Appl Microbiol Biotechnol (2014) 98:297–311
DOI 10.1007/s00253-013-5310-2

Metabolic engineering of *Corynebacterium glutamicum* for 2-ketoisocaproate production

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Received: 15 August 2013 / Revised: 30 September 2013 / Accepted: 1 October 2013 / Published online: 30 October 2013

Abstract 2-Ketoisocaproate (KIC) is used as a therapeutic agent, and a KIC-producing organism may serve as a platform for products deriving from this 2-keto acid. We engineered *Corynebacterium glutamicum* for the production of KIC from glucose by deletion of *ltbR and ilvE*, encoding the transcriptional repressor LtbR and transaminase B, respectively, and additional overexpression of *ilvBNCD*, encoding acetohydroxyacid synthase, acetohydroxyacid isomeroreductase, and dihydroxyacid dehydratase. The KIC-producing strain was improved by deletion of the methylcitrate synthase genes and by decreasing citrate synthase activity by exchange of the native promoter of the citrate synthase gene. In shake-flask fermentations under L-leucine limitation, the newly constructed strain *C. glutamicum* VB (pJC4*ilvBNCD*) produced 31±2 mM (4.0±0.3 g l^{-1}) KIC and showed a product yield of about 0.26±0.02 mol per mole (0.19±0.01 g per gram) of glucose. As by-product, the strain formed about 33 mM 2-ketoisovalerate, which is a precursor of KIC. KIC production was further improved by additional expression of an isopropylmalate synthase allele (*leuA*$^{EC\text{-}G462D}$), encoding an enzyme resistant towards L-leucine inhibition, and by addition of acetate as additional substrate. With glucose and acetate, the newly constructed strain produced 71±3.2 mM (9.2±0.4 g l^{-1}) KIC with a yield of 0.24±0.01 mol C (KIC) per mole C (in both substrates) and with nearly no 2-ketoisovalerate by-product formation (<2 mM). Investigating the activities and regulation of the native isopropylmalate synthase and dehydratase of *C. glutamicum*, we observed competitive and noncompetitive inhibition, respectively, by KIC.

Keywords *Corynebacterium glutamicum* · 2-Ketoisocaproate production · Branched chain keto acids · Acetohydroxyacid synthase · Isopropylmalate synthase · Competitive inhibition

Introduction

Corynebacterium glutamicum is a Gram-positive, facultative anaerobic organism able to use a variety of sugars, alcohols, and organic acids as carbon and energy source (Abe et al. 1967; Kinoshita et al. 1957; Gerstmeir et al. 2003; Eggeling and Bott 2005; Liebl 2006; Nishimura et al. 2007; Takeno et al. 2007; Arndt et al. 2008). So far, the organism was mainly used for the industrial production of amino acids, such as L-glutamate or L-lysine (Eggeling and Bott 2005; Takors et al. 2007); however, recent studies furthermore describe the successful use of *C. glutamicum* for production of further amino acids (e.g., L-valine, L-arginine, or L-proline; Blombach et al. 2008; Schneider and Wendisch 2011; Ikeda et al. 2009; Hasegawa et al. 2013; Jensen and Wendisch 2013) and other commodity chemicals, such as the organic acids pyruvate (Wieschalka et al. 2012), L- or D-lactate (Inui et al. 2004a; Okino et al. 2008) and succinate (Litsanov et al. 2012a, b; reviewed in Wieschalka et al. 2013), the biofuels ethanol (Inui et al. 2004b; Sakai et al. 2007) and isobutanol (Blombach et al. 2011; Yamamoto et al. 2013), xylitol (Sasaki et al. 2010), pantothenate (Hüser et al. 2005), the diamines cadaverin and putrescine (Mimitsuka et al. 2007; Takeno et al. 2007; Schneider and Wendisch 2010, 2011; Kind and Wittmann 2011), polyhydroxybutyrate (Jo et al. 2006), 2-ketoglutarate (Jo et al. 2012) and 2-ketoisovalerate (KIV; Krause et al. 2010a), and lycopene (Heider et al. 2012).

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-5310-2) contains supplementary material, which is available to authorized users.

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Aside from KIV, which is the direct precursor of L-valine, 2-ketoisocaproate (KIC; 2-keto-4-metyl-pentanoate), the direct precursor of L-leucine, might be an interesting target for the biotechnological production with *C. glutamicum*. Both KIV and KIC are used as integral parts of infusion solutions for chronic kidney disease patients, and they serve as nitrogen-free substitutes for their corresponding branched-chain (BC) amino acids (Aparicio et al. 2009; Chang et al. 2009; Feiten et al. 2005; Teschan et al. 1998). Thus, despite reduction of the nitrogen intake and improvement of nitrogen balance, sufficient supply of essential amino acids and protein synthesis can be achieved by transamination of the respective 2-keto acids. This is of special interest since various hepatic disorders are linked to hyperammonemia and an intolerance of dietary protein (Teschner and Heidland 1991). Additionally, KIC is commonly used as supplement in "functional food," since it possesses anticatabolic properties (Zanchi et al. 2011) and may provide benefit in the recovery of muscle function after exercise-induced muscle damage (van Someren et al. 2005; Nunan et al. 2010). A KIC-producing organism may furthermore serve as a platform organism for products deriving from this 2-keto acid, e.g., 3-methylbutanal (isovaleraldehyde, a precursor for vitamin synthesis; Krill 2003) or 3-methyl-1-butanol, a potential future biofuel (Connor and Liao 2008; Connor et al. 2010). To our knowledge, KIC and other 2-keto acids (e.g., 2-ketomethylvalerate) are mainly produced by chemical synthesis, which can be done by several methods (Cooper et al. 1983).

In microorganisms and plants, L-leucine and its precursor KIC are synthesized from KIV (the direct precursor of L-valine) and acetyl-CoA, KIV being derived from two molecules of pyruvate in a pathway comprising three reactions (see Fig. 1). In *C. glutamicum*, these are catalyzed by acetohydroxyacid synthase (AHAS; *ilvBN* gene product), acetohydroxyacid isomeroreductase (AHAIR; *ilvC* gene product), and dihydroxyacid dehydratase (DHAD; *ilvD* gene product) (Pátek 2007). KIV is further converted to either L-valine by transaminase B (TAB; *ilvE* gene product) or, together with one molecule of acetyl-CoA, to L-leucine via the so-called

