codons were replaced by a 21 bp tag.

For the construction of the expression plasmid pAN6-*pyc*^{P458S}, the *pyc*^{P458S} gene was amplified using the oligonucleotides NdeI-*pyc*-for and NheI-SbfI-*pyc*-rev and chromosomal DNA of *C. glutamicum* DM1727 (exchanging the native start codon GTG to ATG). The PCR product was digested with NdeI and NheI and cloned into the vector pAN6. For the construction of the expression plasmid pAN6-*pyc*^{P458S}*ppc*, the coding region of *ppc* together with a 9 bp linker and an artificial ribosome binding site (AAGGA) was amplified using chromosomal DNA of *C. glutamicum* wild type and the oligonucleotide pair SbfI-*ppc*-for/NheI-*ppc*-rev. The PCR product was digested with SbfI and NheI and cloned into pAN6-*pyc*^{P458S}, resulting in pAN6-*pyc*^{P458S}*ppc*. For construction

of pAN6-*aceAaceB*, the *aceA* gene was amplified using the oligonucleotides NdeI-*aceA*-for and NdeI-*aceA*-rev and chromosomal DNA of *C. glutamicum* wild type. The PCR product was digested with NdeI and cloned into the pAN6 vector. The correct orientation of the insert was confirmed by restriction analysis and DNA sequencing. Subsequently the *aceB* gene was amplified from chromosomal DNA of *C. glutamicum* wild type using the oligonucleotides NheI-RBS-*aceB*-for and NheI-*aceB*-rev, which introduce an NheI restriction site and a ribosomal binding site (AAGGA) together with a 9 bp linker or only an NheI restriction site respectively. The PCR product was digested with NheI and cloned into plasmid pAN6-*aceA*. The correct orientation of the *aceB* gene was again confirmed by restriction analysis and DNA sequencing.

Cultivation conditions for aerobic succinate production

For aerobic succinate production, 5 ml of BHI medium supplemented with 2% (w/v) glucose were inoculated with one single colony of the desired *C. glutamicum* strain from a fresh BHI agar plate and incubated on a rotary shaker for 8 h at 30°C. Subsequently the cells were used to inoculate a 500 ml baffled shake flask filled with 50 ml of modified CGXII medium. After approximately 16 h incubation at 120 r.p.m. and 30°C the cells were used to inoculate a 1.4 l bioreactor (Multifors Multi-Fermenter System with six independently controllable bioreactors, Infors, Einsbach, Germany) to an optical density at 600 nm (OD_{600}) of approximately 1. The bioreactor contained 600 ml modified CGXII medium with 4% (w/v) glucose, but without the buffer substance 3-morpholinopropanesulfonic acid. When required, 25 $\mu\text{g ml}^{-1}$ kanamycin was used in the pre- and main cultivations. For induction of the target genes in the pAN6-based expression plasmids, 0.5 mM IPTG was added. The bioreactors were sparged with 0.9 l min^{-1} air. Oxygen saturation was measured online with a polarimetric oxygen electrode (Mettler Toledo, Giessen, Germany) and was held permanently over 30% by gradually increasing stirrer speed from 600 r.p.m. up to 1000 r.p.m. The pH was determined online using a standard pH electrode (Mettler Toledo) and adjusted to pH 7 with 3 M potassium hydroxide and 3 M hydrochloric acid. Foam formation was suppressed automatically by titration of 25% (v/v) Antifoam 204/water suspension (Sigma-Aldrich, Steinheim, Germany). Carbon dioxide in the exit gas flow was measured continuously with an Exit Gas Analyser (Infors, Einsbach, Germany). To explore growth-decoupled succinate production, the maximal biomass formation of *C. glutamicum* was reduced to approximately 50% by decreasing the nitrogen content of the medium. In these experiments, urea was omitted and the $(\text{NH}_4)_2\text{SO}_4$ concentration reduced to 2.71 g l^{-1} . In the control cultures, urea was also omitted, but the $(\text{NH}_4)_2\text{SO}_4$ concentration remained at 20 g l^{-1} .

