

Corynebacterium glutamicum Tailored for Efficient Isobutanol Production^{∇†}

Bastian Blombach,^{1*} Tanja Riester,¹ Stefan Wieschalka,¹ Christian Ziert,² Jung-Won Youn,² Volker F. Wendisch,² and Bernhard J. Eikmanns¹

Institute of Microbiology and Biotechnology, University of Ulm, D-89069 Ulm,¹ and Genetics of Prokaryotes, Faculty of Biology and CeBiTec, University of Bielefeld, D-33501 Bielefeld,² Germany

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

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

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

organic acids, ethanol, and xylitol under oxygen deprivation conditions (24, 38, 39, 42).

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

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

* Corresponding author. Mailing address: Institute of Microbiology and Biotechnology, University of Ulm, 89069 Ulm, Germany. Phone: 49 (0)731 50 22708. Fax: 49 (0)731 50 22719. E-mail: bastian.blombach@uni-ulm.de.

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

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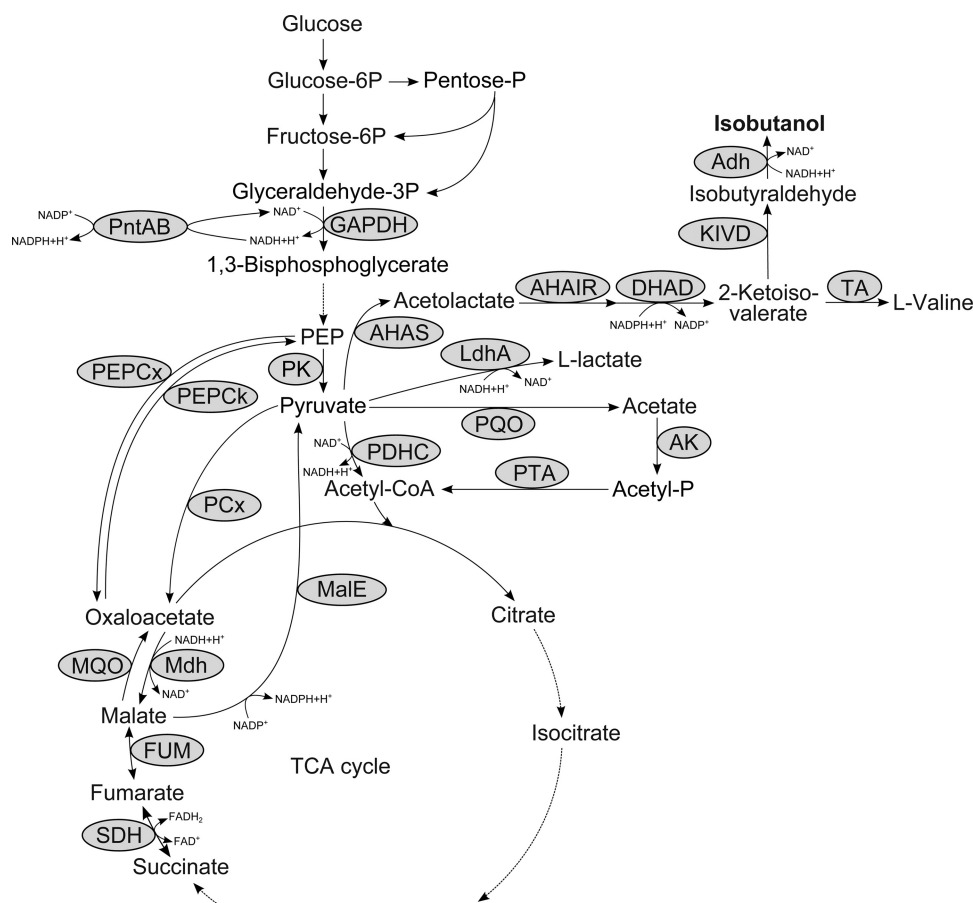


FIG. 1. Enzymes of the central metabolism with the biosynthetic pathway of L-valine in *C. glutamicum* and the synthetic pathway from ketoisovalerate to isobutanol. Abbreviations: Adh, alcohol dehydrogenase; AHAIIR, acetohydroxyacid isomeroreductase; AHAS, acetohydroxyacid synthase; AK, acetate kinase;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

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

(pJC4*ilvBNCD*) seems to be an ideal basis for the production of isobutanol.

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

TABLE 1. Bacterial strains used in this study^a

Strain	Relevant characteristic(s)	Source or reference
<i>E. coli</i> DH5α	F [−] Φ80 <i>lacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) U169 <i>endA1 recA1 hsdR17</i> (rk [−] , mk ⁺) <i>supE44 thi-1 gyrA96 relA1 phoA</i>	22
<i>C. glutamicum</i> WT	WT strain ATCC 13032, biotin auxotrophic	American Type Culture Collection
<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i>	<i>C. glutamicum</i> WT with deletion of <i>aceE</i> , <i>pqo</i> , and <i>ilvE</i> genes, encoding the E1p subunit of the pyruvate dehydrogenase complex, the pyruvate:quinone oxidoreductase, and transaminase B, respectively	28
<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i>	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> with an additional deletion of the <i>ldhA</i> gene, encoding L-lactate dehydrogenase	This work
<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> Δ <i>mdh</i>	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> with an additional deletion of the <i>mdh</i> gene, encoding malate dehydrogenase	This work
<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> Δ <i>malE</i>	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> with an additional deletion of the <i>malE</i> gene, encoding malic enzyme	This work
<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> Δ <i>mdh</i> Δ <i>malE</i>	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> Δ <i>mdh</i> with an additional deletion of the <i>malE</i> gene, encoding malic enzyme	This work
<i>C. glutamicum</i> Iso1	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> (pJC4 <i>ilvBNCD</i>)	This work
<i>C. glutamicum</i> Iso2	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> (pJC4 <i>ilvBNCD</i>)(pBB1 <i>kivd-adh2</i>)	This work
<i>C. glutamicum</i> Iso3	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> (pJC4 <i>ilvBNCD</i>)(pBB1 <i>kivd-adh2</i>)	This work
<i>C. glutamicum</i> Iso4	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> Δ <i>mdh</i> (pJC4 <i>ilvBNCD</i>)(pBB1 <i>kivd-adh2</i>)	This work
<i>C. glutamicum</i> Iso5	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> Δ <i>mdh</i> (pJC4 <i>ilvBNCD</i> - <i>pntAB</i>)(pBB1 <i>kivd-adh2</i>)	This work
<i>C. glutamicum</i> Iso6	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> (pJC4 <i>ilvBNCD</i>)(pBB1 <i>kivd</i>)	This work
<i>C. glutamicum</i> Iso7	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> Δ <i>mdh</i> (pJC4 <i>ilvBNCD</i> - <i>pntAB</i>)(pBB1 <i>kivd-adhA</i>)	This work
<i>C. glutamicum</i> Iso8	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> Δ <i>malE</i> (pJC4 <i>ilvBNCD</i>)(pBB1 <i>kivd-adh2</i>)	This work
<i>C. glutamicum</i> Iso9	<i>C. glutamicum</i> Δ <i>aceE</i> Δ <i>pqo</i> Δ <i>ilvE</i> Δ <i>ldhA</i> Δ <i>mdh</i> Δ <i>malE</i> (pJC4 <i>ilvBNCD</i> - <i>pntAB</i>)(pBB1 <i>kivd-adhA</i>)	This work

^a WT, wild type.

