

Platform Engineering of *Corynebacterium glutamicum* with Reduced Pyruvate Dehydrogenase Complex Activity for Improved Production of L-Lysine, L-Valine, and 2-Ketoisovalerate

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Exchange of the native *Corynebacterium glutamicum* promoter of the *aceE* gene, encoding the E1p subunit of the pyruvate dehydrogenase complex (PDHC), with mutated *dapA* promoter variants led to a series of *C. glutamicum* strains with gradually reduced growth rates and PDHC activities. Upon overexpression of the L-valine biosynthetic genes *ilvBNCE*, all strains produced L-valine. Among these strains, *C. glutamicum aceE* A16 (pJC4 *ilvBNCE*) showed the highest biomass and product yields, and thus it was further improved by additional deletion of the *pqo* and *ppc* genes, encoding pyruvate:quinone oxidoreductase and phosphoenolpyruvate carboxylase, respectively. In fed-batch fermentations at high cell densities, *C. glutamicum aceE* A16 $\Delta pqo \Delta ppc$ (pJC4 *ilvBNCE*) produced up to 738 mM (i.e., 86.5 g/liter) L-valine with an overall yield ($Y_{P/S}$) of 0.36 mol per mol of glucose and a volumetric productivity (Q_P) of 13.6 mM per h [1.6 g/(liter \times h)]. Additional inactivation of the transaminase B gene (*ilvE*) and overexpression of *ilvBNCD* instead of *ilvBNCE* transformed the L-valine-producing strain into a 2-ketoisovalerate producer, excreting up to 303 mM (35 g/liter) 2-ketoisovalerate with a $Y_{P/S}$ of 0.24 mol per mol of glucose and a Q_P of 6.9 mM per h [0.8 g/(liter \times h)]. The replacement of the *aceE* promoter by the *dapA*-A16 promoter in the two *C. glutamicum* L-lysine producers DM1800 and DM1933 improved the production by 100% and 44%, respectively. These results demonstrate that *C. glutamicum* strains with reduced PDHC activity are an excellent platform for the production of pyruvate-derived products.

Corynebacterium glutamicum is a Gram-positive, facultative anaerobic organism that grows on a variety of sugars and organic acids and is the workhorse for the production of a number of amino acids, e.g., L-glutamate, L-lysine, and also L-valine (1–4). Recent studies also showed the successful employment of *C. glutamicum* for the production of the diamines putrescine and cadaverine (5–10), the organic acids D-lactate, succinate, 2-ketoisovalerate, and pyruvate (11–15), the biofuels ethanol and isobutanol (16–18), xylitol (19), and heterologous proteins (20, 21).

Since the common precursor of the products mentioned above is pyruvate (with the exception of xylitol and proteins), the optimization of its availability has a high potential to improve microbial production processes. Radmacher et al. (22) showed that inactivation of D-pantothenate biosynthesis by deleting the *panBC* genes in combination with plasmid-bound overexpression of the genes encoding acetohydroxyacid synthase (AHAS) (*ilvBN* gene product), acetohydroxyacid isomeroreductase (AHAIR) (*ilvC* gene product), dihydroxyacid dehydratase (DHAD) (*ilvD* gene product), and/or transaminase B (TA) (*ilvE* gene product) (Fig. 1) led to increased L-valine production of *C. glutamicum* when cultivated under D-pantothenate-limiting conditions. Later on, Bartek et al. (23) showed that this limitation results in a drastically increased cytoplasmic pyruvate pool, due to reduced coenzyme A (CoA) availability for the reaction of the pyruvate dehydrogenase complex (PDHC). Schreiner et al. (24) identified and functionally characterized the E1p subunit of the PDHC in *C. glutamicum* and showed that the activity of this multienzyme complex is essential for growth of this organism on glucose, pyruvate, or L-lactate. Deletion of the *aceE* gene, encoding the E1p subunit, caused PDHC deficiency, and the resulting strain *C. glutamicum* $\Delta aceE$

strain required either acetate or ethanol as additional carbon source for growth (24, 25). Further characterization of the PDHC-deficient strain *C. glutamicum* $\Delta aceE$ showed that the mutant formed significant amounts of L-valine, L-alanine, and pyruvate from glucose when acetate was exhausted from the medium and growth stopped (26). Plasmid-bound overexpression of the L-valine biosynthesis genes *ilvBNCE* shifted the product spectrum toward L-valine (26), and inactivation of the pyruvate:quinone oxidoreductase (PQO) (*pqo* gene product; Fig. 1) and phosphoglucose isomerase (*pgi* gene product) in *C. glutamicum* $\Delta aceE$ (pJC4 *ilvBNCE*) resulted in even more efficient L-valine production (up to 410 mM, with a maximum yield of 0.86 mol per mol of glucose in the production phase [27]). Based on these results, we engineered the wild type (WT) of *C. glutamicum* for the aerobic, growth-decoupled production of 2-ketoisovalerate (KIV) from glucose by deletion of the *aceE*, *pqo*, and *ilvE* genes and additional overexpression of the *ilvBNCD* genes (11). In fed-batch fermentations at high cell densities, *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE$ (pJC4 *ilvBNCD*) produced up to 188 mM KIV and showed a volumetric productivity of about 4.6 mM KIV per h in the overall production phase (11). In further approaches, we used the PDHC-deficient *C. glutamicum* strain as a platform for the efficient production

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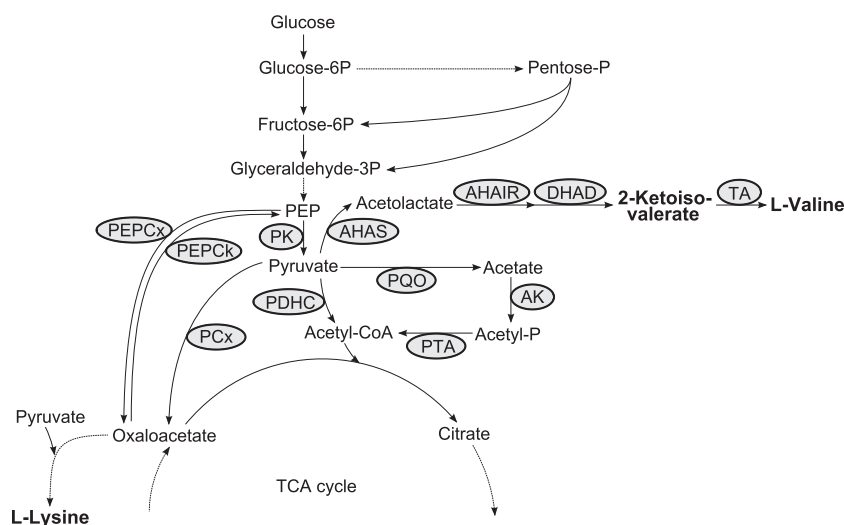


FIG 1 Enzymes of the central metabolism with the biosynthetic pathway of 2-ketoisovalerate/L-valine and L-lysine in *C. glutamicum*. Abbreviations: AHAIR, acetohydroxyacid isomeroreductase; AHAS, acetohydroxyacid synthase; AK, acetate kinase; DHAD, dihydroxyacid dehydratase; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; PEPCK, PEP carboxykinase; PEPCx, PEP carboxylase; PK, pyruvate kinase; PTA, phosphotransacetylase; PQA, pyruvate:quinone oxidoreductase; TA, transaminase B; TCA, tricarboxylic acid.

of about 500 mM pyruvate (i.e., 45 g/liter) or 175 mM isobutanol (i.e., 13 g/liter) and also to improve L-lysine production with *C. glutamicum* (15, 16, 28).

