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Recent advances in the metabolic engineering of *Corynebacterium glutamicum* for the production of lactate and succinate from renewable resources

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Abstract Recent increasing attention to environmental issues and the shortage of oil resources have spurred political and industrial interest in the development of environmental friendly and cost-effective processes for the production of bio-based chemicals from renewable resources. Thus, microbial production of commercially important chemicals is viewed as a desirable way to replace current petrochemical production. *Corynebacterium glutamicum*, a Gram-positive soil bacterium, is one of the most important industrial microorganisms as a platform for the production of various amino acids. Recent research has explored the use of *C. glutamicum* as a potential cell factory for producing organic acids such as lactate and succinate, both of which are commercially important bulk chemicals. Here, we summarize current understanding in this field and recent metabolic engineering efforts to develop *C. glutamicum* strains that efficiently produce L- and D-lactate, and succinate from renewable resources.

Keywords *Corynebacterium glutamicum* · Lactate · Succinate · Metabolic engineering

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Introduction

Starch and lignocellulose are the most abundant renewable resources on earth. Environmental concerns and the depletion of oil reserves have resulted in governmental actions to establish greater energy independence by promoting research into environmentally benign and sustainable production of bio-based chemicals and fuels from those renewable resources. For example, the US Department of Energy selected 30 top chemical candidates that could serve as building blocks for the production of a variety of useful chemicals and polymers from renewable resources [77]. Microbial production of chemicals will help reduce CO_2 accumulation through reduced emissions and through recycling of the CO_2 that is released when bio-based chemicals are combusted. The development of suitable strains of microorganisms is, therefore, critically important for the efficient and cost-effective production of target chemicals from biomass resources. In the post-genomic sequencing era, microbial strains tailored for the production of target compounds can be rationally designed through genetic/metabolic engineering rather than through classical strategies based on mutagenesis and screening. Over the past decade, recombinant strains capable of hydrolyzing starchy and lignocellulosic materials have been developed through genetic/metabolic engineering approaches and used to produce bio-based chemicals and fuels [18, 73].

Corynebacterium glutamicum is a Gram-positive, facultative anaerobic soil bacterium with generally regarded as safe (GRAS) status. This organism was first isolated as a glutamate-producing bacterium in 1956 in Japan [33, 72]. Since that time, the bacterium has been used for industrial production of various amino acids including glutamate and lysine under aerobic conditions [34]. In the first decade of the twenty-first century, the genomic sequences of two *C.*

glutamicum strains, ATCC13032 and R, were revealed [21, 27, 88]. This opened the door for systems metabolic engineering of *C. glutamicum* for rationally designing strains with high productivity of target compounds by combining with knowledge on metabolism and pathway regulation. In addition to its use in the production of various amino acids, *C. glutamicum* has recently attracted attention as a potentially versatile platform for producing bio-based chemicals and fuels.

In 1993, Dominguez et al. [10] showed that *C. glutamicum* produces lactate, succinate and acetate as fermentation products when the oxygen is limited. In 2004, a milestone study was achieved by Inui and colleagues showing that *C. glutamicum* retains its metabolic activity under oxygen deprived conditions when its growth is arrested, and produces L-lactate and succinate at large scale from glucose in minimal medium under oxygen deprivation realized by high-cell density cultivation [23]. The high rate of glucose consumption under oxygen deprivation has been observed only in *C. glutamicum* R, ATCC13032, and the closely related *C. efficiens* YS-314 among *Corynebacterium* relatives tested [80]. After development of the system for producing L-lactate and succinate under oxygen deprivation, the range of target compounds produced by the organism has been expanded by metabolic engineering to include L-valine, L-alanine, and non-natural fermentation products such as ethanol, isobutanol, 1,2-propanediol, and xylitol [4, 16, 17, 24, 26, 45, 59, 62, 81, 82]. Strain optimization for the production of L- and D-lactate and succinate has also been reported (reviewed in this article).

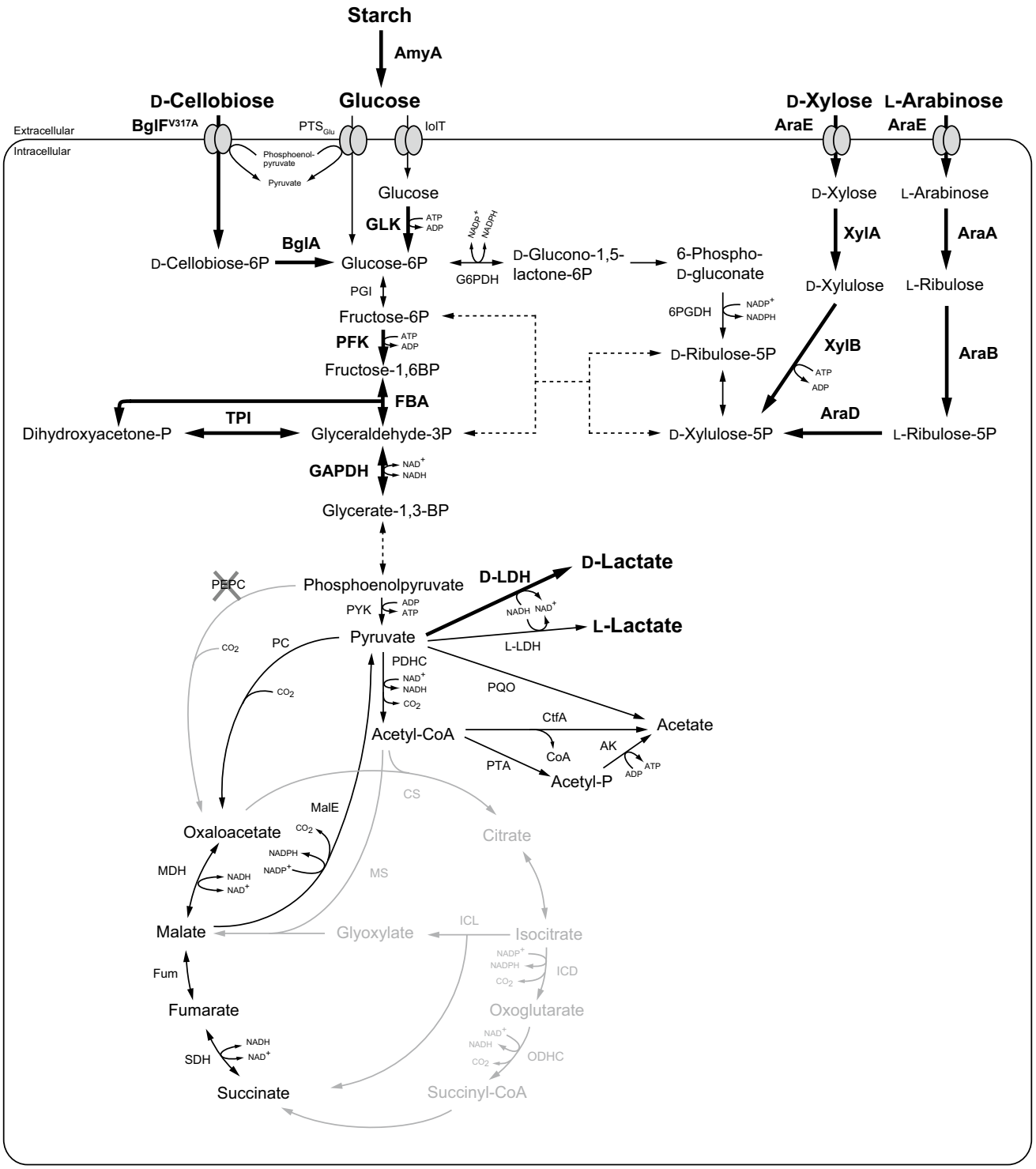
To date, two informative review articles summarizing lactate and succinate production by *C. glutamicum* have been published [75, 79]. After publication of these articles, several interesting studies have been reported regarding the metabolic engineering of *C. glutamicum* for the production of lactate and succinate. The present review focuses on recent advances in the metabolic engineering of *C. glutamicum* for the purpose of optimizing strains to produce L- and D-lactate and succinate from renewable resources.