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

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

MATERIALS AND METHODS

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

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

Conditions for growth and isobutanol formation. *E. coli* was grown aerobically in 2× tryptone-yeast (TY) complex medium (41) at 37°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Precultures of the different *C. glutamicum* strains were grown in 2× TY medium containing 0.5% (wt/vol) potassium acetate. For isobutanol fermentations, cells of an overnight preculture were washed with 0.9% (wt/vol) NaCl and inoculated into CGXII minimal medium (pH 7.4) (16) with 2% (wt/vol) glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-leucine, and L-isoleucine (2 mM each), to give an optical density at 600 nm (OD₆₀₀) of about 15. The cells were cultivated for 4 h at 30°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. The cells were then washed with 0.9% (wt/vol) NaCl, inoculated into the same medium, and incubated at 30°C as 50-ml cultures in 125-ml Müller-Krempel (Müller+Krempel AG, Bülach, Switzerland) bottles on a rotary shaker at 120 rpm. Initially, the gas phase in these bottles was aerobic; however, the cultures became anaerobic by rapidly consuming the oxygen in the gas phase. Antibiotics were added appropriately (kanamycin, 25 μg ml^{−1}; chloramphenicol, 6 μg ml^{−1}). Samples were taken using a needle and syringe to inhibit the penetration of oxygen into the culture. The number of grams of cells (dry weight) was calculated from the OD₆₀₀ using a ratio of 0.3 g of cells (dry weight) liter^{−1} per OD₆₀₀ (7).

Fed-batch fermentations were performed at 30°C in 300-ml cultures in a fed-batch Pro fermentation system from DASGIP (Jülich, Germany). The pH was maintained at 7.4 by online measurement using a standard pH electrode (Mettler Toledo, Giessen, Germany) and the addition of 4 M KOH and 4 M H₂SO₄. Foam development was prohibited by manual injection of about 20 μl of 1:5-diluted Struktol 674 antifoam (Schill und Seilacher, Hamburg, Germany). Dissolved oxygen was measured online using an oxygen electrode (Mettler Toledo, Giessen, Germany) and adjusted in the growth phase to 30% of saturation in a cascade by stirring at 300 to 1,000 rpm and aeration with 1 volume of air per volume of medium per minute (vvm). After complete consumption of acetate, aeration was completely switched off, and the stirring speed was reduced to 300 rpm. The fermentations were carried out in CGXII minimal medium (pH 7.4) (16) initially containing 4% (wt/vol) glucose, 1% (wt/vol) acetate, 0.5% (wt/vol) yeast extract, and L-valine, L-leucine, and L-isoleucine (2 mM each). Antibiotics