As stated above, a common feature of all PDHC-deficient *C. glutamicum* strains is the start of the production phase only after complete consumption of the acetate required for growth. Although the addition of maltose instead of glucose, the use of ethanol instead of acetate, or the inactivation of the transcriptional regulator SugR led to growth-coupled production of L-valine, all strains still require the use of acetate (or ethanol) as an essential carbon source (25, 29), resulting in a production process more laborious than approaches with glucose as a single carbon source. This holds true especially in large-scale production processes, for which rather simple approaches are always preferred.

Promoter engineering is a suitable metabolic engineering strategy to carefully modulate transcription and to alter the resulting enzyme activity avoiding deletion of a corresponding gene and its unwanted effects such as auxotrophies. Holátko et al. (30) reduced activity of the *ilvA* (encoding threonine deaminase) and *leuA* (encoding isopropylmalate synthase) promoters and increased the activities of the *ilvD* and *ilvE* promoters by site-directed mutagenesis of the respective (extended) –10 regions. These modifications in combination with the deletion of *panB* (see above) and expression of *ilvBN* alleles encoding a feedback-resistant variant of the AHAS resulted in an L-isoleucine bradytroph and improved production strain, which is, however, still auxotrophic for D-pantothenate (30). To improve L-lysine production with *C. glutamicum*, several studies employed the strong promoters of the superoxide dismutase gene or of the elongation factor TU gene to replace the native chromosomal promoters of target genes (31–33).

By site-directed mutagenesis of the (extended) –10 region of the dihydrodipicolinate synthase gene (*dapA*) promoter, Vasicová et al. (34) engineered a library with gradually differing promoter activity. Here we made use of this *dapA* promoter library and show that the replacement of the native promoter of the *aceE* gene by mutated *dapA* promoters leads to a series of *C. glutamicum* strains

with gradually reduced PDHC activity and growth on glucose, without a requirement for additional carbon sources. These strains were investigated for their ability to produce L-valine, and the most promising strain was then further engineered for higher L-valine and 2-ketoisovalerate production. Finally, the novel *C. glutamicum* platform with reduced PDHC activity was employed for improving L-lysine production.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains, plasmids, and oligonucleotides used and their relevant characteristics, sequences and sources or purposes are listed in Table 1.

DNA preparation and transformation. Isolation of plasmids from *Escherichia coli* was performed as described previously (35). Plasmid DNA transfer into *C. glutamicum* was carried out by electroporation, and recombinant strains were selected on Luria-Bertani brain heart infusion (LB-BHI) agar plates containing 0.5 M sorbitol, 85 mM potassium acetate, and appropriate concentrations of kanamycin (50 µg/ml) (36). Isolation of chromosomal DNA from *C. glutamicum* was performed as described previously (36). Electroporation of *E. coli* was carried out with competent cells according to the method of Dower et al. (37).

Culture conditions. *E. coli* was grown aerobically in 2× TY complex medium (38) at 37°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Precultures of the different *C. glutamicum* strains were grown in 2× TY medium containing 0.5% (wt/vol) potassium acetate. The L-lysine producer strains *C. glutamicum* DM1800 and DM1933 and their derivatives were grown in BHI medium (Becton Dickinson) (37 g/liter) containing 1% (wt/vol) glucose. For growth and amino acid fermentations in shake flasks, cells of an overnight preculture were washed with 0.9% (wt/vol) NaCl and inoculated into CGXII minimal medium (pH 7.4) (39) with 222 mM glucose to give an optical density at 600 nm (OD₆₀₀) of about 1. The plasmid-carrying strains were grown in the presence of kanamycin (50 µg/ml). *C. glutamicum* was grown aerobically at 30°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm.

Fed-batch fermentations for L-valine production were performed at 30°C in 300-ml cultures in a Fedbatch Pro fermentation system from DASGIP (Jülich, Germany), and those for 2-ketoisovalerate production