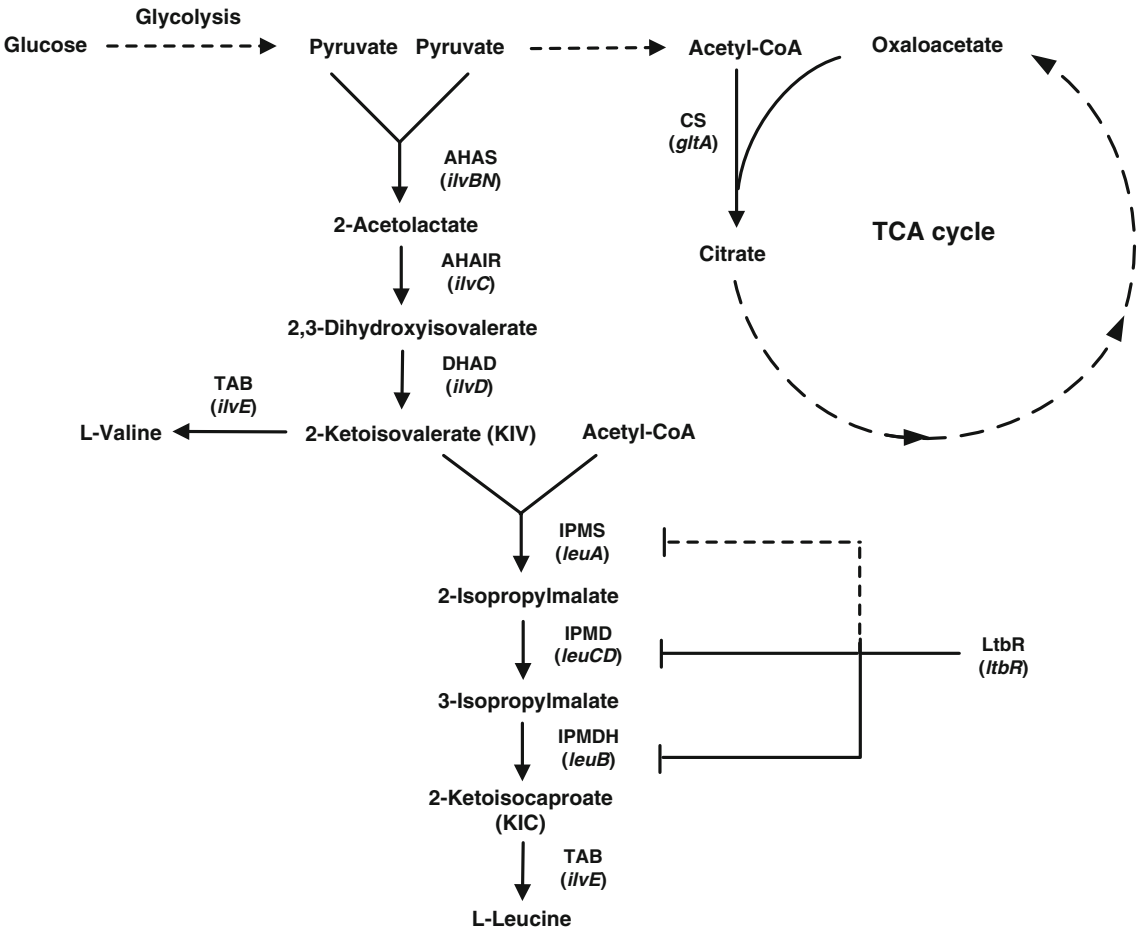

Fig. 1 Pathways from pyruvate towards 2-ketoisovalerate (KIV) and L-valine and towards 2-ketoisocaproate and L-leucine in *C. glutamicum*. Abbreviations: *AHAS*, acetohydroxyacid synthase; *AHAIR*, acetohydroxyacid isomeroreductase; *DHAD*, dihydroxyacid dehydratase; *IPMS*, isopropylmalate synthase; *IMPD*, isopropylmalate dehydratase; *IPMDH*, isopropylmalate dehydrogenase; *TAB*, transaminase B; *LtbR*, leucine and tryptophan biosynthesis regulator. The respective gene names are given in *brackets*. The pathway from KIV and acetyl-CoA to KIC is designated isopropylmalate pathway (IPM). The genes encoding the enzymes of the IMP pathway are directly (*solid line*) or indirectly (*dashed line*) repressed by LtbR

isopropylmalate (IPM) pathway. The three subsequent reactions of the IPM pathway leading to KIC are catalyzed by isopropylmalate synthase (IPMS; *leuA* gene product), isopropylmalate dehydratase (IPMD; *leuCD* gene product), and isopropylmalate dehydrogenase (IPMDH; *leuB* gene product) (Fig. 1). Under physiological conditions, KIC then is further metabolized to L-leucine by TAB (Marienhagen et al. 2005).

AHAS is a key enzyme for the synthesis of BC amino acids L-valine, L-leucine, and L-isoleucine and thus also is a key enzyme for the synthesis of the respective BC 2-keto acids. In *C. glutamicum*, it is subject to an about 50 to 60 % feedback inhibition by the three BC amino acids (Eggeling et al. 1987), and it is competitively inhibited by KIV, leading to an increase of the K_m for pyruvate from about 7.8 mM to about 24.7 mM in the presence of 50 mM KIV (Krause et al. 2010a). The expression of the AHAS (*ilvBN)* genes together with *ilvC* is controlled by an attenuation mechanism, leading to about twofold higher expression in response to a shortage of BC amino acids (Morbach et al. 2000). Furthermore, 2-ketobutyrate, one of the precursors of L-isoleucine, has been shown to increase the expression of *ilvBNC* about tenfold by a so far unknown mechanism (Eggeling et al. 1987; Morbach et al. 2000).

IPMS, the first enzyme of the IPM pathway (see Fig. 1), represents an important key enzyme in L-leucine biosynthesis. It catalyzes the conversion of KIV and acetyl-CoA to 2-isopropylmalate (2-IPM) and free coenzyme A (CoA-SH), and in *C. glutamicum,* it is subject to strong feedback inhibition by L-leucine (K_i=0.4 mM; Pátek et al. 1994). Furthermore, it was observed that the addition of L-leucine to the growth medium leads to a decrease of the specific activity of all three enzymes of the IPM pathway, indicating a negative control of the respective genes at the transcriptional level (Pátek et al. 1994, 1998). Within the *leuA* promoter sequence, there are structures that are indicative for transcription attenuation, i.e., a leader peptide with four consecutive L-leucine residues (Pátek et al. 1994), and additional structures, designated as "LEU element" suggested an alternative type of transcriptional attenuation mechanism (Seliverstov et al. 2005). Regarding expression of the IPMD and IPMDH genes (*leuCD* and *leuB*, respectively), the IclR-type transcriptional regulator LtbR (leucine and tryptophan biosynthesis regulator) has been shown to bind to the *leuCD* and *leuB* promoter regions and to repress expression of these genes more than tenfold (Brune et al. 2007). A moderate differential expression of *leuA* in an LtbR-negative mutant of *C. glutamicum* (about threefold lower expression than in the parental WT strain) was explained by a rather indirect effect of the mutation. On enzymatic level, the regulation of IMPD and IMPDH has not been investigated so far.

The previous successful construction of a KIV-producing strain of *C. glutamicum* (Krause et al. 2010a) prompted us to also approach the generation of a strain for the fermentative production of KIC. In the present study, we rationally engineered *C. glutamicum* wild-type (WT) for the production of this 2-keto acid, analyzed the activities and the regulation of the IPM pathway, and finally optimized KIC production by expression of a *leuA* allele encoding a feedback-resistant IPMS and by providing acetate to the fermentation medium.

Materials and methods

Bacterial strains, plasmids, oligonucleotides, and culture conditions

All bacterial strains and plasmids used in this study and their relevant characteristics and sources are given in Table 1; for oligonucleotides, their nucleotide sequence, and purpose, see Table S1 in the supplementary material.

Escherichia coli was grown aerobically on 2×TY complex medium (Sambrook and Russel 2001) at 37 °C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Precultures of *C. glutamicum* were grown in 2×TY medium at 30 °C. For 2-keto acid fermentations in shake flasks, the cells of an overnight preculture were washed with 0.9 % (*w*/*v*) NaCl and inoculated into CGXII minimal medium (Eikmanns et al. 1991), containing 20 g of ammonium sulfate per l instead of 5 g/l, 4 % (*w*/*v*) glucose, and L-valine, L-leucine, and/or L-isoleucine (2 mM each), to give an initial optical density at 600 nm (OD_{600}) of ~1, corresponding to a biomass concentration of 0.3 g (dry weight)/l (Blombach et al. 2009). The plasmid-carrying strains were cultivated 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.

DNA preparation and transformation

Restriction enzymes, T4 DNA ligase, calf intestinal phosphatase, RNase A, and proteinase K were obtained from MBI-Fermentas and used as instructed by the manufacturer. The isolation of plasmids from *E. coli* was performed as described (Birnboim 1983). Plasmid transfer into *C. glutamicum* was carried out by electroporation, and the recombinant strains were selected on brain heart infusion sorbitol (BHIS) agar plates containing kanamycin (50 or 25 μg/ml) (van der Rest et al. 1999). The isolation of chromosomal DNA from *C. glutamicum* was performed as described by Eikmanns et al. (1994). Electroporation of *E. coli* was carried out with competent cells according to the method of Dower et al. (1988).

Chromosomal deletions and promoter replacement

All deletions and replacements were performed using allelic exchange by homologous recombination for markerless in-frame deletions (Schäfer et al. 1994) with the suicide vector