TABLE 2. Plasmids and oligonucleotides used in this study

Plasmid or oligonucleotide	Relevant characteristic(s) or sequence	Source, reference, or purpose
Plasmids		
pK19 <i>mobsacB</i>	Km ^r , mobilizable (carrying <i>oriT</i> gene), carrying <i>oriV</i> gene	44
pK19 <i>mobsacB</i> - Δ <i>ldhA</i>	pK19 <i>mobsacB</i> carrying a truncated <i>ldhA</i> gene	This work
pK19 <i>mobsacB</i> - Δ <i>mdh</i>	pK19 <i>mobsacB</i> carrying a truncated <i>mdh</i> gene	This work
pK19 <i>mobsacB</i> - Δ <i>malE</i>	pK19 <i>mobsacB</i> carrying a truncated <i>malE</i> gene	This work
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	40
pJC4 <i>ilvBNCD</i> - <i>pntAB</i>	Kan ^r ; plasmid pJC4 carrying the <i>ilvBNCD</i> genes and additionally carrying the <i>pntAB</i> genes from <i>E. coli</i> , encoding the membrane-bound transhydrogenase PntAB; carrying <i>pntAB</i> genes under the control of P _{tac}	This work
pBB1	Cm ^r ; pBB1 is compatible to pJC4 <i>ilvBNCD</i> and harbors the P _{tac} promoter and the T _{imp} terminator; <i>lacI</i> negative	29
pEKEx2- <i>pntAB</i>	Plasmid pEKEx2 carrying the <i>pntAB</i> genes from <i>E. coli</i>	25
pBB1 <i>pntAB</i>	Cm ^r ; plasmid pBB1 carrying the <i>pntAB</i> genes from <i>E. coli</i> ; <i>pntAB</i> under the control of P _{tac}	This work
pSA55	Plasmid for expression of the <i>adh2</i> gene (encoding alcohol dehydrogenase 2) from <i>Saccharomyces cerevisiae</i> and the <i>kivd</i> gene (encoding 2-ketoacid decarboxylase) from <i>Lactococcus lactis</i>	2
pBB1 <i>kivd</i>	Cm ^r ; plasmid pBB1 expressing the <i>kivd</i> gene from <i>L. lactis</i> ; carrying <i>kivd</i> gene under the control of P _{tac}	This work
pBB1 <i>adh2</i>	Cm ^r ; plasmid pBB1 expressing the <i>adh2</i> gene from <i>S. cerevisiae</i> ; carrying <i>adh2</i> gene under the control of P _{tac}	This work
pBB1 <i>kivd</i> - <i>adh2</i>	Cm ^r ; plasmid pBB1 expressing the <i>kivd</i> gene from <i>L. lactis</i> and the <i>adh2</i> gene from <i>S. cerevisiae</i> ; carrying <i>kivd</i> and <i>adh2</i> genes under the control of P _{tac}	This work
pBB1 <i>kivd</i> - <i>adhA</i>	Cm ^r ; plasmid pBB1 expressing the <i>kivd</i> gene from <i>L. lactis</i> and the <i>adhA</i> gene from <i>C. glutamicum</i> ; carrying <i>kivd</i> gene under the control of P _{tac} and <i>adhA</i> gene under the control of the native promoter	This work
Oligonucleotides		
adh4fow	5'-AACTGCAGAACCAATGCATTGGAGGAGACACAACATGTCTATTCCAGAACTCAAAAAAG-3'	Amplification of the <i>adh2</i> gene
adh2rev	5'-CCGCTCGAGCGGTTATTTAGAAGTGTAACAACGAT-3'	Amplification of the <i>adh2</i> gene
kivdfow	5'-AACTGCAGAACCAATGCATTGGAGGAGACACAACATGTATACAGTAGGAGATTACCTAT-3'	Amplification of the <i>kivd</i> gene
kivd2rev	5'-CCAATGCATTGGTTCTGCAGTTTATGATTTATTTGTTTCAGCAAAT-3'	Amplification of the <i>kivd</i> gene
Ptaccheck	5'-CACTCCCGTTCTGGATAATG-3'	Primer to verify orientation of the <i>kivd</i> gene
kivdchkrev	5'-CTGAGAGTGTACCATTATAG-3'	Primer to verify orientation of the <i>kivd</i> gene
adhAfwsoall	5'-ACGCGTCGACGGGAATTGTGTGAATCTTGAAAAG-3'	Amplification of <i>adhA</i> gene; primer to verify orientation of <i>adhA</i> gene
adhArevsall	5'-GCTATGGCCGACGTCGACCAAAGGTCATGCCTTAAGCAGC-3'	Amplification of the <i>adhA</i> gene
pMM36rev	5'-ACTACCGGAAGCAGTGTG-3'	Primer to verify orientation of the <i>adhA</i> gene
pntABfow	5'-CATGCCTGCAGTCATCAATAAAACCG-3'	Amplification of the <i>pntAB</i> genes
pntABrev	5'-GTACGCTGCAGTCTTACAGAGCTTTTCAGG-3'	Amplification of the <i>pntAB</i> genes
transfow2	5'-CTAACATGTATACAAAAAAGCCCGCTCATTAGCGGGGCTGGATGCTCTTACAGAGCTTTCAGGATTGCATCC-3'	Amplification of the <i>pntAB</i> genes
transrev2	5'-CGCCCGGGTTCGGCAACAATGACGGCGAGA-3'	Amplification of the <i>pntAB</i> genes
ldhA1	5'-CCCATCCACTAAACTTAAACAGACGGTTTCTTTCATTTTCGATCC-3'	Primer for deletion of the <i>ldhA</i> gene
ldhA2	5'-TGTTTAAGTTTAGTGGATGGGAAGCAGTTCTTCTAAATCTTTGGCG-3'	Primer for deletion of the <i>ldhA</i> gene
ldhA3	5'-CGCCCGGGGTCATCGACGACATCTGAG-3'	Primer for deletion of the <i>ldhA</i> gene
ldhA4	5'-TGATGGCACCAGTTGCGATGT-3'	Primer to verify deletion of the <i>ldhA</i> gene
ldhfow	5'-CCATGATGCAGGATGGAGTA-3'	Primer to verify deletion of the <i>ldhA</i> gene
ldhrev	5'-CCCAAGCTTGTGCGGTCAGCTGACCTCG-3'	Primer for deletion of the <i>mdh</i> gene
mdh1	5'-CGTCACCGGCGAGCTGGTCCGAATGCTCAGGAATTGCAGG-3'	Primer for deletion of the <i>mdh</i> gene
mdh2	5'-GACCAGCTGCGCCGGTGACGGTGACCTTCTTGGTGGAGACG-3'	Primer for deletion of the <i>mdh</i> gene
mdh3	5'-CGCGGATCCCGCTTGACATGCCAGATGC-3'	Primer for deletion of the <i>mdh</i> gene
mdh4	5'-CCTGATTCCAGGAACGCATC-3'	Primer to verify deletion of the <i>mdh</i> gene
mdhcheckfow	5'-CCTAACATCTTGCAGGTGAG-3'	Primer to verify deletion of the <i>mdh</i> gene
mdhcheckrev	5'-CGGGATCCCTTGCTGCCTACACCTACCTTG-3'	Primer for deletion of the <i>malE</i> gene
malE1	5'-CCATCCACTAAACTTAAACACTGCAGGTCGATGGTCATATC-3'	Primer for deletion of the <i>malE</i> gene
malE2	5'-TGTTTAAGTTTAGTGGATGGGGTCGCCGAAGCGCAAAACGCTTAA-3'	Primer for deletion of the <i>malE</i> gene
malE3	5'-CGGGATCCGAAGTGCTGATCCGCGAAC-3'	Primer for deletion of the <i>malE</i> gene
malE4	5'-CTTCCAGACACGGAATCAGAG-3'	Primer to verify deletion of the <i>malE</i> gene
Co-malE1	5'-GTGATCCTTCCGAGCGTTCC-3'	Primer to verify deletion of the <i>malE</i> gene
Co-malE2		

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

Analytcs. 1 ml of the culture was harvested by centrifugation (13,000 rpm, 10 min, room temperature [RT]) and the supernatant was used for determination of alcohols and glucose and/or organic acid concentrations in the culture fluid. Glucose, acetate, L-lactate, and succinate concentrations were determined by

enzymatic tests (Roche Diagnostics, Penzberg, Germany). The pyruvate concentrations were determined enzymatically according to Lamprecht and Heinz (30). Alcohols in the culture fluid were quantified with a gas chromatograph (GC; PerkinElmer Clarus 600) equipped with a flame ionization detector. Separation of the alcohol compounds was carried out by using a Chromosorb 101 glass column (2-m length, 80/100 mesh) at 130°C, with 10 mM acetone as the internal standard. N₂ was used as the carrier gas. The injector temperature was 200°C,

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

Construction of expression plasmids. For construction of plasmid pBB1adh2, the *adh2* gene from *S. cerevisiae* was amplified from plasmid pSA55 by PCR with primer pair adh4fow/adh2rev. The resulting fragment was digested with PstI/XhoI and ligated into PstI/XhoI-restricted plasmid pBB1. For construction of plasmids pBB1kivd and pBB1kivd-adh2, the *kivd* gene from *L. lactis* was amplified from plasmid pSA55 by PCR with primer pair kivdfow/kivd2rev. The resulting fragment was digested with PstI and ligated into PstI-restricted plasmids pBB1 and pBB1adh2, yielding plasmids pBB1kivd and pBB1kivd-adh2. The correct orientation of the *kivd* gene was verified via PCR with the primer pair Ptacheck/kivdcheckrev. For construction of plasmid pBB1kivd-adhA, the *adhA* gene from *C. glutamicum* was amplified from chromosomal DNA by PCR with the primer pair adhA-fowsall/adhA-revsall. The resulting fragment was digested with SalI and ligated into the SalI-restricted plasmid pBB1kivd. The correct orientation of the *adhA* gene was verified via PCR with the primer pair adhA-fowsall/pMM36rev. For construction of plasmid pBB1pntAB, a 2,985-bp fragment containing the *pntAB* genes from *E. coli* was amplified from plasmid pKEEx2-pntAB via PCR using the primer pair pntABfow/pntABrev. The resulting fragment was cut with PstI and cloned into the PstI-restricted plasmid pBB1. The correct orientation of the *pntAB* genes was verified via restriction with XhoI. For construction of plasmid pJC4ilvBNCD-pntAB, the *pntAB* genes (under the control of the P_{tac} promoter) were amplified from plasmid pBB1pntAB by PCR with the primer pair Transfow2/Transrev2. The resulting fragment was digested with Bst1107I and ligated into the Bst1107I-restricted plasmid pJC4ilvBNCD. All cloned fragments were checked by sequencing (MWG Biotech).