L- and D-lactate production from glucose

Lactate (lactic acid) is currently used in the food, cosmetic, and pharmaceutical industries, and has attracted considerable attention as a renewable resource material for producing next generation plastics. Lactic acid was selected by the US Department of Energy as one of the top 30 candidates for potential use as sugar-derived building blocks to produce a variety of bulk chemicals and commercially important polymers [77]. However, poly-L-lactic acid, which is a commonly used synthetic polymer, has a markedly lower melting temperature than petroleum-based polymers, thus limiting its practical application [28]. Stereocomplex poly-lactic acid (scPLA), which is a 1:1 mixture of polymerized L- and D-lactic acids, is a promising alternative candidate due to its high heat resistance [12, 20]. However, to produce high-quality scPLA, stereoisomers of both L- and D-lactic acid that are of high optical purity are required. Unlike chemical synthesis, microbial production of lactic acid is, therefore, ideal because either optically pure isomer can be synthesized depending on the chiral-specific L- or D-lactate dehydrogenase (LDH) enzyme expressed by the microorganism. Lactic acid bacteria are used exclusively for lactate production because of their high lactate productivity [48]. However, these bacteria require a nutrient-rich medium for growth, which increases both the production and downstream purification costs [67]. Moreover, the presence of D-lactate is essential for the growth of some lactic acid bacteria, which spoils the optical purity [15]. Other microorganisms, such as metabolically engineered *Escherichia coli*, have been developed for large-scale and cost-effective L- and D-lactate production in minimal medium [47, 90].

Corynebacterium glutamicum is a facultative anaerobic bacterium and thus can grow aerobically or anaerobically using either oxygen or nitrate, respectively, as the external electron acceptor [46, 63]. However, Inui et al. [23] showed that even when its growth is arrested in the absence of oxygen or nitrate, *C. glutamicum* maintains its metabolic activity to produce L-lactate as a major product and succinate and acetate as minor products. L-lactate

Fig. 1 The central metabolic pathways of *C. glutamicum* including pathways for the assimilation of carbon sources (glucose, xylose, arabinose, cellobiose) used for the production of either L- or D-lactate. When producing D-lactate, native L-LDH encoding gene is deleted. *Gray arrows* represent pathways not present due to inactivation of genes encoding the enzymes marked with an *X*, or not activated under anaerobic conditions. *Bold lines* represent pathways that are useful for increasing D-lactate production by overexpressing the corresponding genes, or that can be exploited for utilizing new carbon sources by introducing assimilating genes. *6PGDH* 6-phosphogluconate dehydrogenase, *AK* acetate kinase, *AmyA* α-amylase, *AraA* L-arabinose isomerase, *AraB* L-ribulokinase; *AraD* L-ribulose-5-phosphate 4-epimerase, *AraE* L-arabinose transporter, *BglA* phospho-β-glucosidases, $BglF^{V317A}$ mutated PTS permease enabling D-cellobiose import, *CS* citrate synthase, *CtfA* acetyl CoA:CoA transferase, *FBA* fructose bisphosphate aldolase, *FDH* formate dehydrogenase, *Fum* fumarase, *GAPDH* glyceraldehyde phosphate dehydrogenase, *G6PDH* glucose-6-phosphate dehydrogenase, *GLK* glucokinase, *ICD* isocitrate dehydrogenase, *ICL* isocitrate lyase, *IolT* inositol permease, *L-LDH* L-Lactate dehydrogenase, *D-LDH* D-Lactate dehydrogenase, *MalE* malic enzyme, *MDH* malate dehydrogenase, *MS* malate synthase, *ODHC* 2-oxoglutarate dehydrogenase complex, *PC* pyruvate carboxylase, *PDHC* pyruvate dehydrogenase complex, *PEPC* phosphoenolpyruvate carboxylase, *PFK* phosphofructokinase, *PGI* phosphoglucose isomerase, *PQO* pyruvate/quinone oxidoreductase, *PTA* phosphotransacetylase, PTS_{Glu} phosphotransferase system for glucose import, *PYK* pyruvate kinase, *SDH* succinate dehydrogenase, *TPI* triosephosphate isomerase, *XylA* xylose isomerase, *XylB* xylulokinase



is produced from pyruvate by L-LDH (encoded by the *L-ldhA* gene), and succinate is produced mainly through an anaplerotic pathway catalyzed by phosphoenolpyruvate carboxylase (PEPC) and through the reductive branch of the tricarboxylic acid (TCA) cycle via oxaloacetate, malate, and fumarate [23] (Fig. 1). Inui et al. [23] established a *C. glutamicum* two-stage production system consisting of a biomass-production phase under aerobic conditions followed by a production phase under oxygen deprived conditions in minimal medium with a high density of cells in a sealed bottle in which the cells serve as a "biocatalyst". Anaerobic conditions under high cell density are immediately achieved (<5 s), with a dissolved oxygen concentration of <0.01 ppm [19, 49]. Without

Table 1 Summary of lactate and succinate production by the metabolically engineered strains described in this review