Enzyme assay of ICL and MS in cell extracts

Cell cultivation, crude extract preparation and enzyme measurements were performed as described elsewhere (Gerstmeir *et al.*, 2004).

Growth parameter determination

Growth was followed by measuring the OD_{600} with an Ultrospec 500-Pro spectrophotometer (Amersham Biosciences, Freiburg, Germany). The biomass concentration was calculated from OD_{600} values using an experimentally determined correlation factor of 0.25 g (cdw) l^{-1} for an OD_{600} of 1 (Kabus *et al.*, 2007).

Quantification of organic acids and glucose in the culture supernatant

The quantification of glucose and organic acids in the supernatant of the cultures was performed using an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) equipped with a 300 \times 8 mm organic acid column (polystyrol-divinylbenzol resin, CS Chromatographie Service GmbH, Langerwehe, Germany) and a guard cartridge (40 \times 8 mm) filled with the same material. Isocratic elution was performed for 38 min at 40°C with 100 mM sulphuric acid at a flow rate of 0.4 ml min^{-1} . Glucose was detected via an Agilent 1100 Refractive Index Detector and the organic acids were detected via an Agilent 1100 Diode Array Detector at 215 nm. All substances were quantified with the help of an experimentally determined linear correlation factor between the area measured in the chromatogram for a peak with a substance-specific retention time and the concentration of the pure substance in water solution. Quantification was performed using calibration curves obtained with external standards.

Acknowledgements

We thank the Federal Ministry of Food, Agriculture, and Consumer Protection (BMELV) for financial support within the ERA-IB project 'BioProChemBB', Volker F. Wendisch (University of Bielefeld) for providing the plasmid pK19*mobsacB-Δcat* and Brita Weil for technical assistance.

References

- Abe, S., Takayama, K., and Kinoshita, S. (1967) Taxonomical studies on glutamic acid producing bacteria. *J Gen Appl Microbiol* **13**: 279–301.
- Benning, M.M., Wesenberg, G., Liu, R., Taylor, K.L., Dunaway-Mariano, D., and Holden, H.M. (1998) The three-dimensional structure of 4-hydroxybenzoyl-CoA thioesterase from *Pseudomonas* sp. strain CBS-3. *J Biol Chem* **273**: 33572–33579.
- Blombach, B., Riester, T., Wieschalka, S., Ziert, C., Youn, J.W., Wendisch, V.F., and Eikmanns, B.J. (2011) *Corynebacterium glutamicum* tailored for efficient isobutanol production. *Appl Environ Microbiol* **77**: 3300–3310.
- Bott, M. (2007) Offering surprises: TCA cycle regulation in *Corynebacterium glutamicum*. *Trends Microbiol* **15**: 417–425.
- Burkovski, A. (ed.) (2008) *Corynebacteria: Genomics and Molecular Biology*. Norfolk, UK: Caister Academic Press.
- Eggeling, L., and Bott, M. (eds) (2005) *Handbook of Corynebacterium glutamicum*. Boca Raton, Florida, USA: CRC Press, Taylor & Francis Group.