Table 1 Strains and plasmids used in the present work

Strains and plasmids	Relevant characteristics	Source, reference
Strains		
E. coli DH5α	F⁻ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (rk⁻, mk⁺) *supE44 thi-1 gyrA96 relA1 phoA*	Hanahan (1983)
C. glutamicum WT	Wild-type strain ATCC 13032, biotin auxotrophic	Abe et al. (1967)
C. glutamicum Δ*ltbR*	*C. glutamicum* WT with deletion of *ltbR*, encoding the leucine and tryptophan biosynthesis regulator LtbR	This work
C. glutamicum Δ*ltbR* Δ*ilvE*	*C. glutamicum* Δ*ltbR* with deletion of *ilvE,* leucine auxotrophic	This work
C. glutamicum Δ*ltbR* Δ*ilvE* P*gltA*$^{mut\_L1}$	*C. glutamicum* Δ*ltbR* Δ*ilvE* with *gltA* promoter region replaced by an artificial *dapA* promoter (L1 sequence), leucine auxotrophic	This work
C. glutamicum Δ*ltbR* Δ*ilvE* Δ*prpC1* Δ*prpC2* P*gltA*$^{mut\_L1}$=*C. glutamicum* VB	*C. glutamicum* Δ*ltbR* Δ*ilvE* P*gltA*$^{mut\_L1}$ with deletion of *prpC1* and *prpC2*, encoding methylcitrate synthases 1 and 2, leucine auxotrophic	This work
Plasmids		
pK19Δcg1486	pK19*mobsacB* carrying a modified cg1486 (*ltbR*) gene region	Brune et al. (2007)
pK19*mobsacB*Δ	pK19*mobsacB* carrying a truncated *ilvE* gene	Marienhagen et al. (2005)
pK19Δ*PgltA_B-L1*	pK19*mobsacB* carrying a mutated *dapA*-promoter region (L1 sequence)	van Ooyen et al. (2012)
pK19*mobsacB*Δ*prpC1*	pK19*mobsacB* carrying a truncated *prpC1* gene	Radmacher and Eggeling (2007)
pK19*mobsacB*Δ*prpC2*	pK19*mobsacB* carrying a truncated *prpC2* gene	Radmacher and Eggeling (2007)
pJC4*ilvBNCD*	Kan^r; plasmid carrying the *ilvBNCD* genes, encoding the L-valine biosynthetic enzymes acetohydroxyacid synthase, isomeroreductase and dihydroxyacid dehydratase, under the control of their native promoter	Sahm and Eggeling (1999)
pDrive	Kan^r, Amp^r; cloning vector	Qiagen, Hilden
pDrive*leuA*	pDrive carrying the *leuA* gene with *gapA* ribosomal binding site	This work
pBB1	Cm^r; compatible to pJC4*ilvBNCD* and harbors the P_{tac} promoter and the T_{trp} terminator; *lacI* negative	Krause et al. (2010b)
pBB1*leuA*	Cm^r; pBB1 carrying the native *leuA* gene from *C. glutamicum* under control of P_{tac}	This work
pBB1*leuA*$^{EC\text{-}G462D}$	Cm^r;pBB1 carrying a mutated version of the *leuA* gene from *E. coli* K12 under control of P_{tac}	This work
pJC4*ilvBNCDleuA*	Kan^r ; plasmid pJC4*ilvBNCD* carrying the *leuA* gene from *C. glutamicum* under control of P_{tac}	This work
pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$	Kan^r ; plasmid pJC4*ilvBNCD* carrying a mutated version of the *leuA* gene from *E. coli* K12 under control of P_{tac}	This work

pK19*mobsacB*. The chromosomal deletion of *ltbR* in *C. glutamicum* WT was performed by use of pK19Δcg1486 as described (Brune et al. 2007). The subsequent deletion of *ilvE* in *C. glutamicum* Δ*ltbR* was performed with pK19*mobsacB*Δ*ilvE*. The *gltA* promoter region (540 bp) in the resulting strain *C. glutamicum* Δ*ltbR* Δ*ilvE* was replaced by 254 bp of the mutated *dapA* promoter sequence L1 (Vásicová et al. 1999), by homologous recombination with the suicide vectors pK19ΔP*gltA_B-L1* (van Ooyen et al. 2012). The subsequent deletion of the methylcitrate synthase genes *prpC1* and *prpC2* was conducted with the vectors pK19*mobsacB*Δ*prpC1* and pK19*mobsacB*Δ*prpC2*, respectively, as described by Radmacher and Eggeling (2007). After electroporation and selection on BHIS agar plates containing kanamycin (25 μg/ml), the screening for desired mutants was done on 2×TY agar plates containing 10 % (*w*/*v*) sucrose. The replacements at the chromosomal loci were verified by PCR using the oligonucleotide pairs ltbR1/ltbR2, ilvE1/ilvE2, PgltAup/PgltAdown, prpC1fw/prpC1rev, and prpC2fw/prpC2rev, respectively (see Table S1 in the supplementary material).

Homologous and heterologous expression of the *C. glutamicum leuA* and of *leuA*$^{EC\text{-}G462D}$ from *E. coli* K12, respectively

For homologous overexpression of the *C. glutamicum leuA* gene, it was amplified using chromosomal DNA and primer

pair *leuA*-RBS-Ecofor/*leuA*-Ecorev and then firstly cloned into vector pDrive to give plasmid pDrive*leuA*. For cloning of *leuA* into vector pBB1, which carries the *tac* promoter P_{tac} and a terminator, pDrive*leuA* was cut with *Eco*R72I, and after purification, *leuA* was ligated into *Sma*I-restricted pBB1, to give plasmid pBB1*leuA*. For further cloning of *leuA* with P_{tac} and terminator into pJC4*ilvBNCD*, a PCR with primer pair pBB1for*Bst*1107I/pBB1rev*Bst*1107I and with pBB1*leuA* as template was performed. After restriction of the PCR product with *Bst*1107I, it was ligated into *Bst*1107I-restricted pJC4*ilvBNCD* to give plasmid pJC4*ilvBNCDleuA*.

To construct and express a *leuA* gene encoding a feedback-resistant IPMS, the following procedure was applied. The oligonucleotide pairs leuAfor/G462Drev and G462D/leuArev and chromosomal DNA from *E. coli* K12 were used to amplify two fragments of the *E. coli leuA* gene, overlapping by the complementary oligonucleotides G462Drev and G462D. Using these fragments and the oligonucleotides leuAfor and leuArev, a crossover PCR was performed to generate a mutated *leuA* gene with a basepair exchange (G to A) at position 1385, resulting in the replacement of glycine by aspartate at position 462 in the *E. coli* K12 IPMS enzyme. The oligonucleotide leuAfor contains the sequence of the *C. glutamicum* glyceraldehyde-3-phosphate dehydrogenase gene (*gapA*) ribosome binding site and upstream thereof a *Pst*I restriction site. Oligonucleotide leuArev also contains a *Pst*I restriction site. The newly generated *leuA* fragment was cut with *Pst*I and ligated into the multiple cloning site of plasmid pBB1, downstream of the *tac* promoter P_{tac} and upstream of the terminator located on pBB1. A third PCR with the oligonucleotide pair pBB1revStuI/PtacforStuI was done to amplify the mutated *leuA* gene with P_{tac} and terminator sequence. The PCR product was then cut with *Stu*I and ligated into pJC4*ilvBNCD* cut with *Bst*1107I to generate plasmid pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$.

Analytics

For quantification of substrate consumption and product formation, 1-ml samples were taken from the cultures and centrifuged at 13,000×*g* (5 min at room temperature), and the supernatant was used for determination of amino acids, 2-keto acids, and glucose concentrations in the culture fluid. The amino acid concentrations were determined by reversed-phase high-pressure liquid chromatography (RP-HPLC) as described before (Blombach et al. 2008). Glucose concentrations were determined by enzymatic assays with hexokinase/glucose-6-phosphate dehydrogenase from Roche Diagnostics (Penzberg, Germany). The 2-keto acid concentrations were determined by RP-HPLC with a variable wavelength detector (Agilent Technologies). The samples were diluted appropriately, and the separation was performed on a pre- and a main column for organic acids (Multohyp octyldecyl silane; particle size 5 μm; 125×4 mm; from CS Chromatographie, Langerwehe, Germany). As mobile phase, 10 mM H_2SO_4 was used with a constant flow of 0.5 ml/min at 50 °C. Detection occurred with a variable wavelength detector.

Determination of enzyme activities and kinetic parameters

Citrate synthase (CS) catalyzes the condensation of oxaloacetate with acetyl-CoA to form citrate and CoA-SH. A colorimetric standard assay for determination of CS activities was carried out. The principle of the photometric determination is based on the coupling of the reactions mentioned above with the irreversible conversion of CoA-SH with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB)—the so called Ellmans's reagent—which can be detected at a wavelength of 412 nm (extinction coefficient of 14.15 mM^{-1} cm^{-1}) (Srere 1969). *C glutamicum* cells were cultivated in minimal medium containing 4 % (wt/vol) glucose to an OD_{600} of ~5 and harvested by centrifugation (4,200×*g*, 15 min at 4 °C). The cells were washed three times with Tris/HCl 50 mM and sodium glutamate 200 mM, pH 7.5, resuspended in 1 ml of the same buffer, and disrupted with a RyboLyser (Hybaid), four times for 30 s each at speed 6.5 and 4 °C. After centrifugation at 20,800×*g* and 4 °C for 30 min, the supernatant was used for the assays. The protein concentration was quantified with the BCA protein assay (Thermo Fisher Scientific) with bovine serum albumin as standard.

The reaction mixture (1 ml) for determination of the specific CS activities contained 500 μl reaction buffer (400 mM sodium glutamate, 80 mM Tris/HCl, pH 7.5), 100 μl DTNB solution (1 mM DTNB, 50 mM Tris/HCl, pH 7.5), 50 μl oxaloacetate solution (4 mM oxaloacetate, 50 mM Tris/HCl, pH 7.5), 50–200 μl diluted cell extract, and H_2O ad 950 μl. The background extinction at 412 nm (30 °C) was followed for about 1 min. The reaction was started by adding 50 μl of starter solution (3 mM acetyl-CoA, 50 mM Tris/HCl, pH 7.5), and the change in extinction at 412 nm was monitored over a period of 2 min and plotted by the software SWIFT II (version 2.01, 2001 Biochrom Ltd.). A trendline was fitted to the curve, and ΔE/min could be determined from the slope of the line. The assays were linear over time (up to 4 min) and proportional to the protein concentration (up to 0.2 mg ml^{-1}).