Strain	Medium	Titer (g/L)	Yield ($mol_{product}$/ $mol_{substrate}$)	Cultivation time (h)	References
L-Lactate					
C. glutamicum R	Minimal medium, glucose	51.7	1.42	8	[49]
C. glutamicum R	Minimal medium, glucose, bicarbonate	95.6	1.79	6	[49]
C. glutamicum R X5C1	Minimal medium, glucose, xylose, cellobiose	41.4	–	12	[58]
C. glutamicum ATCC13032/pCC-pgsA-amyA	Minimal medium, starch	88.9	1.46	50	[70]
E. coli B0013-090B3	Minimal medium, glucose	142.2	1.74	30.5	[47]
D-Lactate					
C. glutamicum R Δ*ldhA*/pCRB204	Minimal medium, glucose	120.3	1.73	30	[50]
C. glutamicum R LPglc279/pCRB215	Minimal medium, glucose	195.4	1.80	80	Tsuge and Inui unpublished data
E. coli B0013-070B	Minimal medium, glucose	122.8	1.69	28	[90]
Succinate (anaerobic)					
C. glutamicum R Δ*ldhA*/pCRA717	Minimal medium, glucose, bicarbonate	146.3	1.40	46	[51]
C. glutamicum ATCC13032 BOL-3/pAN6-*gap*	Saline, glucose, formate, bicarbonate	133.8	1.67	54	[38]
C. glutamicum ATCC13032 ELB-P	Minimal medium, glucose, bicarbonate	39.0	1.02	59	[79]
C. glutamicum ATCC13032 SA5	Minimal medium, glucose, bicarbonate	109.4	1.32	98	[92]
C. glutamicum ATCC13032 NC-2	Minimal medium, corn cob hydrolysate, bicarbonate	40.8	0.69	48	[74]
E. coli BA305	Sugarcane bagasse hydrolysate	83	0.87	36	[37]
E. coli NZN111	Minimal medium, cassava starch	127.1	–	39	[5]
S. cerevisiae PMCFfg	Minimal medium, glucose	13.0	0.21	120	[84]
Succinate (aerobic)					
C. glutamicum ATCC13032 BL-1/pAN6-*pyc*^{P458S}*ppc*	Minimal medium, glucose	9.7	0.36	22.5	[39]
C. glutamicum ATCC13032 ZX1/pE*acsAgltA*	Minimal medium, glucose	28.1	0.63	67	[91]
C. glutamicum ATCC13032 BL-1/pVWEx1-*glpFKD*	Minimal medium, glycerol	9.3	0.21	22	[40]
C. glutamicum ATCC13032 BL-1/pSb*AmyA*	Minimal medium, microalgal starch	0.5	0.28	24	[36]
C. glutamicum ATCC13032 ZX1/pX*araBAD* pE*acsAgltA*	Minimal medium, arabinose	8.8	0.69	84	[6]

genetic modification, the wild-type *C. glutamicum* strain R produced 574 mM (51.7 g/L) L-lactate in minimal medium after 8 h, with a yield of 1.79 mol/mol glucose when a concentration of 30 g of dry cells/L was used [49] (Table 1). A linear correlation between cell concentration (up to 60 g of dry cells/L) and the rates of lactate and

succinate production was observed. When sodium bicarbonate was added at the beginning of the cultivation, the glucose consumption rate increased. The rate was maximized to 68 mM/h when 100 mM sodium bicarbonate was added [49] due to stimulation of the carbon dioxide-fixing reaction catalyzed by PEPC. Indeed, the succinate yield increased from 0.09 to 0.37 mol/mol glucose. The drawback was that the yield of lactate decreased from 1.79 to 1.56 mol/mol glucose. When 400 mM sodium bicarbonate was added, 1,061 mM (95.6 g/L) L-lactate was produced after 6 h, as mixed organic acids [49] (Table 1). The titer was less advantageous, but the productivity was comparable to metabolically engineered *E. coli* 090B3, which produced 142.2 g/L of L-lactate within 30.5 h, with a yield of 1.74 mol/mol glucose [47] (Table 1).

As mentioned above, because optically pure L- and D-lactate are necessary to synthesize scPLA, large-scale production of D-lactate under oxygen deprivation has also been examined. Interestingly, *C. glutamicum* R does not possess a D-lactate dehydrogenase (Dld)-encoding gene, which enables to grow on D-lactate in *C. glutamicum* ATCC13032 [29]. To produce optically pure D-lactate using *C. glutamicum* R, the native L-LDH-encoding gene *L-ldhA* was deleted, and the D-LDH-encoding *D-ldhA* gene from *Lactobacillus delbrueckii* under the control of the *C. glutamicum* R *ldhA* promoter was introduced using a plasmid. The resultant strain (Δ*ldhA*/pCRB204) produced 1,336 mM (120.3 g/L) D-lactate with >99.9 % optical purity within 30 h when using 60 g of dry cells/L [50] (Table 1). The yield of D-lactate was 1.73 mol/mol glucose. In this study, the pathway for succinate production was untouched to maintain a high glucose consumption rate, with the result that the succinate yield remained high (0.19 mol/mol glucose). To minimize the succinate yield, the *ppc* gene was deleted in the subsequent study. As expected, the yield of succinate from the resultant strain (CRZ2/pCRB203) decreased to 0.05 mol/mol glucose [69]. However, the D-lactate productivity also decreased (to 14.5 mM/h) when using 100 g of wet cells/L (corresponding 25 g of dry cells/L). Replacement of the promoter controlling expression of *D-ldhA* from *ldhA* to the promoter for *gapA* (encoding glyceraldehyde phosphate dehydrogenase [GAPDH]) (CRZ2/pCRB215) increased D-lactate productivity to 19.1 mM/h. Further dramatic improvement in D-lactate productivity was achieved by overexpressing glycolytic genes. Recently, it was shown that overexpression of glycolytic genes is an effective strategy for increasing the production of fermentation products in *C. glutamicum* R under oxygen deprivation. In an alanine-producing strain of *C. glutamicum* R in which the *ldhA* and *ppc* genes were deleted and the alanine dehydrogenase-encoding gene (*alaD*) from *Lysinibacillus sphaericus* was overexpressed, overexpression of the GAPDH-encoding gene increased alanine production by 2.7-fold under oxygen deprivation [26]. Moreover, simultaneous overexpression of the genes encoding GAPDH, phosphoglucose isomerase, phosphofructokinase (PFK), and pyruvate kinase was shown to result in a stepwise 6.4-fold increase in alanine production compared with the control strain [82]. In D-lactate-producing *C. glutamicum* (CRZ2/pCRB215), individual chromosomal overexpression of the 10 glycolytic genes showed that overexpression of the genes encoding glucokinase (GLK), GAPDH, PFK, triosephosphate isomerase (TPI), and fructose bisphosphate aldolase (FBA) increased D-lactate productivity by 98, 39, 15, 13, and 10 %, respectively, compared with the CRZ2/pCRB215 parent strain [69] (Fig. 1). Overexpression of the glycolytic genes also affected the yield of by-products. Overexpression of the GAPDH-encoding gene was shown to increase succinate yield by 59 %. Overexpression of the GLK-encoding gene was shown to dramatically increase the yields of dihydroxyacetone and glycerol, by 9.0- and 6.8-fold, respectively. Upon simultaneous overexpression of the five genes, the strain (LPglc279/pCRB215) produced 2,169 mM (195.4 g/L) D-lactate, with a yield of 1.80 mol/mol glucose after 80 h of cultivation under oxygen deprivation when using 100 g of wet cells/L (corresponding 25 g of dry cells/L) (Tsuge and Inui, Unpublished result; Table 1). The optical purity of the D-lactate produced under these circumstances was >99.9 %. These values are similar to those obtained when using metabolically engineered *E. coli* B0013-070B (122.8 g/L of D-lactate within 28 h, with a yield of 1.69 mol/mol glucose [90] [Table 1]), as strain LPglc279/pCRB215 produced 1,394 mM (125.6 g/L) D-lactate within 32 h.