- Eikmanns, B.J., Follettie, M.T., Griot, M.U., and Sinskey, A.J. (1989) The phosphoenolpyruvate carboxylase gene of *Corynebacterium glutamicum*: molecular cloning, nucleotide sequence, and expression. *Mol Gen Genet* **218**: 330–339.
- Frunzke, J., Engels, V., Hasenbein, S., Gätgens, C., and Bott, M. (2008) Co-ordinated regulation of gluconate catabolism and glucose uptake in *Corynebacterium glutamicum* by two functionally equivalent transcriptional regulators, GntR1 and GntR2. *Mol Microbiol* **67**: 305–322.
- Gerstmeir, R., Cramer, A., Dangel, P., Schaffer, S., and Eikmanns, B. (2004) RamB, a novel transcriptional regulator of genes involved in acetate metabolism of *Corynebacterium glutamicum*. *J Bacteriol* **186**: 2798–2809.
- Guettler, M.V., Rümmler, D., and Jain, M.K. (1999) *Actinobacillus succinogenes* sp. nov., a novel succinic-acid-producing strain from the bovine rumen. *Int J Syst Bacteriol* **49**: 207–216.
- Hanahan, D. (1985) Techniques for transformation of *E. coli*. In *DNA Cloning*. Glover, D.M. (ed.). Oxford, UK: IRL Press, pp. 109–135.
- Hermann, B.G., and Patel, M. (2007) Today's and tomorrow's bio-based bulk chemicals from white biotechnology: a techno-economic analysis. *Appl Biochem Biotechnol* **136**: 361–388.
- Huhn, S., Jolkver, E., Kramer, R., and Marin, K. (2011) Identification of the membrane protein SucE and its role in succinate transport in *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol* **89**: 327–335.
- Ikedda, M., and Nakagawa, S. (2003) The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. *Appl Microbiol Biotechnol* **62**: 99–109.
- Ikedda, M., Ohnishi, J., Hayashi, M., and Mitsuhashi, S. (2006) A genome-based approach to create a minimally mutated *Corynebacterium glutamicum* strain for efficient L-lysine production. *J Ind Microbiol Biotechnol* **33**: 610–615.
- Inui, M., Kawaguchi, H., Murakami, S., Vertes, A.A., and Yukawa, H. (2004a) Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. *J Mol Microbiol Biotechnol* **8**: 243–254.
- Inui, M., Murakami, S., Okino, S., Kawaguchi, H., Vertès, A., and Yukawa, H. (2004b) Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. *J Mol Microbiol Biotechnol* **7**: 182–196.
- Jantama, K., Haupt, M.J., Svoronos, S.A., Zhang, X., Moore, J.C., Shanmugam, K.T., and Ingram, L.O. (2008) Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of *Escherichia coli* C that produce succinate and malate. *Biotechnol Bioeng* **99**: 1140–1153.
- Kabus, A., Niebisch, A., and Bott, M. (2007) Role of cytochrome *bd* oxidase from *Corynebacterium glutamicum* for growth and lysine production. *Appl Environ Microbiol* **73**: 861–868.
- Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., et al. (2003) The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J Biotechnol* **104**: 5–25.
