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

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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, pqq 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 shuttle 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 D\(\Delta pqqD\Delta pta-ackA\Delta sdhCAB\Delta cat/pAN6-pyc\(^{45S8}\)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 \(\gamma\)-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 Basfa 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
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 (McKlnay 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 (McKlnay 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 (Blomback 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 Boll, 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 ΔsdhCAB 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.
The wild-type cells consumed glucose with an uptake rate 5.54 mmol g (cdw)\(^{-1}\)h\(^{-1}\). 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 g (cdw) l\(^{-1}\)] and glucose uptake rates [5.54 mmol g (cdw)\(^{-1}\)h\(^{-1}\)] as the strain C. glutamicum \(\text{sdhCAB}\) (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 mol mol\(^{-1}\) glucose) compared with C. glutamicum \(\text{sdhCAB}\). The specific succinate production rate [1.39 mmol g (cdw)\(^{-1}\)h\(^{-1}\)] was increased about 85% compared with that of the \(\text{sdhCAB}\) strain. The deletion of \(\text{pqo}\) and \(\text{pta-ackA}\) led to an 82% reduction in acetate formation compared with the \(\text{sdhCAB}\) 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...
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<sup>P458S</sup> was constructed. (ii) Simultaneous overproduction 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<sup>-1</sup> kanamycin (plasmid-containing strains only) and 0.5 mM IPTG (plasmid-containing strains only) at pH 7.0 and pO<sub>2</sub> > 30% saturation.

| *C. glutamicum* strain | Growth rate (h<sup>-1</sup>) | Biomass [g (cdw) l<sup>-1</sup>] | Glucose consumed [mM] | Glucose uptake rate [mmol g (cdw)<sup>-1</sup> h<sup>-1</sup>] | Final acetate titre [mM] | Final succinate titre [mM] | Succinate production rate [mmol g<sup>-1</sup> (cdw) h<sup>-1</sup>] | Yield succinate/glucose (mol mol<sup>-1</sup>) |
|------------------------|----------------------------|----------------------------------|-----------------------|---------------------------------|-------------------------|-----------------------------|---------------------------------|-----------------------------|
| 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-pyc<sup>P458S</sup>) | 0.30 | 12.2                             | 242                   | 5.49                            | 20                      | 68                          | 1.44                             | 0.28                         |
| BL-1 (pAN6-pyc<sup>P458S</sup>Ppc) | 0.29 | 12.2                             | 230                   | 5.15                            | 20                      | 82                          | 1.60                             | 0.36                         |
| BL-1 (pAN6-aceAceB)    | 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.

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Phenylpyruvate carboxylase (PEPC): PEPC, which catalyses the carboxylation of PEP to oxaloacetate (Mori and Shioi, 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 PEPC (encoded by the *ppc* gene) with PCP*P458S* was expected to further stimulate the synthesis from oxaloacetate and therefore the expression plasmid pAN6-*pyc*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-*pyc*P458S, BL-1/pAN6-*pyc*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 under constantly control conditions of pH 7.0 and pO2 > 30% using a Multifors bioreactor system. Mean values from two independent cultivations are shown.

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-*pyc*P458S (pyruvate carboxylase, circles), BL-1 with pAN6-*pyc*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.
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 mmol g (cdw)⁻¹ h⁻¹] or the succinate yield (0.28 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 g (cdw) 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 pyc²₄₅₈₈ and ppc in strain BL-1/pAN6-pyc²₄₅₈₈ppc led to an increased succinate production rate [1.60 mmol g (cdw)⁻¹ 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 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 mmol g (cdw)⁻¹ h⁻¹. Overexpression of pyc²₄₅₈₈ 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 g (cdw) l⁻¹] compared with the ΔsdhCAB mutant (Table 1). The glucose uptake rate was reduced compared with the BL-1 strain [5.19 versus 5.54 mmol g (cdw)⁻¹ h⁻¹], whereas the succinate production rate was the same as for the BL-1 strain [1.38 versus 1.39 mmol g (cdw)⁻¹ h⁻¹]. The final succinate titre (72 versus 66 mM) as well as the succinate yield (0.30 versus 0.28 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 ΔsdhCAB deletion mutant resulted in the currently best aerobic succinate producer *C. glutamicum* BL-1/pAN6-pyc²₄₅₈₈ppc. However, the succinate yield (0.36 mol mol⁻¹ glucose) of this strain was still far below the theoretical maximum of 1 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 ΔsdhCAB, BL-1 and BL-1/pAN6-pyc²₄₅₈₈ppc were calculated and analysed (Fig. 4).