IPMS catalyzes the conversion of KIV and acetyl-CoA to 2-isopropylmalate and CoA-SH, IPMD converts 2-isopropylmalate to 3-isopropylmalate, and IPMDH oxidizes 3-isopropylmalate to KIC with NAD^+. For determination of IPMS, IPMD, and IPMDH activities, cell extracts of *C. glutamicum* were prepared as described above, with the exception that 50 mM Tris/HCl pH 8.0 was used as buffer for cell disruption.

IPMS activity was determined by a modified version of the enzymatic assay described by Kohlhaw et al. (1969), i.e., via an end point assay based on the determination of CoA-SH with DTNB (see also above). The reaction mixture (1 ml) contained 500 μl dying solution (1 mM DTNB in 100 mM Tris/HCl, pH 7.5), 40 μl reaction buffer (625 mM Tris/HCl, pH 8.5), 160 μl KCl solution (250 mM), 80 μl acetyl-CoA solution (12.5 mM), 40–80 μl (diluted) cell extract, and H_2O ad 920 μl. After following the background, the reaction was started with 80 μl KIV (6.25 mM). For the inhibition assays, L-leucine, KIC, 2-IPM, and 3-isopropylmalate (3-IPM), respectively, were added as neutralized stock solutions in the concentration indicated in the respective results section.

IPMD activity was determined photometrically by monitoring the increase in absorbance of the intermediate dimethylcitraconate (extinction coefficient of 4.53 mM^{-1} cm^{-1}) at a wavelength of 235 nm (Kohlhaw 1988). Since the IPMD reaction is reversible and the equilibrium of the reaction favors the formation of 2-IPM, 3-IPM was used as substrate. The reaction mixture contained 400 μl potassium dihydrogen phosphate (400 mM, pH 7) and 540 μl H_2O and was preheated for 1 min at 30 °C. Forty microliters of diluted cell extract was added, and after following the background activity for 1 min, the IPMD reaction was started with 20 μl of 100 mM 3-IPM, measured for 2 min, and plotted by the software SWIFT II.

IPMDH activity was determined photometrically by a method described by Schrumpf et al. (1992). In this method, the product of the reaction, KIC, is monitored by derivatization with 2,4-dinitrophenylhydrazine. This compound interacts with the carbonyl groups of KIC, thereby generating 2,4-dinitrophenylhydrazone (2,4-DPH) and H_2O. The absorption of 2,4-DPH can be measured at 540 nm, and the amount of 2,4-DPH corresponds to the amount of KIC formed and can be calculated by means of a standard curve of known concentrations. For measurement, 400 μl buffer (50 mM potassium chloride, 50 mM potassium phosphate, 1 mM magnesium chloride, adjusted to pH 8.0 with potassium hydroxide), 25 μl 40 mM NAD^+, 25 μl diluted cell extract, and 25 μl H_2O were mixed. The enzymatic reaction was started with 25 μl of 20 mM 3-IPM, and the mixture was incubated at 30 °C for 1 min. Then, 250 μl of 2,4-dinitrophenylhydrazine (25 mg/100 ml of 0.5 M HCl) was added. An incubation for 15 min at 30 °C followed to allow formation of 2,4-DPH. After 15 min, the reaction was neutralized with 250 μl of potassium hydroxide (4 g/10 ml), and the extinction was monitored at 540 nm. For each reaction, a blank containing all compounds except substrate was performed. This was necessary due to the fact that the cell extract possibly contained other keto acids such as KIV or KMV which also react with 2,4-dinitrophenylhydrazine.

Since it is not possible to determine a potential KIC inhibition with the IPMDH assay described above (KIC itself is derivatized with 2,4-dinitrophenylhydrazine), we performed the KIC inhibition tests according to a method described by Parsons and Burns (1969). This assay is based on the photometrical determination of NADH formed during the reaction at a wavelength of 340 nm. For measurement, 325 μl H_2O and 500 μl reaction buffer (150 mM potassium chloride, 100 mM potassium dihydrogenphosphate, 3 mM magnesium chloride, adjusted to pH 8.0 with potassium hydroxide) were prewarmed to 30 °C, 50 μl 40 mM NAD^+ and 25 μl cell extract (diluted in 50 mM potassium phosphate, pH 7.0) were added, and the background activity was measured for 1 min at a wavelength of 340 nm. The main reaction was started by addition of 100 μl 20 mM 3-IPM. The increase in absorbance was monitored for 3 min, and ΔE/min was calculated via SWIFT II.

Results

Inactivation of *ltbR* and *ilvE* and overexpression of *ilvBNCD* leads to KIV and KIC accumulation

Brune et al. (2007) previously constructed *C. glutamicum* Δ*ltbR* that carries a deletion in the chromosomal *ltbR* locus, and this deletion has been shown to result in enhanced expression of the L-leucine biosynthetic genes *leuA*, *leuCD*, and *leuB*. Based on this mutant, we constructed the *C. glutamicum* Δ*ltbR* Δ*ilvE* double mutant, which is also devoid of the transaminase B (TAB) gene *ilvE*. TAB catalyzes the last reactions in the synthesis of the branched chain amino acids L-valine, L-leucine, and L-isoleucine, i.e., the amination of the respective 2-keto acids. To test *C. glutamicum* Δ*ltbR* and *C. glutamicum* Δ*ltbR* Δ*ilvE* for their ability to form 2-keto acids, and/or amino acids, shake-flask experiments were performed in minimal medium with 4 % (*w*/*v*) glucose and L-valine, L-leucine, and L-isoleucine as supplement (2 mM each). Growth, glucose utilization, and amino and keto acid accumulation were monitored over the course of the experiment. Both strains grew with a growth rate (μ) of about 0.32 h^{-1} and reached a final OD_{600} of about 63 after 16 h. With the exception of L-valine (2.5 mM), *C. glutamicum* Δ*ltbR* did not produce any of the tested metabolites in measurable concentrations (<0.5 mM). In contrast, after depletion of L-valine, L-leucine, and L-isoleucine in the growth medium after 12 h of incubation, *C. glutamicum* Δ*ltbR* Δ*ilvE* began to produce small amounts of KIC. However, KIC formation stopped after 15 h at a concentration of 2.2 mM, and then the strain began to form L-leucine, L-valine, and KIV in concentrations up to 3 mM.

As shown previously by Krause et al. (2010a), plasmid-bound overexpression of the genes encoding AHAS, AHAIR, and DHAD *(ilvBNCD)* led to more efficient conversion of pyruvate to KIV, which is an intermediate of KIC synthesis

(see Fig. 1). To increase the carbon flux towards KIV and thus also towards KIC synthesis, we transformed *C. glutamicum* Δ*ltbR* Δ*ilvE* with plasmid pJC4*ilvBNCD*. The growth of and product formation by the resulting strain *C. glutamicum* Δ*ltbR* Δ*ilvE* (pJC4*ilvBNCD*) in minimal medium containing glucose was monitored and is shown in Fig. 2a. The strain grew within 15 h to an OD_{600} of about 49 with a μ of 0.27 h^{-1}, consumed glucose completely within 24 h, and formed about 19 mM KIV. In the late exponential growth phase (after 8 h), the cells started KIC secretion which, however, stopped at about 10 mM after 15 h, although there still was glucose left at that time (Fig. 2a). Thus, *C. glutamicum* Δ*ltbR* Δ*ilvE* (pJC4*ilvBNCD*) produced about six- and fivefold higher concentrations of KIV and KIC, respectively, than the same strain without pJC4*ilvBNCD* (see above). These results show that an increase of the flux towards KIV by overexpression of *ilvBNCD* is likely to be an important feature for efficient KIC production with *C. glutamicum*.