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

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence	Source, reference, or purpose
Strains		
<i>E. coli</i> DH5 α	F ⁻ ϕ 80lacZ Δ M15 Δ (lacZYA-argF)U169 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 gyrA96 relA1 phoA</i>	62
<i>C. glutamicum</i> WT	Wild-type strain ATCC 13032, biotin auxotrophic	American Type Culture Collection
<i>C. glutamicum</i> Δ aceE	<i>C. glutamicum</i> WT with deletion of the <i>E1p</i> gene (<i>aceE</i>) of the pyruvate dehydrogenase complex	24
<i>C. glutamicum</i> DM1800	L-Lysine producer; <i>pyc</i> ^{P485S} , <i>lys</i> ^{CT3111} , derived from <i>C. glutamicum</i> WT	63
<i>C. glutamicum</i> DM1800 <i>aceE</i> A16	<i>C. glutamicum</i> DM1800 in which the native <i>aceE</i> promoter was replaced by the <i>dapA</i> -A16 promoter	This work
<i>C. glutamicum</i> DM1933	L-Lysine producer; Δ <i>pck</i> , <i>pyc</i> ^{P485S} ; <i>hom</i> ^{V59A} ; 2 \times <i>lys</i> ^{CT3111} , 2 \times <i>asd</i> , 2 \times <i>dapA</i> , 2 \times <i>dapB</i> , 2 \times <i>ddh</i> , 2 \times <i>lysA</i> , 2 \times <i>lysE</i> , derived from <i>C. glutamicum</i> WT	64
<i>C. glutamicum</i> DM1933 <i>aceE</i> A16	<i>C. glutamicum</i> DM1933 in which the native <i>aceE</i> promoter was replaced by the <i>dapA</i> -A16 promoter	This work
<i>C. glutamicum</i> <i>aceE</i> L1, A23, A25, A16	<i>C. glutamicum</i> WT in which the native <i>aceE</i> promoter was replaced by the <i>dapA</i> -L1, -A23, -A25, or -A16 promoter, respectively	This work
<i>C. glutamicum</i> <i>aceE</i> A16 Δ <i>pqo</i>	<i>C. glutamicum</i> <i>aceE</i> A16 with deleted <i>pqo</i> gene, encoding pyruvate:quinone oxidoreductase	This work
<i>C. glutamicum</i> <i>aceE</i> A16 Δ <i>pqo</i> Δ <i>ppc</i>	<i>C. glutamicum</i> <i>aceE</i> A16 Δ <i>pqo</i> with deleted <i>ppc</i> gene, encoding phosphoenolpyruvate carboxylase	This work
<i>C. glutamicum</i> <i>aceE</i> A16 Δ <i>pqo</i> Δ <i>ppc</i> Δ <i>ilvE</i>	<i>C. glutamicum</i> <i>aceE</i> A16 Δ <i>pqo</i> Δ <i>ppc</i> with deleted <i>ilvE</i> gene, encoding transaminase B	This work
Plasmids		
pK18/19 <i>mobsacB</i>	Km ^r , mobilizable (<i>oriT</i>), <i>oriV</i>	40
pK18 <i>mobsacB</i> aceE-rAc	pK18 <i>mobsacB</i> carrying a truncated promoter region of the <i>aceE</i> gene, encoding the E1 subunit of the PDHC	This work
pK18 <i>mobsacB</i> <i>PaceE</i> <i>dapA</i> -L1, -A23, -A25, -A16	pK19 <i>mobsacB</i> <i>PaceE</i> carrying the <i>dapA</i> -L1, -A23, -A25, or -A16 promoter, respectively	This work
pK19 <i>mobsacB</i> Δ <i>ppc</i>	pK19 <i>mobsacB</i> carrying a truncated <i>ppc</i> gene, encoding phosphoenolpyruvate carboxylase	This work
pK19 <i>mobsacB</i> Δ <i>pqo</i>	pK19 <i>mobsacB</i> carrying a truncated <i>pqo</i> gene, encoding pyruvate:quinone oxidoreductase	41
pK19 <i>mobsacB</i> Δ <i>ilvE</i>	pK19 <i>mobsacB</i> carrying a truncated <i>ilvE</i> gene, encoding transaminase B	61
pK19 <i>mobsacB</i> Δ <i>Pgl</i> A540- <i>PdapA</i> -L1, -A23, -A25, -A16	pK19 <i>mobsacB</i> carrying a truncated promoter region of the <i>gl</i> A gene and the <i>dapA</i> -L1, -A23, -A25, or -A16 promoter, respectively	60
pJc4 <i>ilvBNCD</i>	Kan ^r ; plasmid carrying the <i>ilvBNCD</i> genes encoding the L-valine biosynthetic enzymes acetohydroxyacid synthase, isomeroreductase, and dihydroxyacid dehydratase	65
pJc4 <i>ilvBNCE</i>	Kan ^r ; plasmid carrying the <i>ilvBNCE</i> genes encoding the L-valine biosynthetic enzymes acetohydroxyacid synthase, isomeroreductase, and transaminase B	22
Oligonucleotides		
<i>dapA</i> ow	5'-AACTGCAGAACCAATGCATTTGGTTCTGCAGTTATCACA CCC-3'	Amplification of <i>dapA</i> promoters, NsiI site underlined
<i>dapA</i> rev2	5'-GGGAATTCATATGAGGCTCCTTTAAATCGAGCGGCTCCGGTCTTAGCTGTAAACC-3'	Amplification of <i>dapA</i> promoters/verification of promoter exchange, NdeI site underlined
<i>ace1</i>	5'-CCCAAGCTTGACATTACCGTCCAACC-3'	Sequencing of <i>dapA</i> promoters/verification of promoter exchange
<i>ace2</i>	5'-CGCGGATCCCAGCGTAAACGCTTCTCC-3'	Sequencing of <i>dapA</i> promoters
<i>ilvE1</i>	5'-GCGTTGACTGATTCTTGGTC-3'	Primer to verify deletion of <i>ilvE</i> (11)
<i>ilvE2</i>	5'-CGAGTTCGATGGAATCTTCG-3'	Primer to verify deletion of <i>ilvE</i> (11)
<i>pqo</i> del1	5'-AAGGAATTCGTTTTCGAGGCGACACAGACAG-3'	Primer to verify deletion of <i>pqo</i> (41)
<i>pqo</i> del4	5'-TGGCACAAAGCTTGTTAAGCGCTCGCGGTCAATG-3'	Primer to verify deletion of <i>pqo</i> (41)
<i>ppc1</i>	5'-CCCAGCTTGAGTTGCGCAGCGCAGTG-3'	Primer for deletion of <i>ppc</i> , HindIII site underlined
<i>ppc2</i>	5'-GTGCTGCGCAATGCTGAGGGCATTAGAGCAGTGGATT GG-3'	Primer for deletion of <i>ppc</i> , crossover overlap underlined
<i>ppc3</i>	5'-CCTCAGCATTGCGCAGCACATCGGCCACAGCTTCTGC-3'	Primer for deletion of <i>ppc</i> , crossover overlap underlined
<i>ppc4</i>	5'-CGCGGATCCCGATGACATCAGGTTCTCTC-3'	Primer for deletion of <i>ppc</i> , BamHI site underlined
<i>ppc</i> del1	5'-GGAATAGACTCGCTCGGC-3'	Primer to verify deletion of <i>ppc</i>
<i>ppc</i> del2	5'-GTGAACAGGCTCTCGATGC-3'	Primer to verify deletion of <i>ppc</i>
<i>aceE</i> up-fw	5'-CGGGATCCCGACCCAATGCGTACCGATGTG-3'	Primer for deletion of <i>aceE</i> promoter region, BamHI site underlined
<i>aceE</i> -intrev	5'-GCGCTAGCGCCACCATCGGAGGTGTGTTC-3'	Primer for deletion of <i>aceE</i> promoter region, NheI site underlined
<i>aceE</i> -rAC-soeleft	5'-TTGATCGGCCATATGTATTATGCATCTCTCACGTTTGACG CGAATCG-3'	Primer for deletion of <i>aceE</i> promoter region, crossover overlap underlined, NsiI and NdeI in italic
<i>aceE</i> -rAC-soeright	5'-CAAACGTGAGAGATGCATAATACATATGGCCGATCAAAGC AAAACTTGG-3'	Primer for deletion of <i>aceE</i> promoter region, crossover overlap underlined, NsiI and NdeI in italic

were performed in 1,500-ml cultures in a Bioengineering (Wald, Switzerland) stirred tank reactor with a head pressure of 1.5×10^5 Pa. Batch fermentations for L-lysine production were performed in glass reactors as 600-ml cultures. The pH was maintained at 7.3 by online measurement using a standard pH probe (Mettler Toledo, Giessen, Germany) and ad-

dition of 10% NH₃ and 5 M H₂SO₄ (for L-valine and L-lysine) or 5 M KOH and 5 M H₂SO₄ (for 2-ketoisovalerate). Foam development was prevented by manual injection of about 20 μ l of Struktol 674 antifoam (Schill und Seilacher, Hamburg, Germany). Dissolved oxygen was measured online using a polarometric oxygen electrode (Mettler Toledo, Giessen, Ger-

many) and adjusted to $\geq 20\%$ of saturation in a cascade by stirring at 300 to 1,500 rpm and aeration up to 1 volume per volume per minute. The fermentations were carried out in CGXII minimal medium (pH 7.4) (39) initially containing 222 mM or 333 mM glucose. For 2-ke-tisovalerate fermentations, 1% (wt/vol) yeast extract and 10 mM L-valine, L-isoleucine, and L-leucine were additionally added, and for L-lysine fermentations, 0.5% (wt/vol) BHI powder was additionally added. Antibiotics were added as appropriate (kanamycin, 50 $\mu\text{g}/\text{ml}$). During the fed-batch processes, adequate amounts of 50% (wt/vol) glucose were injected.

Construction of *C. glutamicum* deletion and promoter exchange mutants. Chromosomal inactivation of the phosphoenolpyruvate carboxylase gene *ppc* in *C. glutamicum aceE A16 $\Delta p q o$* was performed using crossover PCR and the suicide vector pK19*mobsacB*. DNA fragments were generated using the primer pairs *ppc1/ppc2* and *ppc3/ppc4*. The two fragments were purified, mixed in equal amounts, and subjected to crossover PCR using primers *ppc1* and *ppc4*. The resulting fusion product (containing the *ppc* gene with an internal deletion of 1,922 bp) was ligated into BamHI/HindIII-restricted plasmid pK19*mobsacB* and transformed into *E. coli*. After isolation and sequencing (MWG Biotech), the recombinant plasmid was electroporated into *C. glutamicum aceE A16 $\Delta p q o$* . Using the method described by Schäfer et al. (40), the intact chromosomal *ppc* gene was replaced by the truncated *ppc* gene via homologous recombination (double crossover). The screening of the *ppc* mutants was done on 2 \times TY agar plates containing 10% (wt/vol) sucrose and 0.5% (wt/vol) potassium acetate. The replacement at the chromosomal locus was verified by PCR using primers *ppcdel1/ppcdel2*.

Chromosomal inactivation of the pyruvate:quinone oxidoreductase gene *pqo* in *C. glutamicum aceE A16* and of the transaminase B gene *ilvE* in *C. glutamicum aceE A16 $\Delta p q o \Delta p p c$* was performed as described before (11, 41). The replacement at the chromosomal locus was verified by PCR using primers *pqodel1/pqodel4* or *ilvE1/ilvE2*, respectively.