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* ΔsdhCAB 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 ΔsdhCAB strain reached 88%. In the

![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* Δsdh after 14 h; (C) *C. glutamicum* BL-1 after 14 h; (D) *C. glutamicum* BL-1 with pAN6-pyc²₄₅₈₈ppc after 14 h.](image-url)
improved succinate producer strain BL-1, the percentage of carbon going into biomass (32%) and CO₂ (27%) did not change compared with the ΔsdhCAB 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 ΔsdhCAB. 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% 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.

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.
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(etyl)transferases/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 over-expression 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 μM acetyl-CoA inhibits PCx activity, whereas PEPCx...
Glucose could not be assigned to products. Third, which 10% and 8% of the consumed carbon equivalents in strains BL-1 and BL-1/pAN6-pyc^K458S,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 CO₂ formation. By limiting biomass formation through a reduced nitrogen supply, CO₂ 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^K458S,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^K458S,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., 2005). 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 (NH₄)₂SO₄ (20 g l⁻¹), urea (5 g l⁻¹), KH₂PO₄ (1 g l⁻¹), K₂HPO₄ (1 g l⁻¹), MgSO₄ × 7 H₂O (0.25 g l⁻¹), CaCl₂ (10 mg l⁻¹), FeSO₄ × 7 H₂O (10 mg l⁻¹), MnSO₄ × H₂O (0.1 mg l⁻¹), ZnSO₄ × 7 H₂O (1 mg l⁻¹), CuSO₄ × 5 H₂O (0.2 mg l⁻¹), NiCl₂ × 6 H₂O (20 mg 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 DH5α was routinely grown in LB medium at 37°C. If appropriate, kanamycin (25 μg ml⁻¹ for C. glutamicum or 50 μg ml⁻¹ for E. coli) and 0.5 mM isopropyl β-D-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 out.
Construction of deletion mutants and plasmids

Corynebacterium glutamicum mutants with in-frame deletions of sdhCAB (ΔsdhCAB), pta-ackA (Δpta-ackA), pqq (Δpqq) and cat (Δcat) 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, 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 pk19mobsacB cut with the same enzymes. The plasmid pk19mobsacB-Δcat was kindly provided by Volker F. Wendisch (University of Bielefeld). The transfer of the resulting plasmids, designated pk19mobsacB-Δgene/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 designed for 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 Δcat 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<sup>Δkss</sup>, the pyc<sup>Δkss</sup> 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 Nhel and cloned into the vector pAN6. For the construction of the expression plasmid pAN6-pyc<sup>Δkss</sup>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/Nhel-ppc-rev. The PCR product was digested with SbfI and Nhel and cloned into pAN6-pyc<sup>Δkss</sup>ppc. For construction...
of pAN6-aceAaceB, the aceA gene was amplified using the oligonucleotides Ndel-aceA-for and Ndel-aceA-rev and chromosomal DNA of C. glutamicum wild type. The PCR product was digested with Ndel 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 Nhel-RBS-aceB-for and Nhel-aceB-rev, which introduce an Nhel restriction site and a ribosomal binding site (AAGGA) together with a 9 bp linker or only an Nhel restriction site respectively. The PCR product was digested with Nhel 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 (OD600) 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 µg ml⁻¹ kanamycin was used in the pre- and main cultivation. 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 2.71 g l⁻¹. In the control cultures, urea was also omitted, but the (NH₄)₂SO₄ 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 (Gerstmair et al., 2004).

Growth parameter determination

Growth was followed by measuring the OD₆₀₀ with an Ultrascpec 500-Pr spectrophotometer (Amersham Biosciences, Freiburg, Germany). The biomass concentration was calculated from OD₆₀₀ values using an experimentally determined correlation factor of 0.25 g (cdw) l⁻¹ for an OD₆₀₀ 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 × 8 mm organic acid column (polystyryl-dvinylbenzol resin, CS Chromatographic Service GmbH, Langerwehe, Germany) and a guard cartridge (40 × 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.

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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 thiopherase from Pseudomonas sp. strain CBS-3. J Biol Chem 273: 33572–33579.

Blombach, B., Riester, T., Wieschelka, 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.

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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., Rumler, 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.

Herrmann, 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.

Ikeda, M., and Nakagawa, S. (2003) The Corynebacterium glutamicum genome: features and impacts on biotechnological processes. Appl Microbiol Biotechnol 62: 99–109.

Ikeda, 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., Vertes, 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., Bischof, 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 Biotecnol 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 succiniproducens 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 succiniproducens 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 Shiio, 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-aa branch of the Corynebacterium glutamicum respiratory chain containing an unusual diheme cytochrome c. 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 succiniproducens 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., Cigler, 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., Zahnow, U., Sahm, H., and Eikmanns, B.J. (1999) Cloning, sequence analysis, 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 xenogenic 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 multiple-purpose cloning vectors derived from the Escherichia coli plasmids pH18 and pH19: 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 pco 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., Bont, 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., Shannugam, 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

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Table S1. Oligonucleotides used in this study.

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