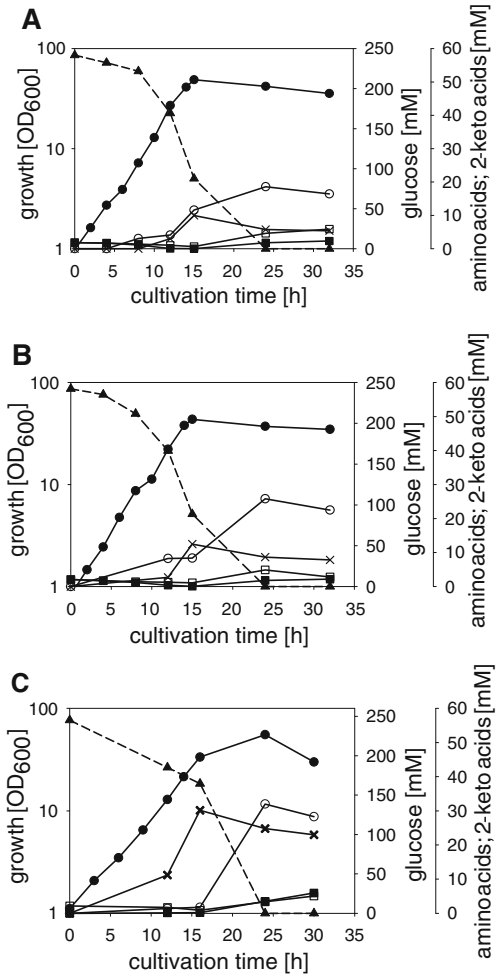

Fig. 2 Growth, glucose consumption, and amino and 2-keto acid accumulation during representative shake-flask batch cultivations of **a** *C. glutamicum* Δ*ltbR* Δ*ilvE* (pJC4*ilvBNCD*) and **b** *C. glutamicum* VB (pJC4*ilvBNCD*) in minimal medium containing glucose (4 %) and all three L-isoleucine, L-leucine, and L-valine (2 mM each), and **c** of *C. glutamicum* VB (pJC4*ilvBNCD*) in minimal medium containing glucose (4 %) and only L-isoleucine and L-valine (2 mM each). Symbols: *closed circles*, growth; closed *triangles*, glucose; *open squares*, L-valine; *filled squares*, L-leucine; *open circles*, KIV; *crosses*, KIC. At least three independent fermentations were performed per set of experiment, all showing comparable results

2-Ketoisocaproate production by *C. glutamicum* Δ*ltbR* Δ*ilvE* with attenuated CS, inactivated methylcitrate synthases, and overexpressed *ilvBNCD* genes

Assuming that an attenuated CS activity will lead to an improved availability of acetyl-CoA and also of pyruvate (both precursors of KIC synthesis; see Fig. 1), we substituted the natural promoter of the CS gene (*gltA*) in *C. glutamicum* Δ*ltbR* Δ*ilvE* by the mutated *dapA* promoter L1 (Vásicová et al. 1999; see also "Materials and methods"), resulting in *C. glutamicum* Δ*ltbR* Δ*ilvE* P*gltA*$^{mut\_L1}$. The exchange of the natural *gltA* promoter with promoter L1 in *C. glutamicum* Δ*ltbR* Δ*ilvE* had no effect on μ or the final OD_{600} in minimal medium plus glucose, although the specific CS activity was only about 30 % of the activity observed in the parental strain with the native *gltA* promoter (0.33±0.03 vs. 1.10±0.10 U/mg protein).

To prevent an interference of methylcitrate synthase (MCS) activities with CS activity [as previously discussed by Radmacher and Eggeling 2007; see "Discussion"], we deleted the MCS genes *prpC1* and *prpC2* in *C. glutamicum* Δ*ltbR* Δ*ilvE* P*gltA*$^{mut\_L1}$. The resulting strain *C. glutamicum* Δ*ltbR* Δ*ilvE* Δ*prpC1* Δ*prpC2* P*gltA*$^{mut\_L1}$ showed the same growth behavior on minimal medium plus glucose (μ = 0.32 h^{-1}; final OD_{600}=63) and the same specific CS activity (0.32±0.02 U/mg protein) as the parental strain, and was designated as *C. glutamicum* VB.

To test the effect of CS attenuation on KIC production, *C. glutamicum* VB was transformed with plasmid pJC4*ilvBNCD*, and shake-flask fermentations with 4 % glucose and with L-isoleucine, L-leucine, and L-valine (2 mM each) in minimal medium were performed. The results of a representative shake-flask experiment with *C. glutamicum* VB (pJC4*ilvBNCD*) are shown in Fig. 2b. In the exponential growth phase, the strain grew within 15 h with a μ of 0.31 h^{-1} to an OD_{600} of ~48. Within 12 h of growth, the strain started to secrete KIV and KIC into the external medium. However, as already observed with *C. glutamicum* Δ*ltbR* Δ*ilvE* (pJC4*ilvBNCD*) (see Fig. 2a), the secretion of KIC stopped after 15 h at about 12.5 mM, in spite of residual glucose in the growth medium. In contrast, KIV was further secreted up to a concentration of 26 mM, until glucose was exhausted. In addition, we observed the formation of up to 6 mM L-valine and 2 mM L-leucine in the medium. This latter result was unexpected since the deletion of the TAB gene (*ilvE*) in *C. glutamicum* has been reported to result in an auxotrophy for L-

isoleucine and L-leucine (Marienhagen et al. 2005; Krause et al. 2010a).

The known inhibition of the IPMS by L-leucine (see "Introduction" and below) and the observations that *C. glutamicum* VB (pJC4*ilvBNCD*) produced significant amounts of KIV and additionally formed L-leucine in the course of the fermentation led to the hypothesis that the addition of L-leucine to the fermentation medium might be counterproductive. Therefore, we analyzed the fermentation behavior of *C. glutamicum* VB (pJC4*ilvBNCD*) in minimal medium with L-valine and L-isoleucine (2 mM each), but without L-leucine. As shown in Fig. 2c, the strain was able to grow without L-leucine; whereas the final OD_{600} was not affected, μ was somewhat lower than with L-leucine (0.21 vs. 0.31 h^{-1}). KIC secretion started earlier than during growth with L-leucine, and within 15 h, *C. glutamicum* VB (pJC4*ilvBNCD*) produced 31±2 mM KIC with a substrate-specific yield ($Y_{P/S}$) of 0.26 mol/mol glucose in the overall production phase. The production of KIC stopped again after 15 h, and then up to 32±2 mM of KIV was formed until glucose was exhausted from the medium. Again, L-leucine and L-valine were formed in significant concentrations (up to 6 mM) as by-products.

We also tested the growth and KIC production of *C. glutamicum* VB (pJC4*ilvBNCD*) in minimal medium without addition of any branched-chain amino acid, with addition of either L-valine, L-leucine, or L-isoleucine alone (2 mM) and with different combinations of two of the three amino acids. Without addition of any amino acid and in minimal medium containing only L-valine and/or L-leucine, *C. glutamicum* VB (pJC4ilvBNCD) did not grow at all (data not shown), indicating that the cells are auxotrophic for L-isoleucine. In minimal medium containing 2 mM L-isoleucine, *C. glutamicum* VB (pJC4ilvBNCD) showed growth (μ of 0.21 to 0.18 h^{-1}), although it was slower than in the presence of L-valine and L-leucine. However, we did not observe any effect of the absence of L-valine on KIC production (data not shown).

Taken together, the results demonstrate that *C. glutamicum* VB (pJC4*ilvBNCD*), in spite of the deletion of the *ilvE* gene, is not auxotrophic for L-leucine and L-valine. Moreover, the results indicate that attenuation of CS activity in combination with limited availability of L-leucine is beneficial for KIC production. Consequently, in the following, all fermentation experiments were performed without L-leucine in the growth medium.

Activities and regulation of enzymes of the IPM pathway

The fermentation pattern of *C. glutamicum* VB (pJC4*ilvBNCD*), in particular the formation of significant amounts of KIV, indicated a bottleneck of the carbon flux towards KIC at the IPM pathway from KIV to KIC (see Fig. 1). This prompted us to study the enzyme activities of the IPM pathway in *C. glutamicum* VB (pJC4*ilvBNCD*) in further detail, especially regarding their inhibition/activation by intermediates of L-leucine and KIC synthesis.

The specific IPMS, IPMD, and IPMDH activities in extracts of glucose-grown cells of *C. glutamicum* WT and of *C. glutamicum* VB (pJC4*ilvBNCD*) are listed in Table 2. In comparison to the WT strain, *C. glutamicum* VB (pJC4*ilvBNCD*) showed about fourfold higher IPMS and about tenfold higher IPMD and IPMDH activities. The higher specific activities in *C. glutamicum* VB (pJC4*ilvBNCD*) can be explained by the deletion of the *ltbR* gene and, thus, the absence of LtbR, which previously was shown to directly or indirectly repress the IPMS, IPMD, and IPMDH genes *leuA*, *leuCD*, and *leuB*, respectively (Brune et al. 2007).