Construction of *C. glutamicum* deletion mutants. Chromosomal inactivation of the *ldhA* L-lactate dehydrogenase gene in *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE$ strain and of the *malE* malic enzyme gene in *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA$ and *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh$ were performed using crossover PCR and the suicide vector pK19mobsacB. DNA fragments were generated using the primer pairs ldhA1/ldhA2 and ldhA3/ldhA4 or primer pairs malE1/malE2 and malE3/malE4, respectively. The two fragments were purified, mixed in equal amounts, and subjected to crossover PCR using primer pairs ldhA1/ldhA4 and malE1/malE4, respectively. The resulting fusion products (containing the *ldhA* gene shortened by 917 bp and the *malE* gene shortened by 1,137 bp) were ligated into SmaI-restricted plasmid pK19mobsacB and transformed into *E. coli*. After isolation and sequencing (MWG Biotech), the recombinant plasmids were introduced by electroporation into the respective *C. glutamicum* strains. By application of the method described by Schäfer et al. (44), the intact chromosomal *ldhA* and *malE* genes were replaced by the truncated genes via homologous recombination (double crossover). The screening of the deletion mutants was performed with 2× TY agar plates containing 10% (wt/vol) sucrose and 0.5% (wt/vol) potassium acetate. The replacements at the chromosomal loci were verified by PCR using primers ldhfow/ldhrev and Co-malE1/Co-malE2, respectively.

Chromosomal inactivation of the *mdh* malate dehydrogenase gene in *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA$ was performed accordingly. DNA fragments were generated using the primer pairs mdh1/mdh2 and mdh3/mdh4, respectively. The two fragments were purified, mixed in equal amounts, and subjected to crossover PCR using primers mdh1 and mdh4. The resulting fusion product (containing the *mdh* gene shortened by 876 bp) was ligated into the BamHI/HindIII-restricted plasmid pK19mobsacB and transformed into *E. coli*. After isolation and sequencing, the recombinant plasmid was introduced by electroporation into *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA$. Double crossover and screening for the correct mutants were performed as described above. The replacement at the chromosomal locus was verified by PCR using the primer pair mdhcheckfow/mdhcheckrev.

Determination of enzyme activities. For determination of enzyme activities, the relevant strains were cultivated aerobically in shake flasks to an OD₆₀₀ of about 5 (for measurement of Mdh, PntAB, and Adh activities). Adh activities were also determined under oxygen deprivation conditions. For this purpose, the cells were cultivated for 6 h with an OD₆₀₀ of about 15 in Müller-Krempel bottles. For both conditions, 50 ml CGXII medium (pH 7.4) (16) with 2% (wt/vol) glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-leucine, and L-isoleucine (2 mM each) was used (see culture conditions). The cells were harvested by centrifugation for 10 min at 4,500 × g, washed once with 25 ml of 0.2 M Tris-HCl (pH 7.4), centrifuged again and resuspended in 1 ml of the same buffer. The cell suspension was transferred into 2-ml screw-cap vials together with 250 mg of glass beads (diameter, 0.1 mm; Roth) and subjected to mechanical disruption four times for 30 s each at speed 6.5 with a RiboLyser (Hybaid) at 4°C

with intermittent cooling on ice for 5 min. Intact cells and cell debris were removed by centrifugation for 15 min at 4,500 × g and 4°C.

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

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

Malate dehydrogenase (Mdh) activity in cell extracts was determined by measuring NAD⁺ reduction at 30°C at 365 nm in 1 ml of 100 mM phosphate buffer (pH 9.2), 4.5 mM MgCl₂, 3 mM NAD⁺, and 25 mM malate, according to Smith (48; modified). One unit of activity is defined as 1 μmol NADH formed per min.

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

RESULTS

Inactivation of LdhA is essential for isobutanol production with *C. glutamicum*. Previously, we demonstrated the ability of *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE$ (pJC4ilvBNCD) (referred to here as *C. glutamicum* Iso1) to produce KIV under aerobic conditions from glucose (28). As KIV is a precursor for isobutanol production, this strain seemed to be ideally suited for production of this alcohol with *C. glutamicum*. Since the formation of 1 mol of isobutanol from 1 mol of glucose requires 1 mol of NADH+H⁺ and 1 mol of NADPH+H⁺ (Fig. 1), we performed the isobutanol fermentations under oxygen deprivation conditions with the aim of increasing NADH+H⁺ availability. We inoculated *C. glutamicum* Iso1 to an OD₆₀₀ of about 15, which remained almost constant in the course of the fermentations. After 48 h, the glucose was completely consumed, and under these conditions *C. glutamicum* Iso1 produced no isobutanol but did produce significant amounts of L-lactate (122 ± 23 mM) and succinate (29 ± 3 mM) as major fermentation products (Fig. 2A). These results show that *C. glutamicum* is naturally not able to produce isobutanol and underline the necessity of implementing a synthetic pathway. Therefore, we cloned the *kivd* gene from *L. lactis* and the *adh2* gene from *S. cerevisiae* on plasmid pBB1, constructed *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE$ (pJC4ilvBNCD)(pBB1kivd-adh2), *C. glutamicum* Iso2, and performed isobutanol fermentations under oxygen deprivation conditions. Within 48 h, *C. glutamicum* Iso2 consumed the glucose completely, but again produced no isobutanol and formed significant amounts of L-lactate and succinate (data not shown). To avoid L-lactate formation and to increase pyruvate and NADH+H⁺ availability, we additionally eliminated LdhA activity by deletion of the corresponding gene in *C. glutamicum* Iso2. The resulting strain, *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA$ (pJC4ilvBNCD)(pBB1kivd-adh2), or *C. glutamicum* Iso3, metabolized the glucose within 48 h completely and produced no L-lactate anymore, but formed 69 ± 8 mM succinate, which is about two times more than that formed by *C. glutamicum* Iso1 and Iso2. Furthermore, *C. glutamicum* Iso3 formed 26 ± 4 mM isobutanol with a substrate-specific yield ($Y_{P/S}$) of 0.22 ± 0.05 mol per mol of glucose (Fig. 2B). These results