Chromosomal replacement of the native *aceE* promoter by mutated *dapA* promoters in *C. glutamicum* WT was performed using the suicide vector pK18*mobsacB*. DNA fragments were generated using primer pairs *aceEup-fw/aceE-rAC-soleft* and *aceE-intrev/aceE-rAC-soeright*. The two fragments were purified, mixed in equal amounts, and subjected to crossover PCR using primers *aceEup-fw* and *aceE-intrev*. The resulting fusion product (containing the *aceE* promoter region shortened by 338 bp) was ligated into BamHI/XbaI-restricted plasmid pK18*mobsacB* and transformed into *E. coli*. After isolation, the nucleotide sequence of the insert region in the recombinant plasmid pK18*mobsacBaceE-rAC* was verified (MWG Biotech), and then the mutated *dapA* promoters (A16, A23, A25, and L1) were amplified via PCR with the primers *dapAfw* and *dapArev2* from plasmids pK19*mobsacB dPglTA540-PdapA-L1*, -A23, -A25, and -A16, respectively, and ligated into NsiI/NdeI-restricted pK18*mobsacBaceE-rAC*. All nucleotide sequences of the inserts in the newly constructed plasmids were verified by sequencing (MWG Biotech). Double crossover and screening for the correct mutants were performed as described above. The replacement of the native *aceE* promoter by the *dapA-L1*, -A16, -A23, and -A25 promoters, respectively, at the chromosomal locus was verified by PCR using primers *ace1/dapArev2*.

Determination of PDHC activities. For determination of PDHC activities, the relevant strains were cultivated aerobically in shake flasks to an OD_{600} of about 5. The cells were harvested by centrifugation for 10 min at 4,500 $\times g$ and 4°C, washed once with 25 ml 0.2 M Tris-HCl (pH 7.4), centrifuged again, and resuspended in 0.5 ml 0.2 M Tris-HCl (pH 7.4), 10 mM MgCl_2 , 3 mM L-cysteine, and 10% (vol/vol) glycerol. The cell suspension was transferred into 2-ml screw-cap vials together with 250 mg of glass beads (0.1-mm diameter; Roth) and subjected to mechanical disruption three times for 30 s at 6,500 rpm with a Precellys 24 instrument (Peqlab) at room temperature (RT) with intermittent cooling on ice for 5 min. Intact cells and cell debris were removed by centrifugation for 15 min at 12,100 $\times g$ and 4°C. The resulting cell extract was then subjected to

ultracentrifugation for 45 min at 45,000 $\times g$ and 4°C, and the supernatant was directly used to determine the PDHC activity as described by Guest and Creaghan (42).

For all tested strains, three biological and two technical replicates were performed. The protein concentration was quantified with the Pierce bicinchoninic acid (BCA) protein assay (Thermo Scientific) with bovine serum albumin as a standard. Assay results were linear over time and proportional to the protein concentration. One unit of activity is defined as 1 μmol NADH formed per min at 30°C.

Analytcs. Biomass formation was followed by determining either the OD_{600} or the cell dry weight (CDW) (in g/liter) at a given time point. Both techniques were correlated for several independent fermentations, resulting in $\text{CDW} = \text{OD}_{600} \times 0.3$.

For determination of glucose and organic and amino acid concentrations in the culture fluid, 1 ml of the culture was harvested by centrifugation (12,100 $\times g$, 10 min, RT) and the supernatant was analyzed. Glucose concentrations were determined by enzymatic tests (Roche Diagnostics). The phosphate concentration was analyzed with the phosphate kit LCK 348 (Hach Lange). The pyruvate concentrations were determined enzymatically as described by Lamprecht and Heinz (43) or by high-pressure liquid chromatography (HPLC) (see below).

The amino acid concentration was determined by reversed-phase HPLC (on an HP 1100 instrument; Hewlett-Packard) with fluorimetric detection (excitation at 230 nm and emission at 450 nm) after automatic precolumn derivatization with *ortho*-phthalaldehyde (44). Separation was carried out at 40°C on a Multohyp octyldecylsilane column (particle size, 5 μm ; 125 by 4 mm; CS-Chromatographie). The elution buffer consisted of a polar phase (0.1 M sodium acetate, pH 7.2) and methanol as a nonpolar phase. Quantification was done by calculation of the concentration using an internal standard (L-ornithine at 100 μM) and with a 10-point calibration curve for each amino acid. Amino acid concentrations were also determined using an Agilent 1200 series apparatus (Agilent Technologies) equipped with an Agilent Zorbax Eclipse Plus C_{18} column (250 by 4.6 mm, 5 μm) protected by an Agilent Zorbax Eclipse Plus C_{18} guard column (12.5 by 4.6 mm, 5 μm). Fluorometric detection (excitation at 230 nm and emission at 450 nm) was carried out after automatic precolumn derivatization with *ortho*-phthalaldehyde. The elution buffer consisted of a polar phase (10 mM Na_2HPO_4 , 10 mM $\text{Na}_2\text{B}_4\text{O}_7$, 0.5 mM NaN_3 , pH 8.2) and a nonpolar phase (45% [vol/vol] acetonitrile, 45% [vol/vol] methanol). Protocol details were as given by Henderson and Brooks (45). Quantification of the analytes was conducted by using L-norvaline as an internal standard to correct for analyte variability and with an 8-point calibration curve for each component as an external reference standard.

Organic acid concentrations were measured via HPLC using an Agilent 1200 series apparatus equipped with a Rezex ROA organic acid H^+ (8%) column (300 by 7.8 mm, 8 μm ; Phenomenex) protected by a Rezex ROA organic acid H^+ (8%) guard column (50 by 7.8 mm). A protocol for phosphate precipitation was applied to each sample and standard prior to measurement. Thus, 45 μl 4 M NH_3 and 100 μl 1.2 M MgSO_4 were added to 1,000 μl sample. After 5 min of incubation, the sample was centrifuged for 5 min at 18,000 $\times g$ and RT, and 500 μl supernatant was then transferred to 500 μl 0.1 M H_2SO_4 . After thorough mixing and 15 min of incubation at RT, samples were finally centrifuged for 15 min at 18,000 $\times g$ at RT. Subsequently, the supernatant was provided for HPLC injection (10- μl injection volume). Separation was performed under isocratic conditions at 50°C (column temperature) for 45 min with 5 mM H_2SO_4 as the mobile phase at a constant flow rate of 0.4 ml/min. Detection of glucose and organic acids was achieved via an Agilent 1200 series refractive index detector at 32°C. Quantification of the analytes was conducted by using L-rhamnose as an internal standard to correct for analyte variability and with an 8-point calibration curve for each component as an external reference standard.