IPMS catalyzes the first reaction of the IPM pathway, namely the conversion of KIV plus acetyl-CoA to 2-IPM (see Fig. 1), and thus, it can be regarded as key enzyme in KIC synthesis. We analyzed the specific IPMS activities in cell extracts of *C. glutamicum* VB (pJC4*ilvBNCD*) in the presence of varying concentrations of acetyl-CoA (0.05 to 4 mM) and KIV (0.05 to 20 mM) and determined the respective K_m values to be 124 and 38 μM, respectively (Fig. S1 in the supplementary material). In accordance with its key position in the IPM pathway, IPMS was previously shown to be strictly regulated via feedback inhibition by L-leucine, and moreover, its synthesis was found to be repressed by L-leucine in the growth medium (Pátek et al. 1994; see

Table 2 Specific activities of IPMS, IPMD, and IPMDH in cell extracts of *C. glutamicum* WT and *C. glutamicum* VB carrying pJC4*ilvBNCD*, pJC4*ilvBNCDleuA*, or pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$. The cells were grown in minimal medium plus 4 % glucose and L-isoleucine and L-valine (2 mM each). The values shown are the means ± SD of at least three independent experiments

Strain	Specific activity [U/mg]		
	IPMS	IPMD	IPMDH
C. glutamicum WT	0.10±0.02	0.03±0.01	0.01±0.01
C. glutamicum VB (pJC4*ilvBNCD*)	0.43±0.08	0.32±0.11	0.09±0.03
C. glutamicum VB (pJC4*ilvBNCDleuA*)	0.87±0.11	n.d.	n.d.
C. glutamicum VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$)	0.39±0.02	n.d.	n.d.

n.d. not determined

"Introduction"). In agreement with these authors, we found strong inhibition of the IPMS activity in cell extracts of *C. glutamicum* VB (pJC4*ilvBNCD*); at 0.05 mM L-leucine, the activity was 44 % inhibited, and at 1 mM L-leucine, it was nearly abolished (Fig. 3). Also in agreement with the previous findings, extracts from *C. glutamicum* VB (pJC4*ilvBNCD*) cells grown with the addition of 2 mM L-leucine exhibited more than tenfold lower specific IPMS activity (0.034± 0.003 U/mg) than the extracts of cells cultivated without L-leucine (0.43±0.08 U/mg), indicating negative transcriptional control of the IPMS gene *leuA* by L-leucine.

To test whether KIC itself inhibits the IPMS in a negative feedback loop, inhibition assays with increasing KIC concentrations were performed with cell extracts of *C. glutamicum* VB (pJC4*ilvBNCD*). In the standard assay (i.e., with 0.5 mM KIV as a substrate), IPMS was half maximally inhibited at about 8 mM KIC. However, it turned out that KIC inhibited the IPMS activity in a competitive manner against its substrate KIV, i.e., the inhibition could be relieved by increasing the KIV concentration in the reaction mixture. As shown in Fig. 4a, the presence of higher KIC concentrations (>1 mM) resulted in increasing K_m values, i.e., in a "right shift" of the reaction curve in the Michaelis–Menten plot. Interestingly, inhibition of the IPMS could not be observed until a KIC/KIV ratio of >10 was reached. At KIC/KIV ratios ≤4, KIC even exerted an activating effect on the enzyme, resulting in enhanced specific IPMS activity and hence a higher V_{max} in the Michaelis–Menten plot (Fig. 4a). However, we cannot exclude that the activating effect is caused by side activities of enzymes contained in the extract. The behavior of the IPMS activity is illustrated exemplarily for the assay conducted with 0.5 mM KIV and increasing concentrations of KIC in Fig. 4b.

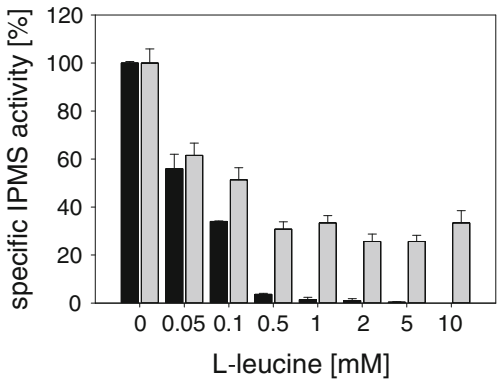

Fig. 3 Percentage of residual specific IPMS activity in cell extracts of *C. glutamicum* VB (pJC4*ilvBNCD*) (*black bars*) and in cell extracts of *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$) (*grey bars*) in the presence of increasing L-leucine concentrations (0.5 mM KIV throughout). One hundred percent corresponds to a specific activity of 0.43 U/mg in the case of *C. glutamicum* VB (pJC4*ilvBNCD*) and of 0.39 U/mg in the case of *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$). The values shown are the means ± SD of at least three independent experiments

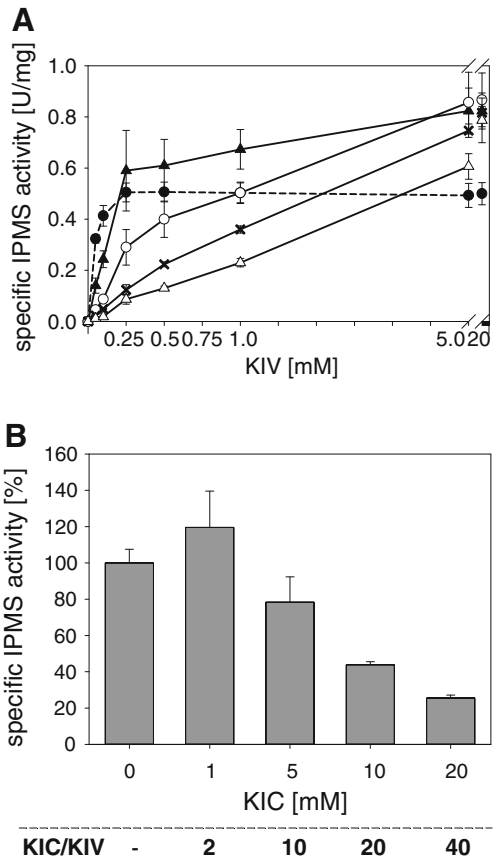

Fig. 4 **a** KIV-dependent specific IPMS activities in cell extracts of *C. glutamicum* VB (pJC4*ilvBNCD*) in the absence (*filled circles*) and in the presence of increasing KIC concentrations. Symbols: *filled triangles*, 1 mM KIC; *open circles*, 5 mM KIC; *crosses*, 10 mM KIC; *open triangles*, 20 mM KIC. The cells were grown in minimal medium containing glucose (4 %, *w*/*v*), L-isoleucine, and L-valine (2 mM each) and harvested at an OD_{600} of about 7. **b** Percentage of residual specific IPMS activity in cell extracts of *C. glutamicum* VB (pJC4*ilvBNCD*) in the presence of increasing KIC concentrations (0.5 mM KIV throughout). A specific activity of 0.43 U/mg corresponds to 100 %. In the last line, the KIC/KIV ratio for each bar is given. The values shown are the means ± SD of at least three independent experiments (three independent crude extracts)

To study the effects of 2-IPM and of 3-IPM on IPMS activity, respective assays were performed with cell extracts of *C. glutamicum* VB (pJC4*ilvBNCD*). Neither 2-IPM nor 3-IPM in concentrations up to 10 mM had a negative effect on the specific IPMS activity (data not shown). However, 2-IPM in concentrations between 0.5 and 2.5 mM and 3-IPM at a concentration of 0.5 mM even led to slightly higher (about 10 %) specific IPMS activities.

IPMD catalyzes the second reaction in the IPM pathway, i.e., the reversible conversion of 2-IPM to 3-IPM. To our knowledge, the regulation of the *C. glutamicum* IPMD on enzymatic level has not been studied so far. Therefore, the influence of KIC and of L-leucine on IPMD activity was investigated in the KIC producer *C. glutamicum* VB (pJC4*ilvBNCD*). Whereas no inhibition of the IMPD activity

was observed with L-leucine concentrations up to 20 mM, the presence of increasing KIC concentrations led to a stepwise decrease of IPMD activity; 5 mM KIC resulted in about 50 % residual activity, and 7.5 mM KIC lowered the activity to less than 10 %. KIC inhibition of IMPD was not competitive since increase of the substrate concentration (up to 30 mM) did not relieve the inhibition (data not shown).

With IMPDH, the inhibition tests did not provide clear and reproducible results, and consequently, no statement on the influence of KIC or L-leucine on its activity can be made.