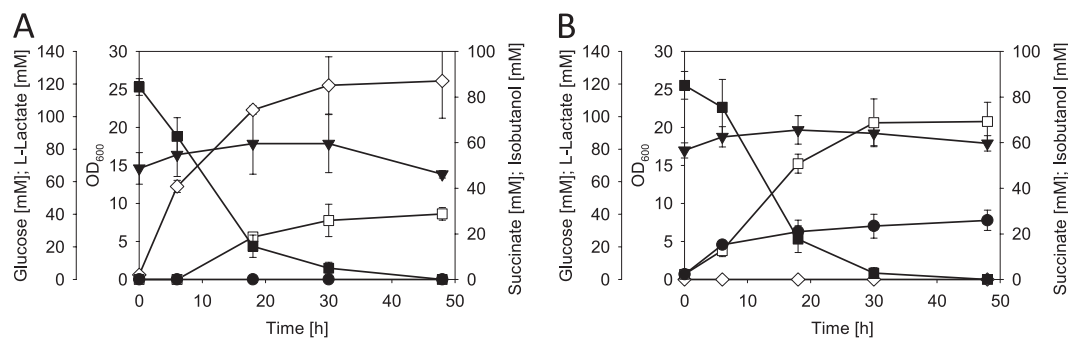


FIG. 2. OD, glucose consumption, and L-lactate, succinate, and isobutanol formation of (A) *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE$ (pJC4ilvBNCD) (*C. glutamicum* Iso1) and (B) *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA$ (pJC4ilvBNCD)(pBB1kivd-adh2) (*C. glutamicum* Iso3) cultivated in Müller-Krempel bottles filled with CGXII medium containing about 100 mM glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-isoleucine, and L-leucine (2 mM each). ▼, OD₆₀₀; ■, glucose; □, succinate; ◇, L-lactate; ●, isobutanol. Three independent fermentations were performed. Error bars show standard deviations.

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

Deletion of the *mdh* Mdh gene in combination with the expression of the *pntAB* transhydrogenase genes further improves isobutanol production. To eliminate succinate as a by-product and to further increase the availability of pyruvate and NADH+H⁺, we eliminated Mdh activity by deletion of the *mdh* gene in *C. glutamicum* Iso3. The resulting strain, *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh$ (pJC4ilvBNCD) (pBB1kivd-adh2), or *C. glutamicum* Iso4, showed no detectable specific Mdh activity, whereas the parental strain *C. glutamicum* Iso3 exhibited 0.46 ± 0.03 U per mg protein. However, in Müller-Krempel bottles, *C. glutamicum* Iso4 consumed only small amounts of glucose (26 mM in 48 h) and produced neither L-lactate nor succinate or isobutanol in significant amounts (Fig. 3A). We speculated that the low glucose consumption is due to a redox imbalance under oxygen deprivation conditions and therefore ligated the *pntAB* operon, encoding the membrane-bound transhydrogenase from *E. coli*, into plasmid pJC4ilvBNCD, and constructed *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh$ (pJC4ilvBNCD-*pntAB*)(pBB1kivd-adh2), or *C. glutamicum* Iso5. To verify the successful expression of *pntAB*, we determined the specific transhydrogenase activities in the membrane fraction of *C.*

glutamicum Iso5. Whereas *C. glutamicum* Iso4 showed no detectable transhydrogenase activity, *C. glutamicum* Iso5 possessed 0.20 ± 0.03 U per mg protein. Isobutanol fermentations of *C. glutamicum* Iso5 under oxygen deprivation conditions revealed that this strain regained the ability to metabolize glucose efficiently and that the cells produced 2 ± 0.1 mM pyruvate (not shown) and 10 ± 1 mM succinate, which is 86% less than that for *C. glutamicum* Iso3. Furthermore, within 48 h, *C. glutamicum* Iso5 produced 42 ± 1 mM isobutanol with a Y_{P/S} of 0.60 ± 0.02 mol per mol of glucose (Fig. 3B), which is about 3-fold higher than that for *C. glutamicum* Iso3. These results demonstrate that, on the one hand, inactivation of Mdh reduces succinate formation and therefore obviously increases pyruvate and/or NADH+H⁺ availability. On the other hand, expression of the *pntAB* transhydrogenase genes probably results in a more balanced redox state, with a regaining of efficient glucose utilization of *C. glutamicum* Iso4, and improves isobutanol production with *C. glutamicum* under oxygen deprivation conditions.

AdhA of *C. glutamicum* is a bottleneck for isobutanol production. To investigate whether *C. glutamicum* possesses isobutyraldehyde-dependent Adh activity, we constructed *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA$ (pJC4ilvBNCD)(pBB1kivd), or *C. glutamicum* Iso6 (without

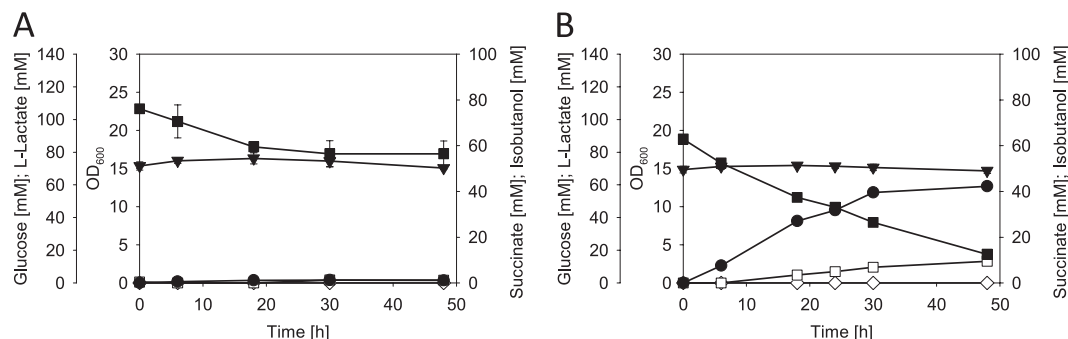


FIG. 3. OD, glucose consumption, and L-lactate, succinate, and isobutanol formation of (A) *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh$ (pJC4ilvBNCD)(pBB1kivd-adh2) (*C. glutamicum* Iso4) and (B) *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh$ (pJC4ilvBNCD-*pntAB*)(pBB1kivd-adh2) (*C. glutamicum* Iso5) cultivated in Müller-Krempel bottles filled with CGXII medium containing about 100 mM glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-isoleucine, and L-leucine (2 mM each). ▼, OD₆₀₀; ■, glucose; □, succinate; ◇, L-lactate; ●, isobutanol. Three independent fermentations were performed. Error bars show standard deviations.