Anaerobic/microaerobic succinate production from glucose

Succinate (Succinic acid), an intermediate of the TCA cycle, is selected by the U.S. Department of Energy as one of the top 12 candidate sugar-derived building blocks [77]. Bio-based succinic acid can be used as a building block for the production of a variety of bulk chemicals including 1,4-butanediol, tetrahydrofuran, and gamma-butyrolactone, that are currently produced from n-butane/butadiene via maleic anhydride using chemical processes [41]. Succinic acid is also useful as a building block for producing commercially important polymers, such as polybutylene succinate adipate [60, 89]. Although the current market for succinic acid is remained in the order of 30–50 kton/year based predominantly on petrochemical-based production, bio-based succinic acid could potentially lead to a market in the order of one to several megatons [25]. The potential for using a microbial bio-based process to replace chemical-based succinic acid production has been extensively studied. Several

microorganisms, including *Anaerobiospirillum succiniciproducens* and *Actinobacillus succinogenes* have been found to produce succinate as the end product of anaerobic fermentation [14, 43]. Metabolically engineered *E. coli* and *Saccharomyces cerevisiae* are also used to produce succinate [5, 54, 84]. Two companies, BioAmber and DSM-Roquette, currently produce succinic acid on a large scale using recombinant *E. coli* and *S. cerevisiae*, respectively [7].

Inui et al. [23] reported that *C. glutamicum* R produces succinate under oxygen deprivation, mainly through an anaplerotic pathway catalyzed by PEPC and through the reductive branch of the TCA cycle via oxaloacetate, malate, and fumarate (Fig. 2). Addition of sodium bicarbonate at the beginning of the process enhances the reaction catalyzed by PEPC and accelerates the rate of succinate production in proportion to the concentration, up to 400 mM [49]. Inui et al. later showed that *C. glutamicum* holds great potential as a host strain for producing succinate by metabolic engineering with the use of the two-stage production system. They tailored *C. glutamicum* R for producing succinate by deleting the *L-ldhA* gene to shut off lactate production and by overexpressing the pyruvate carboxylase (PC)-encoding gene (*pyc*) (Δ*ldhA*-pCRA717). Overexpression of the gene encoding another anaplerotic enzyme, PEPC had no dramatic effect on succinate production. The engineered strain produced 1,240 mM (146.3 g/L) succinate from glucose within 46 h, with a yield of 1.40 mol/mol glucose, when using 50 g of dry cells/L and 400 mM sodium bicarbonate [51] (Table 1). The strain did not produce any lactate. However, a large amount of acetate was still produced as a by-product with a yield of 0.29 mol/mol glucose. Acetate is produced mainly through three pathways catalyzed by phosphotransacetylase (*pta*) plus acetate kinase (*ackA*), pyruvate/quinone oxidoreductase (*pqo*), and acetyl coenzyme A (CoA):CoA transferase (*cat*) [85] (Fig. 2). Litsanov and colleagues further engineered *C. glutamicum* ATCC13032 to minimize acetate production by deleting these four genes. They also chromosomally overexpressed the mutant *pyc* gene encoding PC possessing a P458S amino acid exchange, that reportedly enhanced the reaction [22] (Fig. 2). To increase the reducing equivalents, the NAD^+-coupled formate dehydrogenase-encoding gene (*fdh*) from *Mycobacterium vaccae* was also chromosomally overexpressed (Fig. 2). Last, the GAPDH-encoding gene was overexpressed by introducing plasmid pAN6-*gap*, with the aim of increasing glucose consumption, as was observed with alanine production [26]. The resultant strain, BOL-3/pAN6-*gap*, produced 1,134 mM (133.8 g/L) succinate within 53 h from glucose and formate, with a yield of 1.67 mol/mol glucose when using a culture of cells at an OD_{600} of 50 (approximately 20 g of dry cells/L) and 250 mM sodium bicarbonate [38] (Table 1). Unexpectedly, overexpression of the GAPDH-encoding gene did not increase glucose consumption but instead increased the succinate yield by 10 %, as was observed with D-lactate production (described above).

Because *C. glutamicum* is cultured at neutral pH (6.9–7.5) under oxygen deprived conditions, the dissociated form of succinic acid is the primary product. This indicates that succinate is exported through a protein exporter under these conditions. Two research groups independently indentified the succinate exporter in *C. glutamicum*. Using a comparative genomic analysis approach, Huhn et al. [19] identified the exporter, designated SucE. Later, Fukui et al. [11] identified the same exporter (designated SucE1) through comprehensive transcriptional analysis of *C. glutamicum* under microaerobic conditions. They also demonstrated that overexpression of the SucE1-encoding gene increases succinate production by 1.5-fold at an Eppendorf-tube scale under anaerobic conditions. Zhu et al. [92] investigated the effect of overexpressing the SucE1-encoding gene on succinate production at bioreactor scale and found that succinate productivity and the rate of glucose consumption increased by 19 and 8 %, respectively (Fig. 2). The parent strain they used was SA1 with deletion of *ldhA*, *pta*, *ackA*, *pqo*, and *cat* genes and with plasmid-based overexpression of the native *pyc* gene. They also overexpressed genes encoding isocitrate lyase (*aceA*), malate synthase (*aceB*), and citrate synthase (*gltA*) from a plasmid (strain SA4), with the aim of converting more carbon to succinate through the oxidative TCA cycle and glyoxylate pathway, as well as the reductive branch of the TCA cycle through the anaplerotic pathway (Fig. 2). Overexpression of *aceA*, *aceB*, and *gltA* resulted in a 12.8 ± 0.8 % carbon flux toward the glyoxylate pathway. Strain SA5, in which the succinate transporter-encoding gene (*sucE*) was overexpressed using a plasmid in SA4, produced 926 mM (109.4 g/L) succinate within 98 h, with a yield of 1.32 mol/mol glucose when using 27.5 g of dry cells/L and 230 mM sodium bicarbonate [92] (Table 1). This level of production was highly comparable to that obtained with metabolically engineered *S. cerevisiae* PMCFfg (which produced 13.0 g/L of succinate within 120 h, with a yield of 0.21 mol/mol glucose), even though the process was carried out at pH 3.8, which considerably reduces the cost of downstream processes [84] (Table 1).