Influence of acetate on KIC production

The IPMS K_m value determined for acetyl-CoA (124 μM; see above) is significantly higher than the intracellular acetyl-CoA concentrations of about 24 μM previously determined in *C. glutamicum* WT cells grown in minimal medium containing glucose as carbon and energy source (Wendisch et al. 1997). As the addition of acetate to the growth medium of *C. glutamicum* WT resulted in a significant increase of the intracellular acetyl-CoA concentration (Wendisch et al. 1997), we tested for an effect of acetate supplementation on KIC production with *C. glutamicum* VB (pJC4*ilvBNCD*). For this purpose, 1 % (*w*/*v*) sodium acetate was added to a glucose-grown culture of this strain after 14 h of fermentation, and KIC formation in this culture was compared to the culture incubated without addition of acetate. The cells cultivated without sodium acetate accumulated about 31 mM of KIC and 30 mM of KIV (comparable to the fermentation shown in Fig. 2c) and the cells cultivated with addition of acetate secreted about 42±3 mM KIC and only about 22 mM KIV (data not shown). This result suggests that acetyl-CoA supply is also a limiting factor in KIC formation and that acetate supplementation enables more efficient conversion of KIV to KIC.

Influence of *leuA* overexpression on KIC production

To analyze the effect of *leuA* overexpression on KIC production of *C. glutamicum* VB (pJC4*ilvBNCD*), we cloned the native *C. glutamicum leuA* gene into pJC4*ilvBNCD* under the control of P_{tac} and transformed pJC4*ilvBNCDleuA* into *C. glutamicum* VB. Surprisingly, cell extracts of the resulting strain *C. glutamicum* VB (pJC4*ilvBNCDleuA*) showed only about twofold higher specific IMPS activity than the same strain without *leuA* on plasmid pJC4*ilvBNCD* (Table 2). However, fermentation analysis with *C. glutamicum* VB (pJC4*ilvBNCDleuA*) revealed that this strain secreted only slightly (5–10 %) more KIC than *C. glutamicum* VB (pJC4*ilvBNCD*) (data not shown), indicating that the native IPMS even in higher concentration does not significantly contribute to KIC production.

Influence of a feedback-resistant IPMS on KIC production

Gusyatiner et al. (2002) reported that the replacement of glycine at position 462 in the *E. coli* IPMS by aspartate results in an IPMS enzyme which is resistant towards L-leucine inhibition. To investigate the influence of a feedback-resistant IPMS enzyme on KIC production with *C. glutamicum* VB (pJC4*ilvBNCD*), a mutation leading to the respective amino acid exchange at position 462 was introduced into the *leuA* gene from *E. coli* K12, the resulting $leuA^{EC\text{-}G462D}$ allele was subcloned into plasmid pJC4*ilvBNCD* under the control of P_{tac}, and the resulting plasmid transformed into *C. glutamicum* VB.

To test for the feedback resistance of the $leuA^{EC\text{-}G462D}$ gene product, we determined the specific IPMS activity in cell extracts of *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$) and as a reference also in *C. glutamicum* VB (pJC4*ilvBNCD*) without and with increasing L-leucine concentrations. Without L-leucine, the extracts of cells with $leuA^{EC\text{-}G462D}$ on plasmid pJC4*ilvBNCD* showed even slightly lower specific IPMS activity than the reference cells without $leuA^{EC\text{-}G462D}$ (Table 2). As shown in Fig. 3, the specific IPMS activities in the extracts of both strains were decreasing with increasing L-leucine concentrations in the range between 0.05 and 0.5 mM L-leucine. However, this decrease was much more pronounced in extracts of *C. glutamicum* VB (pJC4*ilvBNCD*); a concentration of 0.5 mM L-leucine resulted in only 0.018 U/mg, i.e., <5 % of the original IPMS activity, and at a concentration of 10 mM L-leucine, the enzyme activity was below the detection limit. In contrast, extracts of *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$) showed still about 0.12–0.13 U/mg protein (i.e., about 31 % of the original activity) in the presence of 0.5 mM L-leucine, and even at higher L-leucine concentrations, this activity did not fall under this value (Fig. 3). It can be assumed that the residual IPMS activity is based on expression of $leuA^{EC\text{-}G462D}$, whereas the inihibitable activity corresponds to expression of the native (chromosomal) *leuA* gene. However, these findings confirmed that $leuA^{EC\text{-}G462D}$ codes for a feedback-resistant version of the IPMS enzyme.

C. glutamicum VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$) then was analyzed for growth and production behavior and compared to its parental strain *C. glutamicum* VB (pJC4*ilvBNCD)*. In shake-flask fermentations in minimal medium with 4 % glucose and L-isoleucine and L-valine (2 mM each), the newly constructed strain showed a slightly reduced μ of 0.17 h^{-1} (parental strain, 0.21 h^{-1}) and reached an OD_{600} of 54 after 24 h. As shown in Fig. 5a, *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$) secreted almost twice as much KIC (54±4 mM=7.0±0.5 g l^{-1}) into the external medium as its parental strain *C. glutamicum* VB (pJC4*ilvBNCD*) (Fig. 2c), the $Y_{P/S}$ was about 0.22 mol/mol glucose. Furthermore, the formation of KIV (2.1±0.5 mM) was nearly

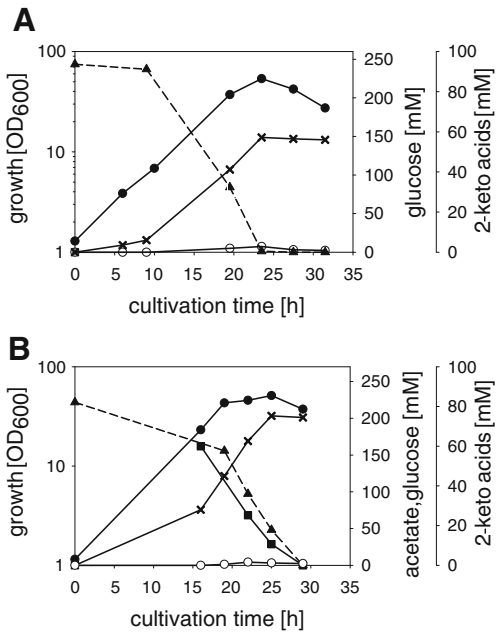

Fig. 5 Growth, substrate consumption, and 2-keto acid accumulation during representative shake-flask batch cultivations of *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$) in **a** minimal medium containing glucose (4 %, *w*/*v*), L-isoleucine, and L-valine (2 mM each) or **b** minimal medium containing glucose (4 %, *w*/*v*), acetate (1 %, *w*/*v*; added after 16 h of growth), L-isoleucine, and L-valine (2 mM each). Symbols: *closed circles*, growth; *closed triangles*, glucose; *open circles*, KIV; *crosses*, KIC; filled *squares*, acetate. At least three independent fermentations were performed per set of experiments, all showing comparable results

abolished in the culture of *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$), indicating that the additional KIC has been synthesized at the expense of KIV.

Since we observed a positive effect of acetate on KIC formation of *C. glutamicum* VB (pJC4*ilvBNCD*) (see above), *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G426D}$) was cultivated in minimal medium with 4 % glucose, and after 18 h of cultivation, 1 % of acetate was added, and the product spectrum was monitored. As shown in Fig. 5b, *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G426D}$) secreted after 25 h about 71 ±3 mM (9.2±0.4 g l^{-1}) of KIC, which represents an increase of about 33 % when compared to the KIC production by cells cultivated without acetate (see above). The substrate-specific yield amounted to 0.24±0.01 mol C (KIC) per mole C (in glucose and acetate).

Discussion

We here constructed a *C. glutamicum* strain efficiently producing KIC with nearly no by-product formation. Aside from the fact that KIC itself is an interesting product (see "Introduction"), it is a precursor for the synthesis of the potential biofuel 3-methyl-1-butanol. Using the L-valine and L-leucine biosynthetic pathways (including a feedback inhibition-resistant IPMS encoded by *leuA*$^{EC\text{-}G462D}$) and a subsequent decarboxylation and reduction step in a BC amino acid transferase-deficient *E. coli* strain, Connor and Liao (2008) recently engineered this organism for the production of 3-methyl-1-butanol. The direct progenitor for the 3-methyl-1-butanol producer produced KIC in concentrations of up to 2.3 g l^{-1}, which is about one third of that observed with our optimized *C. glutamicum* producer with glucose as a substrate and only one fourth of that with glucose and acetate as substrates. Thus, it seems that *C. glutamicum* is very well suited as a KIC-producing organism, and it is tempting to speculate that the combination of the engineering strategies for KIC and 3-methyl-1-butanol production in *C. glutamicum* will result in an optimized 3-methyl-1-butanol producer strain with high yield and productivity.