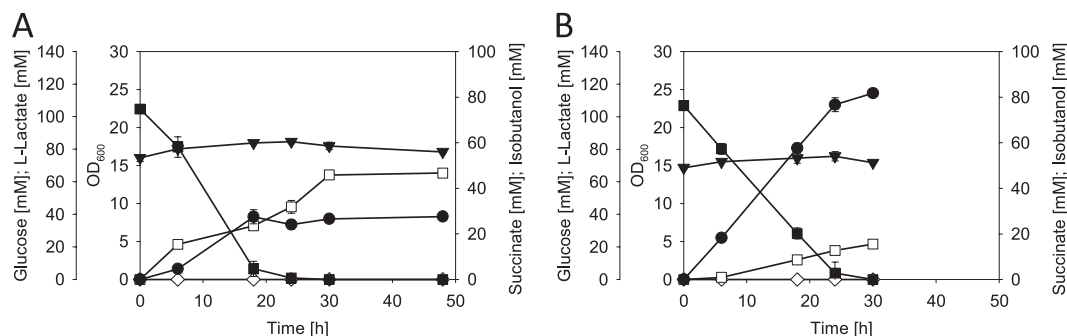


FIG. 4. OD, glucose consumption, and L-lactate, succinate, and isobutanol formation of (A) *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA$ (pJC4ilvBNCD)(pBB1kivd) (*C. glutamicum* Iso6) and (B) *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh$ (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA) (*C. glutamicum* Iso7) cultivated in Müller-Krempel bottles filled with CGXII medium containing about 100 mM glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-isoleucine, and L-leucine (2 mM each). ▼, OD₆₀₀; ■, glucose; □, succinate; ◇, L-lactate; ●, isobutanol. Three independent fermentations were performed. Error bars show standard deviations.

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

Smith et al. (49) already observed that overexpression of the *adhA* gene is favorable for isobutanol production with *C. glutamicum*. Therefore, we cloned the *adhA* gene of *C. glutamicum* on plasmid pBB1kivd and constructed *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh$ (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA), or *C. glutamicum* Iso7. To verify successful expression of the *C. glutamicum* *adhA* gene, we determined the specific isobutyraldehyde-dependent Adh activity of *C. glutamicum* Iso7. *C. glutamicum* Iso7 showed 0.94 ± 0.11 U per mg protein, which is about 3-fold higher than that for *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh$ (pJC4ilvBNCD-pntAB)(pBB1kivd-adh2) (0.33 ± 0.04 U per mg protein). To test for the effect of *adhA* gene overex-

pression on isobutanol formation, we cultivated *C. glutamicum* Iso7 in Müller-Krempel bottles. As shown in Fig. 4B, *C. glutamicum* Iso7 consumed the glucose rapidly within 30 h and produced 16 ± 1 mM succinate and 82 ± 1 mM isobutanol, with a $Y_{P/S}$ of 0.77 ± 0.01 mol per mol of glucose. Taken together, these results show that plasmid-encoded ADH2 from *S. cerevisiae* does not contribute to isobutanol production with *C. glutamicum*. In accordance with the results by Smith et al. (49), we show that AdhA of *C. glutamicum* is a bottleneck and that plasmid-bound overexpression of the *adhA* gene significantly improves isobutanol production with *C. glutamicum*.

The role of malic enzyme (MalE) for isobutanol production with *C. glutamicum*. The improvement of isobutanol production by expression of the *pntAB* transhydrogenase genes (*C. glutamicum* Iso5 and *C. glutamicum* Iso7) (Fig. 3B and 4B) indicated that NADPH+H⁺ supply might be a critical factor for isobutanol production with *C. glutamicum*. However, *C. glutamicum* Iso3 produced isobutanol without expression of transhydrogenase genes; thus, this strain should have the ability to convert NADH+H⁺ to NADPH+H⁺. As outlined in a review by Sauer and Eikmanns (43), one proposed transhydrogenase-like route consists of the combined reactions of PCx and/or PEPCx, Mdh, and MalE (Fig. 1). To test this hypothesis, we inactivated MalE by deletion of the corresponding gene in *C. glutamicum* Iso3, yielding *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta malE$ (pJC4ilvBNCD)(pBB1kivd-adh2), here called *C. glutamicum* Iso8. Under oxygen deprivation conditions, *C. glutamicum* Iso8 consumed only about half of the glucose within 48 h and produced 59 ± 3 mM succinate; however, no isobutanol was observed (Fig. 5A). This result suggests that inactivation of MalE interrupts the transhydrogenase-like cycle consisting of PCx/PEPCx, Mdh, and MalE, and therefore is essential for providing NADPH+H⁺ for isobutanol production. We also investigated the role of MalE in a *pntAB* gene-expressing strain and deleted the *malE* gene in *C. glutamicum* Iso7 to obtain *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh \Delta malE$ (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA), *C. glutamicum* Iso9. Within 48 h, *C. glutamicum* Iso9 consumed the glucose almost completely and produced 15 ± 2 mM succinate and 24 ± 4 mM isobutanol (Fig. 5B), which is about half of the concentration of isobutanol observed with *C. glutamicum* Iso7. These results again underline the importance of the transhy-

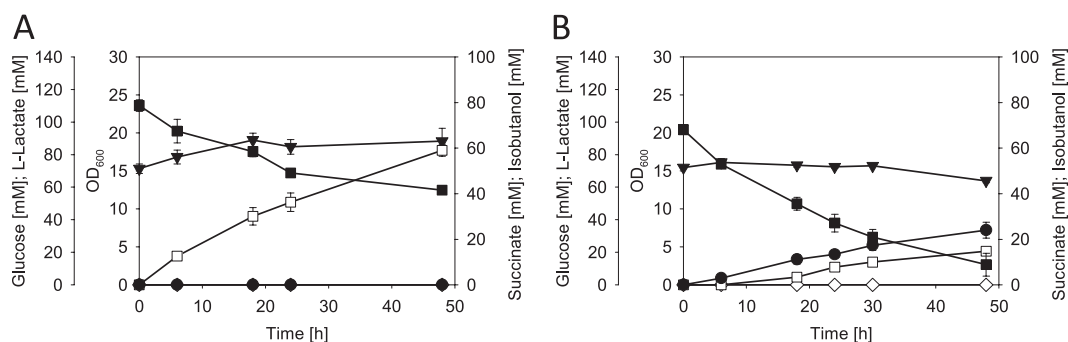


FIG. 5. OD, glucose consumption, and L-lactate, succinate, and isobutanol formation of (A) *C. glutamicum* $\Delta aceE$ Δpqq $\Delta ilvE$ $\Delta ldhA$ $\Delta malE$ (pJC4ilvBNCD)(pBB1kivd-adh2) (*C. glutamicum* Iso8) and (B) *C. glutamicum* $\Delta aceE$ Δpqq $\Delta ilvE$ $\Delta ldhA$ Δmdh $\Delta malE$ (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA) (*C. glutamicum* Iso9) cultivated in Müller-Krempel bottles filled with CGXII medium containing about 100 mM glucose, 0.5% (wt/vol) yeast extract, and L-valine, L-isoleucine, and L-leucine (2 mM each). ▼, OD₆₀₀; ■, glucose; □, succinate; ◇, L-lactate; ●, isobutanol. Three independent fermentations were performed. Error bars show standard deviations.