The above-mentioned studies involved a two-stage bioprocess consisting of aerobic growth in complex or minimal medium followed by anaerobic succinate production in a sealed bottle or a fermenter after harvesting and resuspension of the cells in fresh minimal medium at high cell density. To overcome the practical disadvantages of such two-stage processes, a one-stage fermentation process in a single bioreactor was demonstrated by Wieschalka et al. This process includes three phases: (1)

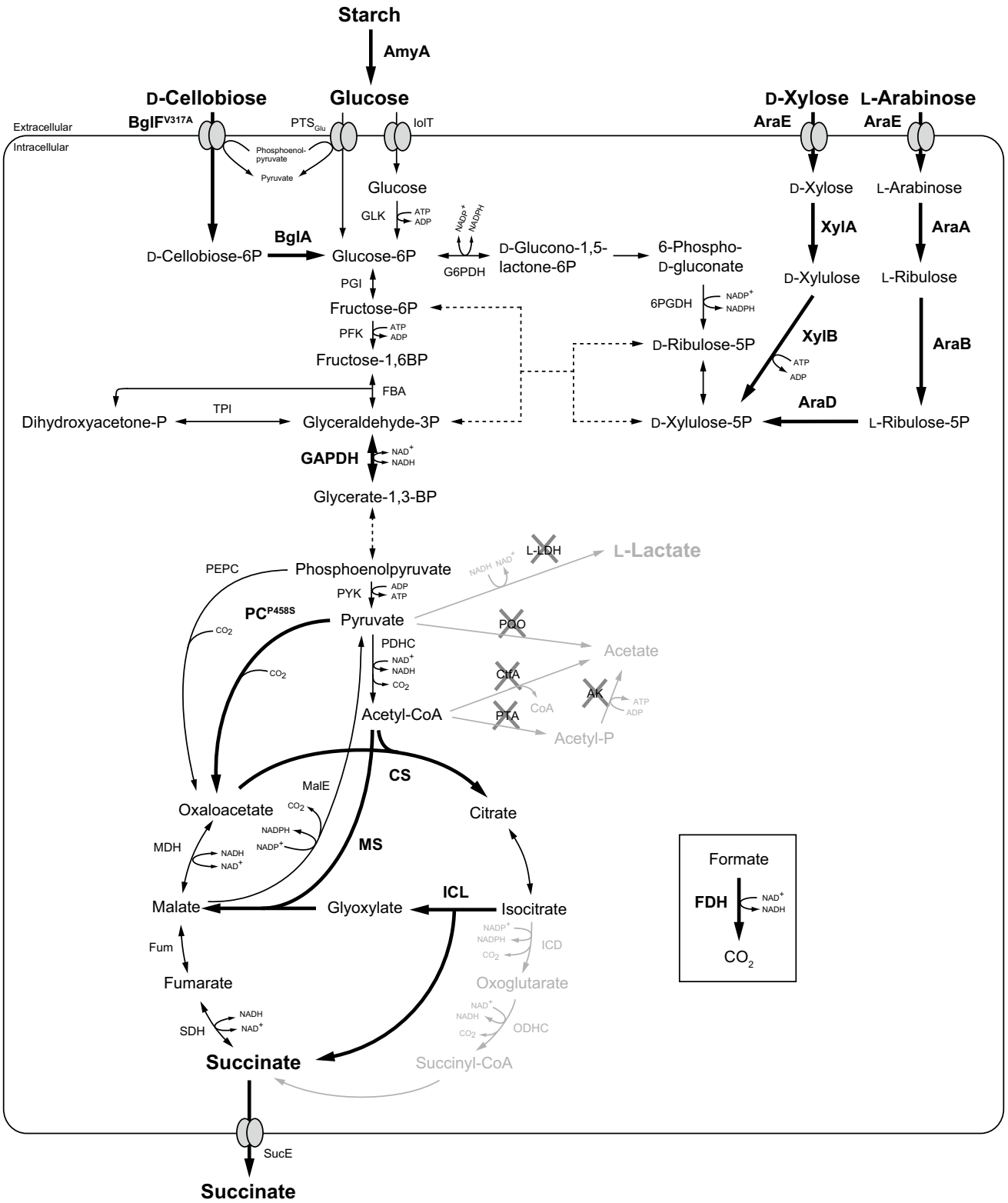


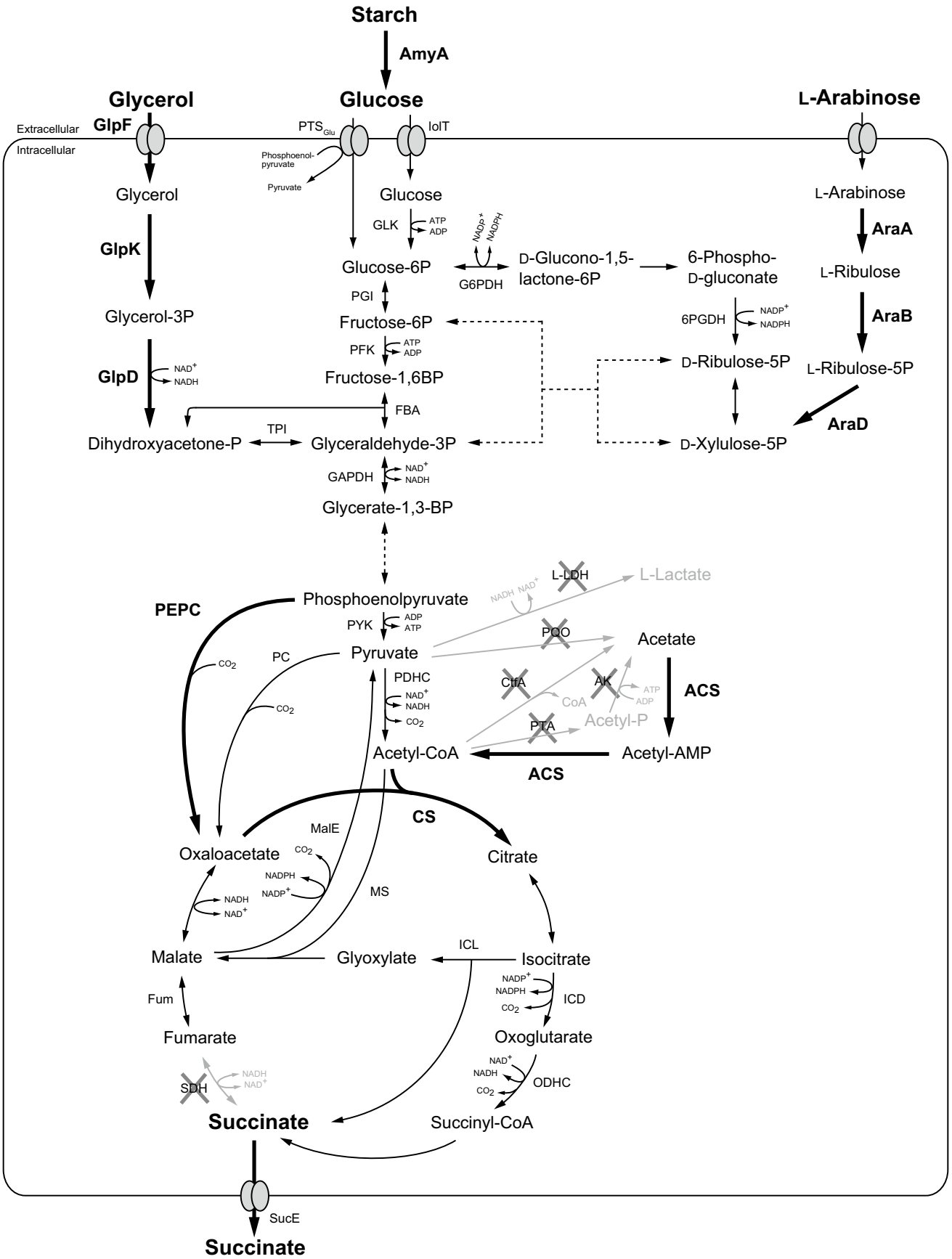
Fig. 2 The central metabolic pathways of *C. glutamicum* including pathways for the assimilation of carbon sources (glucose, xylose, arabinose, cellobiose) used for the production of succinate under anaerobic conditions. *Gray arrows* represent pathways not present due to inactivation of genes encoding the enzymes marked with an *X*, or not activated under anaerobic conditions. *Bold lines* represent pathways that are useful for increasing succinate production by overexpressing the corresponding genes, or that can be exploited for utilizing new carbon sources by introducing assimilating genes