- Kirchner, O., and Tauch, A. (2003) Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. *J Biotechnol* **104**: 287–299.
- Lee, P.C., Lee, W.G., Lee, S.Y., and Chang, H.N. (2001) Succinic acid production with reduced by-product formation in the fermentation of *Anaerobiospirillum succiniciproducens* using glycerol as a carbon source. *Biotechnol Bioeng* **72**: 41–48.
- Lee, P.C., Lee, S.Y., Hong, S.H., and Chang, H.N. (2002) Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen. *Appl Microbiol Biotechnol* **58**: 663–668.
- Lin, H., Bennett, G.N., and San, K.Y. (2005a) Metabolic engineering of aerobic succinate production systems in *Escherichia coli* to improve process productivity and achieve the maximum theoretical succinate yield. *Metab Eng* **7**: 116–127.
- Lin, H., Bennett, G., and San, K. (2005b) Genetic reconstruction of the aerobic central metabolism in *Escherichia coli* for the absolute aerobic production of succinate. *Biotechnol Bioeng* **89**: 148–156.
- McKinlay, J.B., Vieille, C., and Zeikus, J.G. (2007) Prospects for a bio-based succinate industry. *Appl Microbiol Biotechnol* **76**: 727–740.
- Mori, M., and Shio, I. (1985) Purification and some properties of phosphoenolpyruvate carboxylase from *Brevibacterium flavum* and its aspartate-overproducing mutant. *J Biochem* **97**: 1119–1128.
- Niebisch, A., and Bott, M. (2001) Molecular analysis of the cytochrome *bc₁-aa₃* branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome *c₁*. *Arch Microbiol* **175**: 282–294.
- Oh, I.J., Kim, D.H., Oh, E.K., Lee, S.Y., and Lee, J. (2009) Optimization and scale-up of succinic acid production by *Mannheimia succiniciproducens* LPK7. *J Microbiol Biotechnol* **19**: 167–171.
- Okino, S., Noburyu, R., Suda, M., Jojima, T., Inui, M., and Yukawa, H. (2008) An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. *Appl Microbiol Biotechnol* **81**: 459–464.
- Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., et al. (1992) The alpha/beta hydrolase fold. *Protein Eng* **5**: 197–211.
- Petersen, S., de Graaf, A.A., Eggeling, L., Mollney, M., Wiechert, W., and Sahm, H. (2000) *In vivo* quantification of parallel and bidirectional fluxes in the anaplerosis of *Corynebacterium glutamicum*. *J Biol Chem* **275**: 35932–35941.
- Peters-Wendisch, P.G., Kreutzer, C., Kalinowski, J., Patek, M., Sahm, H., and Eikmanns, B.J. (1998) Pyruvate carboxylase from *Corynebacterium glutamicum*: characterization, expression and inactivation of the *pyc* gene. *Microbiology* **144**: 915–927.
- Peters-Wendisch, P.G., Schiel, B., Wendisch, V.F., Katsoulidis, E., Möckel, B., Sahm, H., and Eikmanns, B.J. (2001) Pyruvate carboxylase is a major bottleneck for glutamate and lysine production by *Corynebacterium glutamicum*. *J Mol Microbiol Biotechnol* **3**: 295–300.
- Raab, A.M., Gebhardt, G., Bolotina, N., Weuster-Botz, D., and Lang, C. (2010) Metabolic engineering of *Saccharo-*