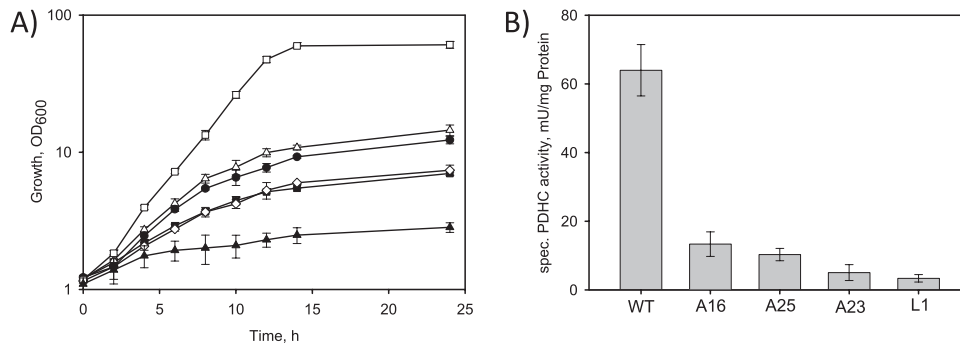


FIG 2 (A) Growth of *C. glutamicum* WT and its derivatives with reduced (A16, A23, A25, and L1) or abolished (*C. glutamicum* $\Delta aceE$) PDHC activity in shake flasks containing CGXII medium with 222 mM glucose. \square , *C. glutamicum* WT; \blacktriangle , *C. glutamicum* $\Delta aceE$; \triangle , *C. glutamicum* *aceE* A16; \bullet , *C. glutamicum* *aceE* A25; \diamond , *C. glutamicum* *aceE* A23; \blacksquare , *C. glutamicum* *aceE* L1. (B) Specific PDHC activities of *C. glutamicum* WT and its derivatives with reduced PDHC activity grown in shake flasks containing CGXII medium with 222 mM glucose. Three independent fermentations were performed. Error bars show standard deviations.

RESULTS

Replacement of the *aceE* promoter by mutated *dapA* promoters results in reduced growth and PDHC activity.

Recently, we identified and functionally characterized the E1p subunit of the PDHC in *C. glutamicum*, located the promoter region, and identified the transcriptional start site of the *aceE* gene at 121 nucleotides upstream of the translational start (24). To replace the native *aceE* promoter, we first cloned the flanking promoter regions into the suicide vector pK18*mobsacB*, deleting 338 nucleotides upstream of the translational start site. We then cloned the four *dapA* promoter variants A16, A23, A25, and L1 (34) between the flanking regions. Applying homologous recombination, we were able to replace the native *aceE* promoter in *C. glutamicum* WT by the mutated *dapA* promoters (see Materials and Methods). Subsequently, the growth of the resulting strains *C. glutamicum* *aceE* A16, A23, A25, and L1 was compared with that of *C. glutamicum* WT and the PDHC-deficient strain *C. glutamicum* $\Delta aceE$ in minimal medium with 4% (wt/vol) glucose (Fig. 2A). As already shown by Schreiner et al. (24), *C. glutamicum* $\Delta aceE$ showed almost no growth (OD₆₀₀ of 2.8 after 24 h), whereas *C. glutamicum* WT reached an OD₆₀₀ of about 61 after 24 h, with a growth rate of $0.32 \pm 0.01 \text{ h}^{-1}$. *C. glutamicum* strains with the exchanged *dapA* promoters A16, A25, A23, and L1 reached significantly lower final OD₆₀₀s of about 14.5, 12.3, 7.4, and 7.0, respectively. Compared to that of the parental WT strain, the growth rates of the mutant strains were gradually decreased to $\mu = 0.22, 0.20, 0.15,$ and 0.14 h^{-1} for strains A16, A25, A23, and L1, respectively.

To further analyze the replacement of the native *aceE* promoter by mutated *dapA* promoters, we determined the overall PDHC

activities in the respective strains grown in minimal medium with 4% (wt/vol) glucose. *C. glutamicum* WT showed in the exponential growth phase a PDHC activity of $64 \pm 7 \text{ mU per mg protein}$. In agreement with the decreased growth rates, *C. glutamicum* *aceE* A16, A25, A23, and L1 showed significantly lower PDHC activities of 13, 10, 5, and 3 mU per mg protein, respectively (Fig. 2B).

Overexpression of the *ilvBNCE* genes in strains with reduced PDHC activity results in improved L-valine overproduction.

To analyze the suitability of the engineered *C. glutamicum* strains with reduced PDHC activity for L-valine production, we transformed *C. glutamicum* *aceE* A16, A25, A23, and L1 and the WT strain with plasmid pJC4 *ilvBNCE*, carried out shake flask cultivations in minimal medium with 222 mM glucose, and investigated growth, substrate consumption, and amino acid formation (Table 2). Overexpression of the L-valine biosynthetic pathway genes resulted in L-alanine and L-valine overproduction in all strains. *C. glutamicum* WT (pJC4 *ilvBNCE*) showed a substrate-specific biomass yield ($Y_{X/S}$) of about 0.066 g CDW per mmol of glucose, a biomass-specific glucose consumption rate (q_s) of about 0.80 g glucose per g CDW and h, a substrate-specific L-alanine yield of about 0.02 mol L-alanine per mol of glucose consumed. Interestingly, among the modified *C. glutamicum* strains, the one with lowest $Y_{X/S}$ but with the uppermost q_s did not exhibit the highest $Y_{P/S}$. With stepwise increasing $Y_{X/S}$ (L1, A23, A25, and A16), the $Y_{P/S}$ increased from about 0.21 (L1) to 0.37 (A16) mol L-valine per mol of glucose, and the substrate-specific L-alanine yield dropped from about 0.06 (L1) to 0.02 (A16) mol L-alanine per mol of glucose (Table 2).

TABLE 2 Substrate-specific biomass yield ($Y_{X/S}$), biomass-specific glucose consumption rate (q_s), and substrate-specific L-alanine and L-valine yields ($Y_{P/S}$) of *C. glutamicum* L-valine producer strains cultivated in CGXII medium with 4% (wt/vol) glucose in shake flasks^a

Strain	$Y_{X/S}$ (g CDW/mmol glucose)	q_s [g glucose/(g CDW × h)]	$Y_{P/S}$ (mol/mol glucose)	
			L-Alanine	L-Valine
<i>C. glutamicum</i> WT (pJC4 <i>ilvBNCE</i>)	0.066 ± 0.012	0.80 ± 0.15	0.02 ± 0.01	0.11 ± 0.04
<i>C. glutamicum</i> <i>aceE</i> L1 (pJC4 <i>ilvBNCE</i>)	0.024 ± 0.004	1.00 ± 0.17	0.06 ± 0.01	0.21 ± 0.01
<i>C. glutamicum</i> <i>aceE</i> A23 (pJC4 <i>ilvBNCE</i>)	0.027 ± 0.003	0.91 ± 0.10	0.07 ± 0.01	0.25 ± 0.01
<i>C. glutamicum</i> <i>aceE</i> A25 (pJC4 <i>ilvBNCE</i>)	0.040 ± 0.003	0.80 ± 0.06	0.04 ± 0.01	0.35 ± 0.02
<i>C. glutamicum</i> <i>aceE</i> A16 (pJC4 <i>ilvBNCE</i>)	0.042 ± 0.002	0.77 ± 0.04	0.02 ± 0.01	0.37 ± 0.01
<i>C. glutamicum</i> <i>aceE</i> A16 Δp_{qo} (pJC4 <i>ilvBNCE</i>)	0.042 ± 0.003	0.72 ± 0.05	0.02 ± 0.01	0.40 ± 0.03
<i>C. glutamicum</i> <i>aceE</i> A16 Δp_{qo} Δp_{pc} (pJC4 <i>ilvBNCE</i>)	0.039 ± 0.005	0.80 ± 0.11	0.02 ± 0.01	0.42 ± 0.02

^a Values represent the arithmetic mean \pm standard deviation from at least three independent experiments.