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

Fed-batch fermentations with *C. glutamicum* $\Delta aceE$ Δpqq $\Delta ilvE$ $\Delta ldhA$ Δmdh (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA). To test the suitability of *C. glutamicum* $\Delta aceE$ Δpqq $\Delta ilvE$ $\Delta ldhA$ Δmdh (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA), or *C. glutamicum* Iso7, for an improved isobutanol production process, we established a fed-batch fermentation based on mixed substrate divided in an aerobic growth phase and a production phase under oxygen deprivation conditions (Fig. 6). These fermentations were carried out using CGXII medium initially containing 4% (wt/vol) glucose, 1% (wt/vol) acetate, 0.5% (wt/vol)

yeast extract, and L-valine, L-leucine, and L-isoleucine (2 mM each). To allow growth to a high cell density, after 6.5 h, an adequate amount of a 50% (wt/vol) acetate stock solution was added to the growing cells, resulting in an OD₆₀₀ of about 45 after 9.5 h (Fig. 6). During the growth period, about 60 mM glucose were consumed in addition to acetate; however, no isobutanol, pyruvate, L-lactate, or succinate was excreted into the medium. After complete consumption of acetate (at 9.5 h), we added about 330 mM glucose (applied as 50% [wt/vol] stock solution) into the medium, switched off aeration, and reduced the stirring speed to 300 rpm. The pO₂ dropped to 0% within less than 1 min (and remained at 0% during the rest of the experiment), and the cells started to excrete isobutanol into the medium. As shown in Fig. 6, the cells accumulated about 175 mM isobutanol within 39.5 h with a volumetric productivity of 4.4 mM h⁻¹ and an overall yield in the production phase

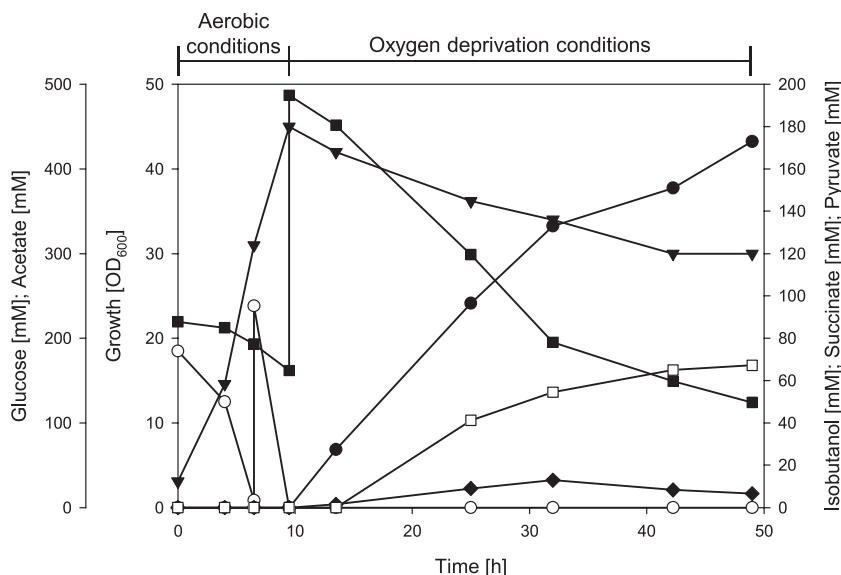


FIG. 6. Isobutanol accumulation during a representative fed-batch fermentation of *C. glutamicum* $\Delta aceE$ Δpqq $\Delta ilvE$ $\Delta ldhA$ Δmdh (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA) (*C. glutamicum* Iso7) on CGXII medium initially containing 4% (wt/vol) glucose, 1% (wt/vol) acetate, 0.5% (wt/vol) yeast extract, and 2 mM L-valine, L-isoleucine, and L-leucine, respectively. After 9.5 h, the aeration was switched off and the stirring speed was reduced to 300 rpm. ▼, OD₆₀₀; ■, glucose; ○, acetate; □, succinate; ◇, pyruvate; ●, isobutanol. Three independent fed-batch fermentations were performed, all three showing comparable results.

(between 9.5 h and 49 h) of about 0.48 mol of isobutanol per mol of glucose. In addition to isobutanol, the cells excreted about 7 mM pyruvate and 67 mM succinate into the medium, indicating that isobutanol production by *C. glutamicum* Iso7 can be further increased. Taken together, these results demonstrate that *C. glutamicum* $\Delta aceE \Delta p q o \Delta i l v E \Delta l d h A \Delta m d h$ (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA) is a very useful platform for optimizing isobutanol production with *C. glutamicum* under oxygen deprivation conditions.

DISCUSSION

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

C. glutamicum $\Delta aceE \Delta p q o \Delta i l v E$ (pJC4ilvBNCD)(pBB1kivd-adh2) excreted significant amounts of L-lactate and succinate, but no isobutanol, indicating that pyruvate was not effectively directed toward KIV and isobutanol. Consequently, we inactivated the *LdhA* gene in this strain, resulting in isobutanol production with a $Y_{P/S}$ of about 0.22 ± 0.05 mol per mol glucose. A beneficial effect on isobutanol formation by inactivation of *LdhA* was observed before by Smith et al. (49). To further increase pyruvate and/or $\text{NADH} + \text{H}^+$ availability and to avoid succinate formation, we additionally inactivated *Mdh*. Interestingly, the resulting strain showed a severe reduction of glucose consumption, possibly due to an unbalanced redox state of the cell under the oxygen deprivation conditions applied. One possibility to regenerate NAD^+ and simultaneously increase $\text{NADPH} + \text{H}^+$ availability in *C. glutamicum* would be the expression of the *pntAB* genes encoding the membrane-bound transhydrogenase from *E. coli*. This enzyme uses the proton gradient across the cytoplasmic membrane to drive the reduction of NADP^+ by oxidizing $\text{NADH} + \text{H}^+$ (Fig. 1) and previously was shown to improve L-lysine production with *C.*

glutamicum under aerobic conditions (25). Expression of the *pntAB* genes in *C. glutamicum* $\Delta aceE \Delta p q o \Delta i l v E \Delta l d h A \Delta m d h$ (pJC4ilvBNCD)(pBB1kivd-adh2) in fact recovered efficient glucose utilization, led in combination with the inactivation of *Mdh* to efficient reduction of succinate formation, and strongly improved isobutanol production ($Y_{P/S}$ of 0.60 ± 0.02 mol per mol of glucose). The results indicate that under oxygen deprivation conditions, the expression of the *pntAB* genes results in the conversion of $\text{NADH} + \text{H}^+$ to $\text{NADPH} + \text{H}^+$ and therefore contributes to maintaining a balanced redox state for isobutanol production. Also, Smith et al. (49) tried to increase $\text{NADPH} + \text{H}^+$ -availability for isobutanol production, by redirecting the carbon flux in the *C. glutamicum* $\Delta aceE \Delta l d h A$ strain (pKS167) through the pentose phosphate pathway by inactivation of *PGI*. Unfortunately, this attempt to increase $\text{NADPH} + \text{H}^+$ availability did not improve isobutanol production, probably generating an imbalance in the redox state of the cell (49).