an aerobic growth phase on glucose and acetate; (2) a self-induced microaerobic phase at the end of the exponential growth phase with minimal aeration to assist in physiological adaptation from the aerobic to subsequent anaerobic conditions; and (3) an anaerobic production phase, achieved by supplying the fermenter with CO_2 gas. They used the pyruvate-overproducing strain ELB-P, in which *aceE* (encoding the E1p subunit of the pyruvate dehydrogenase complex), *ldhA*, *pqo*, the last 249 bp of the C-terminal domain of *ilvN* (encoding the small subunit of acetohydroxyacid synthase), *alaT* (encoding the alanine aminotransferase), and *avtA* (encoding the valine:pyruvate aminotransferase) were deleted [78]. This growth-decoupled one-stage bioprocess produced 330 mM (39.0 g/L), succinate with a yield of 1.02 mol/mol glucose [79] (Table 1).

Aerobic succinate production from glucose

One-stage bioprocesses involving aerobic growth and anaerobic succinate production require delicate regulation of the bioreactor. Moreover, in anaerobic cultivation, by-products must be produced to generate reducing equivalents. To circumvent these problems, aerobic succinate production was considered. Because succinate must be produced through the oxidative TCA cycle under aerobic conditions, the key target of metabolic engineering for aerobic succinate production is deletion of the succinate dehydrogenase-encoding genes (*sdhCAB*) (Fig. 3). Litsanov and colleagues showed that although the growth rate and biomass formation of a Δ*sdhCAB* strain were 9 and 28 % lower than the wild type, the Δ*sdhCAB* strain accumulated 40 mM succinate after 22.5 h of aerobic cultivation [39]. Because the major by-product formed during cultivation of the Δ*sdhCAB* strain was acetate (125 mM after 22.5 h), the four genes responsible for acetate production (*pqo*, *pta*, *ackA*, *cat*; see above) were also deleted to minimize production of this by-product (Strain BL-1). Acetate accumulation decreased to 22 mM in BL-1, whereas succinate accumulation increased to 66 mM. Overexpression of the anaplerotic enzymes-encoding genes pyc^{P458S} and *ppc* (BL-1/pAN6-pyc^{P458S}*ppc*) to increase the availability of oxaloacetate as a substrate for citrate synthase resulted in production of 82 mM (9.7 g/L) succinate after 22.5 h aerobic cultivation, with a yield of 0.36 mol/mol glucose [39] (Fig. 3; Table 1). Interestingly, overexpression of the pyc^{P458S} gene increased the succinate titer by only 3 %, whereas overexpression of the *ppc* gene increased the titer by 21 %. In contrast, during anaerobic cultivation, overexpression of the *pyc* gene resulted in a twofold increase in succinate production, whereas overexpression of the *ppc* gene had no significant effect [23]. Previous studies showed that PC-catalyzed pathway from pyruvate to oxaloacetate is responsible for 91 % of the oxaloacetate replenishment during aerobic growth of *C. glutamicum* on glucose [53], whereas the PEPC-catalyzed pathway from phosphoenolpyruvate to oxaloacetate is primarily responsible for producing succinate under oxygen deprived conditions [23]. These results demonstrate that the genes encoding anaplerotic enzymes that play a minor role in oxaloacetate replenishment under both conditions (PEPC under aerobic and PC under anaerobic) can increase the availability of oxaloacetate when overexpressed. Low theoretical yield is the primary disadvantage of aerobic succinate production, because a major proportion of the carbon source is converted into biomass and CO_2. Therefore, Litsanov et al. tried to restrict growth by decreasing the level of the nitrogen source, which is essential for growth but not for succinate formation. As a result, the maximal biomass concentration under nitrogen-limiting conditions was 45 % lower than that under conditions of nitrogen excess, but succinate production was 43 % higher under nitrogen-limiting conditions than under conditions of nitrogen excess. As expected, the succinate yield under nitrogen-limiting conditions increased to 0.45 mol/mol glucose [39]. Although all known genes involved in acetate production were deleted, a relatively large amount of acetate was still produced (15 mM). Zhu and colleagues tried to solve this problem by introducing the gene encoding acetyl-CoA synthase (*acsA*) from *Bacillus subtilis*, which converts acetate to acetyl-CoA through acetyladenylate (Fig. 3). Plasmid-based expression of *acsA* in a strain in which the *sdhCAB, ldhA*, *pqo*, *cat*, and *pta* genes were deleted and in which the native *pyc* and *ppc* genes were overexpressed (strain ZX1) resulted in elimination of acetate production and an increase in the yield of succinate to 0.5 mol/mol glucose [91]. Overexpression of the gene encoding citrate synthase (*gltA*) as well (strain ZX1/pE*acsAgltA*) increased the succinate concentration by 23 % and increased the yield to 0.61 mol/mol glucose (Fig. 3). In contrast to anaerobic conditions, under aerobic conditions, the overexpression of succinate exporter-encoding gene had no effect on succinate productivity. In fed-batch cultivation, strain ZX1/pE*acsAgltA* produced approximately 238 mM (28.1 g/L) succinate within 67 h, with a yield of 0.63 mol/mol glucose [91] (Table 1).