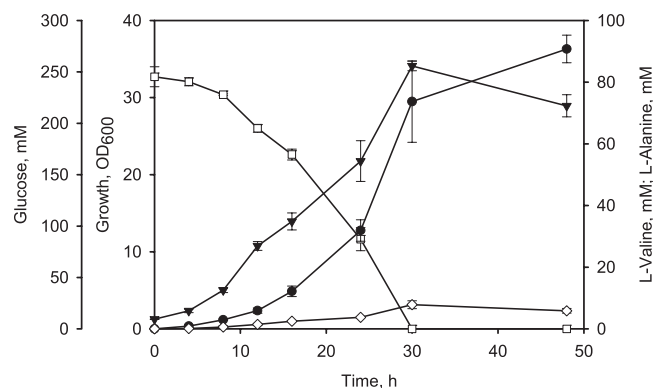


FIG 3 Growth, glucose consumption, and L-alanine and L-valine formation by *C. glutamicum aceE A16* (pJC4 *ilvBNCE*) cultivated in shake flasks with CGXII medium containing 222 mM glucose. ▼, OD₆₀₀; □, glucose; ◇, L-alanine; ●, L-valine. Three independent fermentations were performed. Error bars show standard deviations.

Inactivation of the pyruvate:quinone oxidoreductase and phosphoenolpyruvate carboxylase further improves L-valine production. Among the engineered *C. glutamicum* L-valine producers, *C. glutamicum aceE A16* (pJC4 *ilvBNCE*) showed the best performance, i.e., the highest $Y_{X/S}$ and $Y_{P/S}$ combined with the lowest substrate-specific L-alanine yield (Table 2). In shake flask experiments, this strain consumed 245 mM glucose within 30 h and produced about 90 mM L-valine after 48 h (Fig. 3). Thus, *C. glutamicum aceE A16* (pJC4 *ilvBNCE*) formed the optimal basis for further improvement, and therefore we stepwise deleted the *pqo* and *ppc* genes to further increase pyruvate availability. Both modifications did not significantly alter the $Y_{X/S}$, q_S , or substrate-specific L-alanine yield, but they cumulatively led to about a 14% increased $Y_{P/S}$, from 0.37 to 0.42 mol L-valine per mol of glucose (Table 2).

Fed-batch fermentations with *C. glutamicum aceE A16 Δpqo Δppc* (pJC4 *ilvBNCE*). In order to test the suitability of *C. glutamicum aceE A16 Δpqo Δppc* (pJC4 *ilvBNCE*) for an improved L-valine production process, we carried out fed-batch fermentations in minimal medium initially containing about 333 mM glucose (Fig. 4). Under these conditions, *C. glutamicum aceE A16 Δpqo Δppc* (pJC4 *ilvBNCE*) grew exponentially with a growth rate of 0.15 h⁻¹ to an OD₆₀₀ of about 158 (i.e., 47.4 g CDW/liter) and produced about 378 mM L-valine (i.e., 44.3 g/liter) within 33.5 h. Phosphate in the medium then became limiting; however, despite

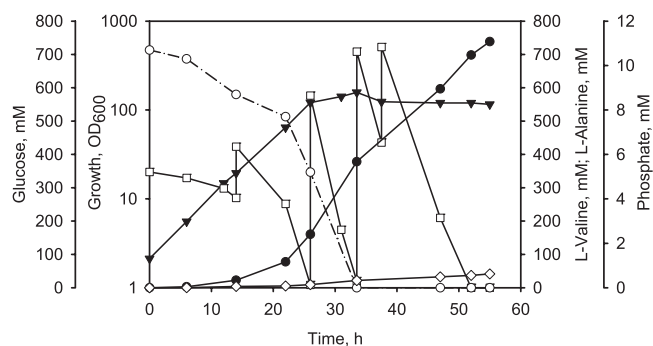


FIG 4 Representative fed-batch fermentation of *C. glutamicum aceE A16 Δpqo Δppc* (pJC4 *ilvBNCE*) in CGXII medium initially containing about 333 mM glucose. ▼, OD₆₀₀; □, glucose; ◇, L-alanine; ●, L-valine; ○, phosphate.

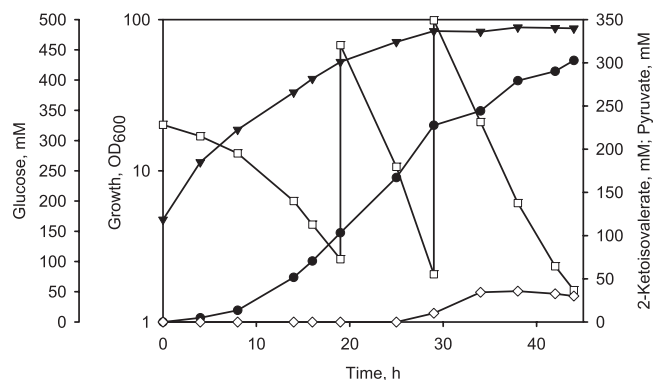


FIG 5 Representative fed-batch fermentation of *C. glutamicum aceE A16 Δpqo Δppc ΔilvE* (pJC4 *ilvBNCD*) in CGXII medium initially containing about 333 mM glucose, 1% (wt/vol) yeast extract, and 10 mM L-valine, L-isoleucine, and L-leucine. ▼, OD₆₀₀; □, glucose; ◇, pyruvate; ●, 2-ketoisovalerate.

the transition to the stationary phase, the cells continued to metabolize glucose and to produce L-valine (Fig. 4). Until the end of the fermentation after 55 h, *C. glutamicum aceE A16 Δpqo Δppc* (pJC4 *ilvBNCE*) produced about 41 mM L-alanine and up to 738 mM L-valine (86.5 g/liter), with a $Y_{P/S}$ of 0.36 mol L-valine per mol of glucose and a volumetric productivity of about 13.6 mM per h [1.6 g/(l × h)]. Taken together, the results show that *C. glutamicum aceE A16 Δpqo Δppc* (pJC4 *ilvBNCE*) with reduced PDHC activity represents an excellent production platform. In contrast to the previously developed producer strains, *C. glutamicum aceE A16 Δpqo Δppc* (pJC4 *ilvBNCE*) did not require any additional carbon source (such as acetate or ethanol) for growth (see the introduction).

Inactivation of transaminase B and overexpression of *ilvBNCD* in *C. glutamicum aceE A16 Δpqo Δppc* results in efficient 2-ketoisovalerate production. Recently, we demonstrated the ability of PDHC-deficient *C. glutamicum* strains to produce 2-ketoisovalerate from glucose when acetate was exhausted from the culture broth (11). To investigate the applicability of the novel production platform for 2-ketoisovalerate production, we inactivated the transaminase B by deletion of the *ilvE* gene in *C. glutamicum aceE A16 Δpqo Δppc* and transformed the resulting strain with plasmid pJC4 *ilvBNCD*. With the final strain, *C. glutamicum aceE A16 Δpqo Δppc ΔilvE* (pJC4 *ilvBNCD*), we carried out fed-batch fermentations in minimal medium initially containing 333 mM glucose, 1% (wt/vol) yeast extract, and 10 mM L-valine, L-isoleucine, and L-leucine. In contrast to the corresponding (transaminase B-positive) L-valine producer, *C. glutamicum aceE A16 Δpqo Δppc ΔilvE* (pJC4 *ilvBNCD*) showed no exponential growth but showed a steadily decreasing growth rate. After 29 h and at an OD₆₀₀ of about 84 (25.2 g CDW/liter) (Fig. 5) growth stopped, although neither phosphate nor L-valine, L-isoleucine, or L-leucine became limiting in the culture broth (not shown). In the growth phase, *C. glutamicum aceE A16 Δpqo Δppc ΔilvE* (pJC4 *ilvBNCD*) produced about 228 mM (26 g/liter) 2-ketoisovalerate. After the growth arrest, the strain continued to consume glucose and further excreted 2-ketoisovalerate into the medium. At the end of the fermentation after 44 h, the strain reached an OD₆₀₀ of about 88 (26.4 g CDW/liter) and produced about 30 mM pyruvate, 33 mM L-valine, and up to 303 mM (35 g/liter) 2-ketoisovalerate (Fig. 5), with a $Y_{K/S}$ of 0.24 mol per mol of glucose