- myces cerevisiae* for the biotechnological production of succinic acid. *Metab Eng* **12**: 518–525.
- Reinscheid, D.J., Eikmanns, B.J., and Sahm, H. (1994a) Characterization of the isocitrate lyase gene from *Corynebacterium glutamicum* and biochemical analysis of the enzyme. *J Bacteriol* **176**: 3474–3483.
- Reinscheid, D.J., Eikmanns, B.J., and Sahm, H. (1994b) Malate synthase from *Corynebacterium glutamicum*: sequence analysis of the gene and biochemical characterization of the enzyme. *Microbiology* **140**: 3099–3108.
- Reinscheid, D.J., Schnicke, S., Rittmann, D., Zahnnow, U., Sahm, H., and Eikmanns, B.J. (1999) Cloning, sequence analysis, expression and inactivation of the *Corynebacterium glutamicum* *pta-ack* operon encoding phosphotransacetylase and acetate kinase. *Microbiology* **145**: 503–513.
- van der Rest, M.E., Lange, C., and Molenaar, D. (1999) A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. *Appl Microbiol Biotechnol* **52**: 541–545.
- Sambrook, J., MacCallum, P., and Russell, D. (2001) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.
- Samuelov, N.S., Lamed, R., Lowe, S., and Zeikus, J.G. (1991) Influence of CO₂-HCO₃⁻ levels and pH on growth, succinate production, and enzyme activities of *Anaerobiospirillum succiniciproducens*. *Appl Environ Microbiol* **57**: 3013–3019.
- Samuelov, N.S., Datta, R., Jain, M.K., and Zeikus, J.G. (1999) Whey fermentation by *Anaerobiospirillum succiniciproducens* for production of a succinate-based animal feed additive. *Appl Environ Microbiol* **65**: 2260–2263.
- San, K.Y., Bennett, G.N., Berrios-Rivera, S.J., Vadali, R.V., Yang, Y.T., Horton, E., *et al.* (2002) Metabolic engineering through cofactor manipulation and its effects on metabolic flux redistribution in *Escherichia coli*. *Metab Eng* **4**: 182–192.
- Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**: 69–73.
- Schneider, J., and Wendisch, V.F. (2010) Putrescine production by engineered *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol* **88**: 859–868.
- Scholten, E., and Dagele, D. (2008) Succinic acid production by a newly isolated bacterium. *Biotechnol Lett* **30**: 2143–2146.
- Scholten, E., Renz, T., and Thomas, J. (2009) Continuous cultivation approach for fermentative succinic acid production from crude glycerol by *Basfia succiniciproducens* DD1. *Biotechnol Lett* **31**: 1947–1951.
- Schreiner, M.E., Riedel, C., Holatko, J., Patek, M., and Eikmanns, B.J. (2006) Pyruvate:quinone oxidoreductase in *Corynebacterium glutamicum*: molecular analysis of the *pqo* gene, significance of the enzyme, and phylogenetic aspects. *J Bacteriol* **188**: 1341–1350.
- Veit, A., Rittmann, D., Georgi, T., Youn, J.W., Eikmanns, B.J., and Wendisch, V.F. (2009) Pathway identification combining metabolic flux and functional genomics analyses: acetate and propionate activation by *Corynebacterium glutamicum*. *J Biotechnol* **140**: 75–83.
- Wendisch, V.F., Bott, M., and Eikmanns, B.J. (2006) Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for biotechnological production of organic acids and amino acids. *Curr Opin Microbiol* **9**: 268–274.
- Werpy, T., and Peterson, G. (eds) (2004) *Top Value Added Chemicals from Biomass*. Oak Ridge, USA: US Department of Energy, pp. 1–76.
- Yao, W., Deng, X., Zhong, H., Liu, M., Zheng, P., Sun, Z., and Zhang, Y. (2009) Double deletion of *dtsR1* and *pyc* induce efficient L-glutamate overproduction in *Corynebacterium glutamicum*. *J Ind Microbiol Biotechnol* **36**: 911–921.
- Yasuda, K., Jojima, T., Suda, M., Okino, S., Inui, M., and Yukawa, H. (2007) Analyses of the acetate-producing pathways in *Corynebacterium glutamicum* under oxygen-deprived conditions. *Appl Microbiol Biotechnol* **77**: 853–860.
- Youn, J.W., Jolkver, E., Kramer, R., Marin, K., and Wendisch, V.F. (2008) Identification and characterization of the dicarboxylate uptake system DccT in *Corynebacterium glutamicum*. *J Bacteriol* **190**: 6458–6466.
- Youn, J.W., Jolkver, E., Kramer, R., Marin, K., and Wendisch, V.F. (2009) Characterization of the dicarboxylate transporter DctA in *Corynebacterium glutamicum*. *J Bacteriol* **191**: 5480–5488.
- Yukawa, H., Omumasaba, C.A., Nonaka, H., Kos, P., Okai, N., Suzuki, N., *et al.* (2007) Comparative analysis of the *Corynebacterium glutamicum* group and complete genome sequence of strain R. *Microbiology* **153**: 1042–1058.
- Yuzbashev, T.V., Yuzbasheva, E.Y., Sobolevskaya, T.I., Laptev, I.A., Vybornaya, T.V., Larina, A.S., *et al.* (2010) Production of succinic acid at low pH by a recombinant strain of the aerobic yeast *Yarrowia lipolytica*. *Biotechnol Bioeng* **107**: 673–682.
- Zeikus, J.G., Jain, M.K., and Elankovan, P. (1999) Biotechnology of succinic acid production and markets for derived industrial products. *Appl Environ Microbiol* **51**: 545–552.
- Zhang, X., Jantama, K., Shanmugam, K.T., and Ingram, L.O. (2009) Reengineering *Escherichia coli* for succinate production in mineral salts medium. *Appl Environ Microbiol* **75**: 7807–7813.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligonucleotides used in this study.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.