Although KIV is a precursor of KIC (see Fig. 1), we could not use our previously constructed KIV producer of *C. glutamicum* (Krause et al. 2010a) as a parental strain for a KIC-producing derivative. The basis to engineer *C. glutamicum* for KIV production was the inactivation of the pyruvate dehydrogenase complex (PDHC) (Krause et al. 2010a). However, inactivation of the PDHC prevents the formation of acetyl-CoA, which is the second substrate of the IPMS (see Fig. 1) and therefore is essential for KIC formation. Thus, we started the construction of a KIC-producing *C. glutamicum* strain with *C. glutamicum* WT by deletion of the *ltbR* and *ilvE* genes, overexpression of the *ilvBNCD* genes, and exchange of the native *gltA* promoter for an artificial one. The promoter exchange resulted in reduction of CS activity to about 30 % of the parental activity. This, on the one hand, should still ensure a reasonable operation of the citric acid cycle for energy conservation and for anabolic purposes and, on the other hand, was expected to increase the availability of pyruvate and acetyl-CoA, the substrate and cosubstrate for AHAS and IPMS, respectively. However, we speculated that the decreased CS activity in the long run might result in expression of the genes *prpC1* and *prpC2*, both coding for MCS enzymes with CS side activity. Double mutants of *C. glutamicum*, which lacked *gltA* and *prpC1*, regained CS activity due to the expression of *prpC2* (Radmacher and Eggeling 2007). The authors showed that this phenotype in several suppressor mutants was due to different mutations in the respective *prpR* gene, encoding an activator of the *prpC2* gene. To avoid suppressor mutations in our KIC producer, we deleted *prpC1* and *prpC2*.

The appearance of L-leucine in the culture of *C. glutamicum* VB (pJC4*ilvBNCD*), the better KIC production and the higher IPMS activity of cells grown in minimal medium without L-leucine (Fig. 2b and c), and the sensitivity of the IPMS towards L-leucine (Pátek et al. 1994; Fig. 3) pointed to the L-leucine inhibition of IPMS as one main reason for the limitation of the carbon flux from KIV to KIC in *C. glutamicum*. This assumption was verified by the

finding that the plasmid-bound expression of *leuA*$^{EC\text{-}G462D}$, coding for a feedback-resistant IPMS (Gusyatiner et al. 2002), in *C. glutamicum* VB (pJC4*ilvBNCD*) drastically improved the production behavior. *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$) did not only produce significantly more KIC, but also exhibited a strong reduction of formation of KIV as byproduct, indicating highly efficient conversion of KIV towards KIC.

It has to be mentioned that the specific IPMS activity in extracts of *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$) was about twofold lower than in extracts of *C. glutamicum* VB (pJC4*ilvBNCDleuA*) and at about the same level as in extracts of cells without plasmid-encoded *leuA*. The absolute specific IPMS activities observed in the three strains suggest that (1) *leuA* overexpression from pJC4*ilvBNCDleuA* is relatively weak and (2) *leuA*$^{EC\text{-}G462D}$ overexpression from pJC4*ilvBNCDleuA*^{G462D} is even weaker and somehow exerts a negative effect on expression of the native *leuA* gene. However, at present, we do not have an explanation for this result.

Apart from inhibition of IPMS by L-leucine, the supply of the IPMS with acetyl-CoA is another critical and limiting factor for KIC production. The K_m value of 124 μM determined for acetyl-CoA for the IPMS in *C. glutamicum* VB (pJC4*ilvBNCD*) was significantly higher than the intracellular acetyl-CoA concentration of about 24 μM detected in *C. glutamicum* WT, when grown on glucose (Wendisch et al. 1997). Due to the reduced CS activity in *C. glutamicum* VB (pJC4*ilvBNCD*), it can be assumed that the intracellular acetyl-CoA concentration is higher than in the WT strain; however, the acetyl-CoA concentration in *C. glutamicum* VB (pJC4*ilvBNCD*) is probably still not high enough to allow maximum specific activity of the IPMS. This assumption is in good accordance with the fact that cultivation of *C. glutamicum* VB (pJC4*ilvBNCD*) and of *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$) with glucose plus acetate resulted in higher KIC production. An increase of the intracellular acetyl-CoA concentration from about 24 to 155 μM was observed when *C. glutamicum* WT cells were cultivated with acetate as additional carbon source (Wendisch et al. 1997).

C. glutamicum VB (pJC4*ilvBNCD*) was able to grow without L-leucine in the culture medium. This observation is in contrast to the L-leucine auxotrophy of *C. glutamicum* Δ*ilvE* reported by Marienhagen et al. (2005) and Krause et al. (2010a). A potential reason for the L-leucine prototrophy might be the formation of L-leucine as observed during cultivation of *C. glutamicum* VB (pJC4*ilvBNCD*) without L-leucine. Since *ilvE* is deleted in this strain, formation of L-leucine can only be explained by the activity of unspecific transaminases such as AlaT and AvtA (Marienhagen et al. 2005; Marienhagen and Eggeling 2008). It is conceivable that these enzymes can only use KIC as substrate when it is present in comparably high concentrations. Due to the lower CS activity, the overexpression of *ilvBNCD*, and the deletion of *ltbR*, it is likely that the flux towards KIC and also the concentration of KIC in *C. glutamicum* VB (pJC4*ilvBNCD*) are higher than in *C. glutamicum* Δ*ilvE*, and thus, the former strain is able to form enough L-leucine for growth.

Feedback inhibition of IPMS by L-leucine in general is a common feature among diverse bacteria (Stieglitz and Calvo 1974), and this probably is a regulatory feature to allow for balanced synthesis of BC amino acids. For *Salmonella typhimurium* and *Alcaligenes eutrophus*, it was reported that also KIC inhibits the IPMS, in a competitive manner with respect to KIV (Kohlhaw et al. 1969; Wiegel and Schlegel 1977a). Similarly, the IPMS in cell extracts of *C. glutamicum* VB (pJC4*ilvBNCD*) was competitively inhibited by KIC. At a KIC/KIV ratio of 20, for instance, the enzyme displayed about 40 % residual activity (see Fig. 4b). In contrast, the IPMS of *S. typhimurium* was inhibited to about 50 % already at a KIC/KIV ratio of 5 (Kohlhaw et al. 1969). However, although the KIC inhibition of IPMS seems to be relatively weak, it might be that this regulation is critical for further improvement of KIC production with *C. glutamicum* VB (pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$) or other KIC-producing strains with improved carbon flux from KIV to KIC.

In addition to L-leucine inhibition, competitive product inhibition of IPMS by 2-IPM was reported for *A. eutrophus* (Wiegel and Schlegel 1977b). However, in *C. glutamicum* VB (pJC4*ilvBNCD*), both the intermediates 2-IPM and 3-IPM did not influence IPMS activity. These findings suggest that although the reaction catalyzed and also the amino acid sequences of IPMS enzymes are highly conserved among different organisms (Yindeeyoungyeon et al. 2009), the regulatory mechanisms present in these organisms differ significantly from each other.

IPMD is present in many bacteria and yeasts (e.g., Lee et al. 2012; Kohlhaw 2003), and the respective *leuCD* genes are subject to negative transcriptional control by L-leucine in many organisms, such as *Saccharomyces cerevisiae* (Hsu et al. 1982) and *C. glutamicum* (Pátek et al. 1994). Furthermore, there is an indication for positive transcriptional control of *leuCD* in presence of the IPMS gene product 2-IPM in *Neurospora crassa* (Gross 1965). Due to the instability of IPMD in cell extracts isolated from various organisms, the enzyme and its regulatory properties have not been extensively studied in the past (Bigelis and Umbarger 1976). For *C. glutamicum* VB (pJC4*ilvBNCD*), we found that the specific IPMD activity was not affected by L-leucine. The addition of KIC, however, resulted in a noncompetitive inhibition of the enzyme. This inhibition might be a regulatory feature to control L-leucine biosynthesis via sensing the carbon flux through the IPM pathway. The physiological importance of IPMD inhibition by KIC in *C. glutamicum* VB (pJC4*ilvBNCD*) or in *C. glutamicum* VB

(pJC4*ilvBNCDleuA*$^{EC\text{-}G462D}$) remains unclear. However, for further improvement of KIC production with *C. glutamicum*, IMPD inhibition by KIC has to be taken into account.

Having in mind the final titers and yields of our KIV-producing strain of *C. glutamicum* (188±28 mM and 0.47± 0.05 mol/mol of glucose; Krause et al. 2010a), there clearly is room for further improvement of KIC production with *C. glutamicum*. One target certainly is the KIC inhibition of IPMS and of IMPD. Other approaches might include a further reduction of CS activity by use of a weaker promoter than the L1 version, an increase of the IPMS$^{EC\text{-}G462D}$ activity or the optimization of process parameters, such as substrate feeding, pH, or oxygen control.

Acknowledgments We thank J. Marienhagen, J. van Ooyen, and L. Eggeling (all three IBG-1, Research Center jülich) for providing plasmids pJC4*ilvBNCD*, pK19mobsacBΔilvE, pK19ΔPgltA_B-L1, pK19mobsacBΔprpC1, and pK19mobsacBΔprpC1. The support of the State Baden Württemberg (MINT programme, grant 31-655.056-1/145) is gratefully acknowledged.

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