TABLE 3 Substrate-specific biomass yield ($Y_{X/S}$), final titer, substrate-specific L-lysine yield ($Y_{P/S}$), and biomass-specific production rate (q_p) of *C. glutamicum* L-lysine producers cultivated in CGXII medium with 4% (wt/vol) glucose and 0.5% (wt/vol) BHI in shake flasks (DM1800) or in bioreactors (DM1933)^a

Strain	$Y_{X/S}$ (g CDW/mmol glucose)	Titer (mM L-lysine)	$Y_{P/S}$ (mol L-lysine/mol glucose)	q_p [g L-lysine/(g CDW × h)]
<i>C. glutamicum</i> DM1800	0.072 ± 0.001	22 ± 2	0.10 ± 0.01	0.068 ± 0.007
<i>C. glutamicum</i> DM1800 <i>aceE</i> A16	0.048 ± 0.001	45 ± 2	0.20 ± 0.02	0.091 ± 0.009
<i>C. glutamicum</i> DM1933	0.069 ± 0.004	35 ± 3	0.16 ± 0.03	0.078 ± 0.016
<i>C. glutamicum</i> DM1933 <i>aceE</i> A16	0.043 ± 0.006	52 ± 6	0.23 ± 0.04	0.138 ± 0.028

^a Values represent the arithmetic mean ± standard deviation from three independent experiments.

and a volumetric productivity of about 6.9 mM per h [0.8 g (l × h)].

Reducing PDHC activity improves L-lysine production.

Since pyruvate is also the precursor for L-lysine synthesis (see Fig. 1), we analyzed the effect of reduced PDHC activity on L-lysine production with *C. glutamicum*. For this purpose, we replaced the native *aceE* promoter in the defined L-lysine producer *C. glutamicum* DM1800 by the *dapA*-A16 promoter. With the resulting strain, *C. glutamicum* DM1800 *aceE* A16, we carried out shake flask cultivations in minimal medium with 222 mM glucose and 0.5% (wt/vol) BHI and analyzed growth and L-lysine production. In comparison to the parental strain *C. glutamicum* DM1800, the newly constructed *C. glutamicum* DM1800 *aceE* A16 showed a 34% reduced $Y_{X/S}$ of about 0.048 g CDW per mmol glucose; however, it produced two times more L-lysine and showed a 34% increased biomass-specific production rate (q_p) (Table 3). We also introduced the *dapA*-A16 promoter in *C. glutamicum* DM1933, which produces about 60% more L-lysine than strain DM1800, and carried out batch fermentations in aerated and stirred bioreactors. Under these conditions, *C. glutamicum* DM1933 *aceE* A16 reached a $Y_{X/S}$ of about 0.043 g CDW per mmol glucose, secreted the by-products L-valine (5 mM) and L-alanine (3 mM), and showed a $Y_{P/S}$ of 0.23 mol L-lysine per mol of glucose and a q_p of 0.138 g L-lysine per g CDW and h (Table 3), which are about 44% and 77% higher than the values for the parental *C. glutamicum* DM1933.

Platform comparison: PDHC deficiency versus reduction of PDHC activity. Table 4 gives an overview of the production characteristics of L-valine and 2-ketoisovalerate producers based on PDHC deficiency or on PDHC with reduced activity. The genetic backgrounds of the respective strains are not completely identical; however, as shown above, the additional deletion of the *ppc* gene improved the $Y_{P/S}$ of L-valine production with *C. glutamicum* *aceE* A16 $\Delta p q o \Delta p p c$ (pJC4 *ilvBNCE*) by only 5% compared to that with the parental strain with an active phosphoenolpyruvate carboxy-

lase. With the given improvements taken into account, the contribution of the *ppc* deletion is low, and therefore, a comparison of the respective producers is acceptable.

For L-valine production, the reduction of the PDHC activity in *C. glutamicum* in combination with the deletion of the *pqo* and *ppc* genes and overexpression of the *ilvBNCE* genes led to growth-coupled L-valine production of 83.6 g/liter in fed-batch fermentations. Compared to the value for *C. glutamicum* $\Delta aceE \Delta p q o$ (pJC4 *ilvBNCE*) (27), this titer is 3.4 times higher. Furthermore, *C. glutamicum* *aceE* A16 $\Delta p q o \Delta p p c$ (pJC4 *ilvBNCE*) showed a 43% higher overall $Y_{P/S}$ and a 3-times-higher volumetric productivity (Q_p) (Table 4).

For 2-ketoisovalerate, the effect is not as drastic as for L-valine production. Compared to the $Y_{P/S}$ of the PDHC-deficient producer strain, that of *C. glutamicum* *aceE* A16 $\Delta p q o \Delta p p c \Delta ilvE$ (pJC4 *ilvBNCD*) was reduced by about 19%; however, the final titer and the Q_p were improved by 55% and 40%, respectively (Table 4).

Taken together, our results demonstrate that the novel engineered *C. glutamicum* platform with reduced PDHC activity has characteristics which are superior compared to its PDHC-deficient counterpart.

DISCUSSION

Several recent studies with *C. glutamicum* aimed to improve the pyruvate availability for L-valine, L-lysine, L-alanine, 2-ketoisovalerate, or isobutanol production or to engineer *C. glutamicum* for the production of pyruvate itself. These approaches included (i) inactivating enzymes of D-pantothenate synthesis to limit CoA availability for the PDHC reaction (22, 30, 46), (ii) applying anaerobic conditions to abolish/reduce oxidative tricarboxylic acid (TCA) flux (47–49), (iii) using an ATPase-defective mutant leading to increased pyruvate availability (50, 51), and (iv) deleting the *aceE* gene, encoding the E1p subunit of the PDHC (11, 15, 16, 25–28). However, all these approaches either resulted

TABLE 4 Final product concentrations (c_p), substrate-specific product yields ($Y_{P/S}$), and volumetric productivities (Q_p) of fed-batch fermentations of *C. glutamicum* L-valine and 2-ketoisovalerate producers with either inactivated PDHC or PDHC with reduced activity^a

Condition and strain	c_p (g/liter)	$Y_{P/S}$ (mol C/mol C)	Q_p [g/(liter × h)]
L-Valine overproduction			
<i>C. glutamicum</i> $\Delta aceE \Delta p q o$ (pJC4 <i>ilvBNCE</i>) ^b	24.6 ± 2.6	0.23 ± 0.02	0.5 ± 0.1
<i>C. glutamicum</i> <i>aceE</i> A16 $\Delta p q o \Delta p p c$ (pJC4 <i>ilvBNCE</i>)	83.6 ± 2.6	0.33 ± 0.01	1.5 ± 0.1
2-Ketoisovalerate overproduction			
<i>C. glutamicum</i> $\Delta aceE \Delta p q o \Delta ilvE$ (pJC4 <i>ilvBNCD</i>) ^c	21.8 ± 3.2	0.26 ± 0.02	0.5 ± 0.1
<i>C. glutamicum</i> <i>aceE</i> A16 $\Delta p q o \Delta p p c \Delta ilvE$ (pJC4 <i>ilvBNCD</i>)	33.7 ± 1.0	0.21 ± 0.04	0.7 ± 0.1

^a Given values represent the arithmetic mean ± standard deviation of at least three independent experiments.

^b Data are from reference 27.