Since *C. glutamicum* possesses no chromosomally encoded transhydrogenases (25), we speculated that in *C. glutamicum* $\Delta aceE \Delta p q o \Delta i l v E \Delta l d h A$ (pJC4ilvBNCD)(pBB1kivd-adh2), a transhydrogenase-like route consisting of the enzymes PCx/PEPCx, $\text{NADH} + \text{H}^+$ -dependent *Mdh*, and NADP^+ -dependent *MalE* (Fig. 1) is responsible for $\text{NADPH} + \text{H}^+$ supply. Such a cycle was previously assumed to play a role in $\text{NADPH} + \text{H}^+$ supply for aerobic L-lysine production (13). In fact, inactivation of *MalE* in our strain led to a complete inability to form isobutanol and, thus, gives further indication of the functionality of a transhydrogenase-like cycle in *C. glutamicum*. Even in the *pntAB* gene-expressing strain *C. glutamicum* $\Delta aceE \Delta p q o \Delta i l v E \Delta l d h A \Delta m d h$ (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA), inactivation of *MalE* reduced the $Y_{P/S}$ for isobutanol about 2-fold. This finding is surprising, since inactivation of the *Mdh* should interrupt the proposed transhydrogenase-like route. However, since *C. glutamicum* $\Delta aceE \Delta p q o \Delta i l v E \Delta l d h A \Delta m d h$ (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA) still produced succinate, the presence of malate as substrate for *MalE* is likely. Therefore, these results indicate that *MalE* is an important enzyme for $\text{NADPH} + \text{H}^+$ generation. This, in consequence, means that *MalE* does not work in the reverse (malate-forming) direction under these conditions, as was previously proposed (5, 21), and indicates the existence of an alternative route for the formation of succinate and/or malate, as proposed by Inui et al. (24). However, due to the finding that *MalE* plays a crucial role for the generation of $\text{NADPH} + \text{H}^+$, it is obvious that overexpression of the *malE* gene might be an opportunity to replace expression of the *pntAB* genes, thereby reducing the amount of the undesired by-product succinate and improving isobutanol production with *C. glutamicum*.

Atsumi et al. (4) investigated the role of different Adhs on isobutanol production with *E. coli* and showed that the chromosomally encoded *YqhD* is the major isobutyraldehyde-converting enzyme, and *ADH2* from *S. cerevisiae* contributes only to a minor extent to isobutanol production with *E. coli*. We found that under aerobic and also under oxygen deprivation conditions, *ADH2* does not contribute at all to the isobutyraldehyde-dependent *Adh* activity in *C. glutamicum*. *C. glutamicum* $\Delta aceE \Delta p q o \Delta i l v E$ (pJC4ilvBNCD)(pBB1kivd) produced as much isobutanol as the same strain additionally expressing

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

A suitable production process on the industrial scale might be the combination of biomass formation and isobutanol production in a single reactor. Therefore, we established a fed-batch fermentation with *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh$ (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA). During the aerobic growth phase, neither isobutanol nor pyruvate or succinate were formed. This is in accordance with previous results obtained with PDHC-deficient *C. glutamicum* L-valine producer strains, which also did not secrete L-valine during growth (6, 8). The nonproduction phenotype is due to reduced glucose uptake in the presence of acetate, mediated by the global regulator SugR (9, 18). Inactivation of SugR or replacement of acetate by ethanol resulted in L-valine production during growth (9) and might be also useful to improve isobutanol production with PDHC-deficient *C. glutamicum* strains. However, production during the aerobic growth phase probably is not very useful, since aeration would result in a loss of isobutanol by gas stripping. Furthermore, we applied oxygen deprivation conditions to improve NADH+H⁺ availability. The presence of succinate as a (major) by-product gives evidence for a surplus of pyruvate and of NADH+H⁺ and indicates that isobutanol production with *C. glutamicum* can be further improved by, e.g., overexpression of the *malE* gene (see above). Between 9.5 h and 32 h, the *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh$ strain (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA) showed a volumetric productivity of about 5.9 mM h⁻¹, which is similar to that of 1-butanol production with different *Clostridium* strains (31). After 32 h of fermentation, the glucose consumption rate dropped from 1.1 to 0.4 mmol of glucose h⁻¹ (g of cells [dry weight])⁻¹, and the volumetric productivity decreased to about 4.4 mM h⁻¹ (between 9.5 and 49 h) (Fig. 6). However, the $Y_{P/S}$ remained constant in the course of the whole fermentation. The reason for this behavior remains unclear but might be attributed to isobutanol toxicity for the cells. Cell toxicity might be avoided by integrated product removal by gas stripping with N₂ and product recovery by continuous condensation, which was successfully applied for 1-butanol production with *Clostridium beijerinckii* (19). Such a process will probably allow the system to maintain its high productivity.

The fact that the fed-batch fermentations of *C. glutamicum* $\Delta aceE \Delta pqo \Delta ilvE \Delta ldhA \Delta mdh$ (pJC4ilvBNCD-pntAB)(pBB1kivd-adhA) showed a significantly reduced $Y_{P/S}$ compared to that from the Müller-Krempel bottles indicates that the physiological state of the cells during the transition from aerobic to oxygen-deprived conditions may have an impact on the overall production behavior. In favor of this hypothesis, Vemuri et al. (52) found in a combined (consecutive) aerobic/anaerobic succinate production process with *E. coli* that $Y_{P/S}$ for succinate changed in response to altered culture conditions in the growth phase. The authors attributed this

observation to the physiological state of the cells entering the transition from aerobic to anaerobic conditions. Recently, Martínez et al. (35) investigated more precisely the role of the physiological state of the cell in a similar approach for succinate production with *E. coli*. These authors found that the introduction of a microaerobic phase at the end of the aerobic growth phase led to an adjusted enzymatic machinery for the anaerobic production phase, which resulted in increased succinate yields, and they concluded that besides the genetic modification of a strain, process optimization is crucial for reaching high yields in such a system. This, in consequence, opens the possibility of improving our *C. glutamicum* production process, e.g., by the introduction of oxygen-limited conditions at the end of the growth phase.

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