Fig. 3 The central metabolic pathways of *C. glutamicum*, including pathways for the assimilation of carbon sources (glucose, arabinose, glycerol) used for the production of succinate under aerobic conditions. *Gray arrows* represent pathways not present due to inactivation of genes encoding the enzymes marked with an *X*. *Bold lines* represent pathways that are useful for increasing succinate production by overexpressing the corresponding genes or that can be exploited for utilizing new carbon sources by introducing assimilating genes. *ACS* acetyl-CoA synthase, *GlpD* glycerol-3-phosphate dehydrogenase, *GlpF* glycerol facilitator, *GlpK* glycerol kinase ▶


Starch
AmyA
Glycerol
Glucose
L-Arabinose
Extracellular
Intracellular
GlpF
PTS Glu
IolT
Phosphoenol-pyruvate
Pyruvate
Glycerol
Glucose
L-Arabinose
GLK
ATP
ADP
NADP+
NADPH
GlpK
Glucose-6P
D-Glucono-1,5-lactone-6P
6-Phospho-D-gluconate
G6PDH
AraA
L-Ribulose
PGI
Glycerol-3P
Fructose-6P
6PGDH
NADP+
NADPH
AraB
PFK
ATP
ADP
GlpD
NAD+
NADH
Fructose-1,6BP
D-Ribulose-5P
L-Ribulose-5P
FBA
AraD
TPI
Dihydroxyacetone-P
Glyceraldehyde-3P
D-Xylulose-5P
GAPDH
NAD+
NADH
Glycerate-1,3-BP
L-LDH
L-Lactate
PEPC
Phosphoenolpyruvate
PYK
ADP
ATP
PQO
Acetate
PC
CO2
Pyruvate
PDHC
NAD+
NADH
CO2
CtfA
AK
ACS
CoA
ATP
ADP
PTA
Acetyl-P
Acetyl-CoA
Acetyl-AMP
ACS
CS
MalE
Oxaloacetate
CO2
Citrate
NADPH
NADP+
NADH
NAD+
MS
ICL
Malate
Glyoxylate
Isocitrate
NADP+
NADPH
CO2
ICD
Fum
Fumarate
Oxoglutarate
NAD+
NADH
CO2
ODHC
SDH
NADH
NAD+
Succinate
Succinyl-CoA
SucE
Succinate

Production of lactate and succinate from alternative resources

So far, we have focused on studies examining the production of lactate and succinate primarily from purified glucose, as this is the simplest way to examine the effect of metabolic engineering. In terms of production costs, economically feasible microbial production of chemicals depends heavily on the use of low-cost carbon sources, especially when the products are commodity chemicals. Because wild-type *C. glutamicum* cannot directly utilize low-cost carbon sources such as lignocellulose, glycerol, and starch, considerable research has focused on metabolically engineering strains to expand the substrate spectrum. A particularly promising carbon source for microbial production of commodity chemicals is lignocellulosic biomass, which consists largely of cellulose, hemicellulose, and lignin, since it is the most abundant renewable organic resource on earth [3]. Cellulose, a polysaccharide consisting of a linear chain of glucose molecules, becomes a suitable carbon source following depolymerization by chemical or enzymatic processes. Hemicellulose, which consists primarily of glucose and the C5 sugars xylose and arabinose, can also be utilized as a carbon source after the pretreatment. While some microorganisms, such as *E. coli*, naturally utilize xylose and arabinose, *C. glutamicum* must be engineered to assimilate these C5 sugars.

The first *C. glutamicum* strain capable of utilizing xylose was developed by introducing the plasmid pCRA811, which carries the xylose isomerase (*xylA*) and xylulokinase (*xylB*) genes from *E. coli* [30] (Figs. 1, 2). The resultant strain, CRX2, grew on xylose as a sole carbon source. Interestingly, when organic acids were produced under oxygen deprivation, the L-lactate yield was higher when using glucose (1.36 mol/mol glucose) than when using xylose (1.06 mol/mol glucose), whereas the succinate yield was lower when using glucose (0.28 mol/mol glucose) than when using xylose (0.50 mol/mol glucose). When using a mixture of glucose and xylose at a mass ratio of 2:1, which is typical of lignocellulosic hydrolysates [3], xylose was consumed only after the glucose was depleted under aerobic growth conditions, showing apparent carbon catabolite repression [8]. Under oxygen deprivation, the carbon catabolite repression was partially deregulated, but the rate of xylose consumption doubled once the glucose was exhausted, showing that metabolism of xylose is still affected by the presence of glucose [30]. Carbon catabolite repression under anaerobic conditions was later fully deregulated when five copies of the *xylA* and *xylB* genes were integrated into the chromosome of *C. glutamicum* R (strain X5C1). Strain X5C1 consumed xylose at a constant rate, regardless of the presence or absence of glucose [57]. The strain also carried one copy each of the $bglF^{V317A}$ and *bglA* genes (encoding the β-glucoside-specific enzyme IIBCA component of the phosphotransferase system and phospho-β-glucosidase, respectively), which enabled *C. glutamicum* R to utilize cellobiose [35] (Figs. 1, 2). Strain X5C1 produced approximately 460 mM (41.4 g/L) L-lactate and 125 mM (14.8 g/L) succinate within 12 h from a mixture of 222 mM (40 g/L) glucose, 134 mM (20 g/L) xylose, and 29.3 mM (10 g/L) cellobiose [58] (Table 1). Another C5 sugar contained in hemicellulose, arabinose, could be utilized by *C. glutamicum* R upon introduction of plasmid pCRA820, which carried the *ara* operon, consisting of the *araB*, *araA*, and *araD* genes (encoding L-ribulokinase, L-arabinose isomerase, and L-ribulose-5-phosphate 4-epimerase, respectively) from *E. coli* (Figs. 1, 2, 3; strain CRA1) [31]. Around the same time, Kawaguchi and colleagues reported that of 10 *C. glutamicum* strains tested, only strain ATCC31831 could utilize arabinose as a sole carbon source [32]. The strain possessed arabinose-utilization genes (*araB*, *araD*, *araA*) analogous to those in *E. coli*, forming an operon with a different gene order than *E. coli*. The strain also possessed an arabinose transporter gene (*araE*) located upstream of the *araBDA* operon. Unexpectedly, chromosomal integration of *araE* increased the rate of both xylose and arabinose consumption by 2.7- and 2.8-fold, respectively, under oxygen deprivation [58] (Figs. 1, 2). Strain X5C1 containing an integrated *araE* gene (strain ACX-araE) completely consumed xylose (17.5 g/L), arabinose (7 g/L), and cellobiose (7 g/L) simultaneously with glucose (35 g/L) within 14 h under oxygen deprivation (the amount of organic acids produced is unknown) [58]. Aerobic succinate production from arabinose was recently demonstrated by Chen et al. [6], who introduced plasmid pX*araBAD* (which carries the *E. coli araBAD* operon) into strain ZX1/pE*acsAgltA* (see above), and the resultant strain produced 74.4 mM (8.8 g/L) succinate within 84 h, with a yield of 0.58 mol/mol arabinose (Fig. 3; Table 1).