^c Data are from reference 11.

in a requirement for D-pantothenate, ethanol, or acetate, did not allow an adequate adjustment of the PDHC activity and the carbon flux into the TCA cycle, and/or required a cleverly devised redox state of the cell (under anaerobic conditions). In this study, we applied promoter engineering to develop *C. glutamicum* strains with gradually decreased PDHC activity by exchange of the native *aceE* promoter by a series of mutated *dapA* promoters, allowing the screening of the most promising variant for further optimization by metabolic engineering. Since the PDHC is a multienzyme complex consisting of three subunits (AceE, AceF, and Lpd), which are arranged in a tightly defined stoichiometric composition (52, 53), it is difficult to predict the effects of tuning the promoter activity of one of the respective genes (*aceE*, *aceF*, or *lpd*) on the overall PDHC activity. Therefore, we randomly choose four *dapA* promoter variants (A16, A23, A25, and L1) (34) to replace the native *aceE* promoter and analyzed growth and overall PDHC activity. Shake flask cultivations of the different engineered strains (*C. glutamicum aceE* A16, A23, A25, and L1) revealed that with decreasing PDHC activity the growth rate coherently decreased but the final biomass concentration also did so (Fig. 2A and B), although glucose was still present in the medium (data not shown). The reduced biomass formation is surprising, but it might be attributed to metabolic inhibition since all strains excreted significant amounts of L-valine, L-alanine, and pyruvate (data not shown). However, overexpression of the *ilvBNCE* genes in these strains, leading to a drain of pyruvate toward L-valine, did not improve the final biomass concentration (data not shown). Moreover, compared to the wild type, *C. glutamicum aceE* A23 and L1 (pJC4 *ilvBNCE*) showed 14% and 25% increased q_s (Table 2), indicating energy limitation, which might have led to the observed growth.

For the promoter variants *dapA*-A16, -A23, and -A25, we found a linear correlation of the overall PDHC and the respective promoter activity (data not shown). However, the *dapA*-A16 promoter showed the highest activity (34), but interestingly, this promoter was unable to completely compensate for the activity of the native *aceE* promoter, since *C. glutamicum aceE* A16 showed 79% lower PDHC activity and a 31% decreased growth rate compared to those of *C. glutamicum* WT (Fig. 2). It is noteworthy that the -10 region (TATCCT) of the native *aceE* promoter corresponds to that of the *dapA*-A14 promoter, which shows lower activity than the *dapA*-A16 promoter (34). As a consequence, other enhancing elements such as the extended -10 region or the action of transcriptional regulators such as RamB (54) give the *aceE* promoter its strength. Since *C. glutamicum aceE* A16 showed the highest $Y_{P/S}$ and $Y_{X/S}$, it might be anticipated that other promoters, stronger than the *dapA*-A16 promoter but weaker than the native *aceE* promoter, might be even more suitable for production purposes. Alternatively to or in combination with our approach, modulation of translation initiation by altering the sequence length between the ribosome binding site (RBS) and the translational start codon (TSC), using different TSCs, or deleting the RBS as recently done to improve putrescine production with *C. glutamicum* (9) as well as self-cloning (55) or deletion of repressor/activator binding sites (for, e.g., RamB) might be promising strategies to adjust expression of the *aceE* gene more exactly. However, taking into account the numbers for current aerobic production processes for L-valine with *E. coli* (61 g/liter) (56) or *C. glutamicum* (48 g/liter) (27) and for 2-ketoisovalerate with *C. glutamicum* (22 g/liter) (11), the novel engineered *C. glutamicum* platform with

reduced PDHC activity (84 g/liter L-valine and 34 g/liter 2-ketoisovalerate) (Table 4) is highly competitive or even superior.

In contrast to the L-valine producer (Fig. 4), the 2-ketoisovalerate producer *C. glutamicum aceE* A16 $\Delta p q o \Delta p p c \Delta i l v E$ (pJC4 *ilvBNCD*) showed no constant growth but a steadily decreasing growth rate (Fig. 5). This phenomenon might be attributed to an inhibiting effect of the product, since neither phosphate nor one of the supplemented amino acids (L-valine, L-isoleucine, or L-leucine) became limiting (data not shown). However, the decreasing growth rate of the 2-ketoisovalerate producer shows the general advantage of the PDHC-deficient platform (production does not start before acetate is depleted and growth stops) for the production of cytotoxic products. In any case, despite the continuously decreasing growth rate, *C. glutamicum aceE* A16 $\Delta p q o \Delta p p c \Delta i l v E$ (pJC4 *ilvBNCD*) reached a significantly higher 2-ketoisovalerate titer than the PDHC-deficient counterpart (Table 4). For the production of more toxic/growth-inhibiting products, the situation might be different, and a growth-decoupled production process will then be advantageous.

C. glutamicum is the workhorse for industrial L-lysine production, and several improvements have been made by metabolic engineering (reviewed in references 57, 58, and 59). Recently, we inactivated the PDHC in the defined L-lysine producer *C. glutamicum* DM1729, which led to an auxotrophy for acetate; however, it also led to 44% improved L-lysine production compared to that of the parental strain (28). Here we found that introduction of the *dapA*-A16 promoter in *C. glutamicum* DM1800, which shows an $Y_{P/S}$ identical to that of DM1729, led to prototrophic growth and increased the $Y_{P/S}$ by 100%, indicating that reduction of PDHC activity might be more useful to optimize L-lysine production with *C. glutamicum* than a complete shutdown of the PDHC. The reason for reduced improvement of the PDHC-deficient counterpart might be reduced expression of relevant genes under nongrowing conditions, limiting efficient L-lysine production.

Van Ooyen et al. (60) also made use of the *dapA* promoter library from Vasicová et al. (34) to adjust citrate synthase (encoded by *gltA*) flux for optimized L-lysine production with *C. glutamicum*. The most promising variant, *C. glutamicum* DM1800 $\Delta p r p C 1 \Delta p r p C 2$ with *gltA* under the control of the C7-*dapA* promoter, improved L-lysine production by 82% compared to that in the parental strain (60), which is in the same range as the improvement by reduction of PDHC activity (see above). In any case, both examples demonstrate that reducing the TCA flux by optimizing pyruvate and oxaloacetate supply is highly beneficial for efficient L-lysine production with *C. glutamicum*. *C. glutamicum* DM1933 *aceE* A16 excreted L-alanine and L-valine in significant amounts in batch fermentations. L-Alanine formation is catalyzed mainly by the aminotransferase AlaT from pyruvate (61). This reaction is in equilibrium, and therefore formation of L-alanine and also L-valine is a good indicator for an increased intracellular pyruvate concentration, suggesting that L-lysine production in this strain is limited by the reactions from pyruvate to L-lysine and not by pyruvate availability. The PDHC-deficient L-lysine producer DM1729 $\Delta a c e E$ also excreted L-alanine, L-valine, and pyruvate into the medium, and the additional overexpression of the *ddh* gene (encoding diaminopimelate dehydrogenase) reduced the formation of these by-products and increased the $Y_{P/S}$ by 60% (28). Here, we found that introducing a second copy of the *ddh* gene and additionally of all L-lysine biosynthetic genes (*lysC*^{T3111}, *asd*, *dapA*, *dapB*, *ddh*, *lysA*, and *lysE*) in *C. glutamicum* DM1800

aceE A16 resulted in only about a 15% improved $Y_{p/s}$ (DM1800 *aceE* A16 versus DM1933 *aceE* A16), indicating that either *ddh* expression is still too low or other obstacles such as a sufficient NADPH availability have to be overcome in *C. glutamicum* DM1933 *aceE* A16. However, the surplus of the precursor pyruvate in *C. glutamicum* DM1933 *aceE* A16 opens the possibility for further optimization of L-lysine production by metabolic engineering.

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