The production of L-lactate and succinate from C5 sugars in the studies described above involved the use of purified sugars, which is not industrially feasible. Lignocellulosic biomass requires dilute acid pretreatment to accelerate the accessibility of enzymes and subsequent hydrolysis of the polysaccharide [13]. During acid pretreatment, some amount of side products, such as furfural, 5-hydroxymethyfurfural, vanillic acid, and acetic and formic acids are released, which usually inhibits microbial growth and fermentation [2, 42]. Although *C. glutamicum* is highly tolerant to such fermentation inhibitors under anaerobic conditions when they are individually added to the medium [56, 71], the production of organic acids using lignocellulose hydrolysates under these conditions has not been demonstrated. Recently, Wang et al. [74] demonstrated succinate production from hydrolysates of corn cob, which is a significant lignocellulosic by-product of

industrial corn production. The corn cob hydrolysates were found to contain toxic compounds, such as 5-hydroxymethyfurfural, vanillic acid, and *p*-hydroxybenzoic acid. Succinate production was indeed inhibited when using corn cob hydrolysates, but the inhibition could be reversed by addition of activated charcoal to absorb the toxins. Strain NC-2, in which the *ldhA* gene was deleted and the *xylA* and *xylB* genes from *E. coli* were introduced, produced 40.8 g/L of succinate within 48 h, with a yield of 0.69 g/g total sugar under anaerobic conditions in two-stage fermentation [74] (Table 1). Metabolically engineered *E. coli* BA305 produced 83 g/L of succinate within 36 h from sugarcane bagasse hydrolysates, with a yield of 0.87 g/g total sugar [37] (Table 1). Although *C. glutamicum* produced less succinate from hydrolysates than did *E. coli*, further metabolic engineering to accelerate succinate production (as described above) could make *C. glutamicum* a superior host strain for producing succinate from lignocellulose hydrolysates (Table 1).

Glycerol is another attractive carbon source for microbial production of fermentation products, because it is produced in abundance as a major by-product of biodiesel production [86]. Another advantage is that glycerol requires no complicated pretreatment before use as a raw material for fermentation. Although *C. glutamicum* cannot naturally utilize glycerol as a carbon source, introduction of genes of the *E. coli glpFKD* operon, which encode glycerol facilitator, glycerol kinase, and glycerol-3-phosphate dehydrogenase, respectively, enabled *C. glutamicum* to grow on glycerol as a sole carbon source [55] (Fig. 3). A plasmid carrying the *glpFKD* genes was introduced into strain BL-1 (see above) for aerobic succinate production (BL-1/pVWEx1-*glpFKD*). The strain produced 79 mM (9.3 g/L) succinate under aerobic conditions, with a yield of 0.21 mol/mol glycerol [40] (Table 1), a level of production comparable to that obtained when using glucose. Thus, glycerol must be considered a useful resource for succinate production.

Hydrolyzed starch is widely used as a carbon source for microbial production of fermentation products. Although *C. glutamicum* cannot naturally utilize starch, several strains have been developed that are capable of directly utilizing starch by secretion or cell-surface display of α-amylase encoded by the *amyA* gene from *Streptococcus bovis* 148 or *Streptomyces griseus* [61, 65, 66] (Figs. 1, 2, 3). Using AmyA-displaying *C. glutamicum* ATCC13032 (ATCC13032/pCC-*pgsA*-*amyA*), a mixture of 88.9 g/L of L-lactate and 10.4 g/L of succinate was directly produced from 128.5 g/L of soluble starch through consolidated bioprocessing after five cycles of anaerobic cultivation using 60 g of dry cells/L [70] (Table 1). Studies of succinate production from starch have advanced through the use of *E. coli*. Metabolically engineered *E. coli* NZN111 produced 127.1 g/L of succinate within 39 h from cassava starch by simultaneous saccharification and fermentation [5] (Table 1). Recently, microalgae have attracted attention as a host strain for producing bio-fuels, gas, and chemicals because these organisms grow and produce fermentation products using only CO_2 [9]. Microalgae have also attracted attention as a potential feedstock for microbial production after acid hydrolysis or even without pretreatment because they contain large amounts of carbohydrates such as starch or glycogen [1, 44]. To test the utility of using microalgal biomass as a carbon source for *C. glutamicum*, Lee et al. [36] used *Chlamydomonas reinhardtii* as a feedstock for CO_2-derived succinate production by metabolically engineered *C. glutamicum* after simply disrupting the cells by glass bead beating. Strain BL-1 (see above) harboring a plasmid carrying the *amyA* gene from *S. bovis* (BL-1/pSb*AmyA*) produced 0.5 g/L of succinate from microalgal starch without the need for complicated pretreatments, with a yield of 0.28 g/g starch under aerobic conditions (Table 1).

Conclusions and perspectives

Driven by metabolic engineering, *C. glutamicum* is now an attractive platform for producing lactate and succinate with industrial feasibility because of its high productivity in minimal medium, especially under anaerobic conditions. Although further strain optimization to improve the titer and yield of lactate and succinate is desirable, research is now moving to the next stage and focusing on areas, such as the optimization of bioreactor conditions, or the use of lignocellulosic hydrolysates rather than on the use of purified sugars.

An as yet unexploited target for constructing an optimized strain, especially for succinate production, is optimizing the redox state. A well-balanced redox state is critically important for the production of fermentation products under anaerobic conditions. Recently, it was reported that overexpression of the *E. coli* transhydrogenase-encoding gene *pntAB* in *C. glutamicum* ATCC13032 (the aim of which was to increase the amount of NADH available to accelerate succinate production) increased the NADH/NAD^+ ratio and decreased the NADPH/$NADP^+$ ratio during prolonged aerobic growth. Although the growth rate was unchanged, the succinate productivity increased 2.2-fold in the transition phase, which was 16–24 h after growth was initiated [83]. Optimizing the redox state through simple genetic engineering could further improve succinate production under anaerobic conditions. Another expectation for improving process productivity derives from recently revealed research on the global regulators governing the expression of genes involved in sugar uptake, glycolysis, the pentose phosphate pathway, and the TCA

cycle [64, 68, 76]. On L-isoleucine production, overexpression of the gene encoding the global regulator Lrp (which regulates the expression of several operons involved in the metabolism of branched-chain amino acid) was shown to increase the productivity [87].

In addition to metabolic engineering to increase process productivity, physiological alteration of *C. glutamicum*, such as render the organism highly tolerant to low pH, high temperatures, and fermentation inhibitors, is an ideal strategy for industrial production. Since *C. glutamicum* is cultivated at neutral pH under both aerobic and anaerobic conditions, altering the organism so that it would exhibit a high tolerance to low pH would be beneficial. Purifying organic acids from low-pH fermentation broth is usually significantly more cost efficient than purifying these compounds from neutral-pH broth [7]. This makes yeast an attractive platform for the production of organic acids due to its high acid tolerance. In addition, cultivation at low pH reduces the risk of contamination. Tolerance to high temperatures is also beneficial for reducing the risk of contamination. Moreover, engineering strains to be high-temperature tolerant could reduce the costs associated with cooling during fermentation. Lastly, even though *C. glutamicum* has high tolerance to fermentation inhibitors under anaerobic conditions, it is expected that the organism would acquire complete tolerance to fermentation inhibitors contained in lignocellulosic hydrolysates through genetic engineering of, for example, sigma factors which regulate the expression of genes involved in tolerance to various stresses [52]. Such acquired phenotypes can be advantageous for producing other fermentation products as well as the organic acids.

Overall, *C. glutamicum* is now a major candidate microorganism for use as a platform for industrial production of lactate and succinate, and the potential exists for the use of *C. glutamicum* as a cell factory for producing fine, value-added chemicals as well as bulk chemicals.

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