

# Effect of pyruvate dehydrogenase complex deficiency on L-lysine production with *Corynebacterium glutamicum*

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**Abstract** Intracellular precursor supply is a critical factor for amino acid productivity of *Corynebacterium glutamicum*. To test for the effect of improved pyruvate availability on L-lysine production, we deleted the *aceE* gene encoding the E1p enzyme of the pyruvate dehydrogenase complex (PDHC) in the L-lysine-producer *C. glutamicum* DM1729 and characterised the resulting strain DM1729-BB1 for growth and L-lysine production. Compared to the host strain, *C. glutamicum* DM1729-BB1 showed no PDHC activity, was acetate auxotrophic and, after complete consumption of the available carbon sources glucose and acetate, showed a more than 50% lower substrate-specific biomass yield (0.14 vs 0.33 mol C/mol C), an about fourfold higher biomass-specific L-lysine yield (5.27 vs 1.23 mmol/g cell dry weight) and a more than 40% higher substrate-specific L-lysine yield (0.13 vs 0.09 mol C/mol C). Overexpression of the pyruvate carboxylase or diaminopimelate dehydrogenase genes in *C. glutamicum* DM1729-BB1 resulted in a further increase in the biomass-specific L-lysine yield by 6 and 56%, respectively. In addition to L-lysine, significant amounts of pyruvate,

L-alanine and L-valine were produced by *C. glutamicum* DM1729-BB1 and its derivatives, suggesting a surplus of precursor availability and a further potential to improve L-lysine production by engineering the L-lysine biosynthetic pathway.

**Keywords** *Corynebacterium glutamicum* · L-lysine · Pyruvate dehydrogenase complex · L-lysine production

## Introduction

*Corynebacterium glutamicum* is an aerobic, Gram-positive organism that grows on a variety of sugars and organic acids. The organism is used for the fermentative production of amino acids, such as L-glutamate and L-lysine (Kelle et al. 2005; Kimura 2005; Leuchtenberger et al. 2005), recently amounting to more than 1,500,000 and 750,000 t/a, respectively (Shimizu and Hirasawa 2007; Wittmann and Becker 2007). These impressive production numbers became possible because of tremendous advances in fermentation technology on the one side and in sophisticated strain improvement on the other (Sahm et al. 2000; de Graaf et al. 2001; Pfefferle et al. 2003; Ikeda 2003; Kelle et al. 2005). However, due to the growing world market for amino acids (Leuchtenberger et al. 2005), there is an increasing interest in further strain development and optimisation of efficient fermentative L-lysine production processes.

As shown in Fig. 1, *C. glutamicum* synthesises L-lysine in a split pathway from oxaloacetate via L-aspartate, L-aspartate semialdehyde and D, L-diaminopimelate as the main intermediates (Schrumpf et al. 1991). At high ammonium concentrations (and thus, under L-lysine production conditions), the organism uses the diaminopimelate dehydrogenase (DDH in Fig. 1) variant of the pathway, whereas the succinyl transferase (in Fig. 1) variant is only

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This study is dedicated to Prof. Dr. Hermann Sahm on the occasion of his 65th birthday.

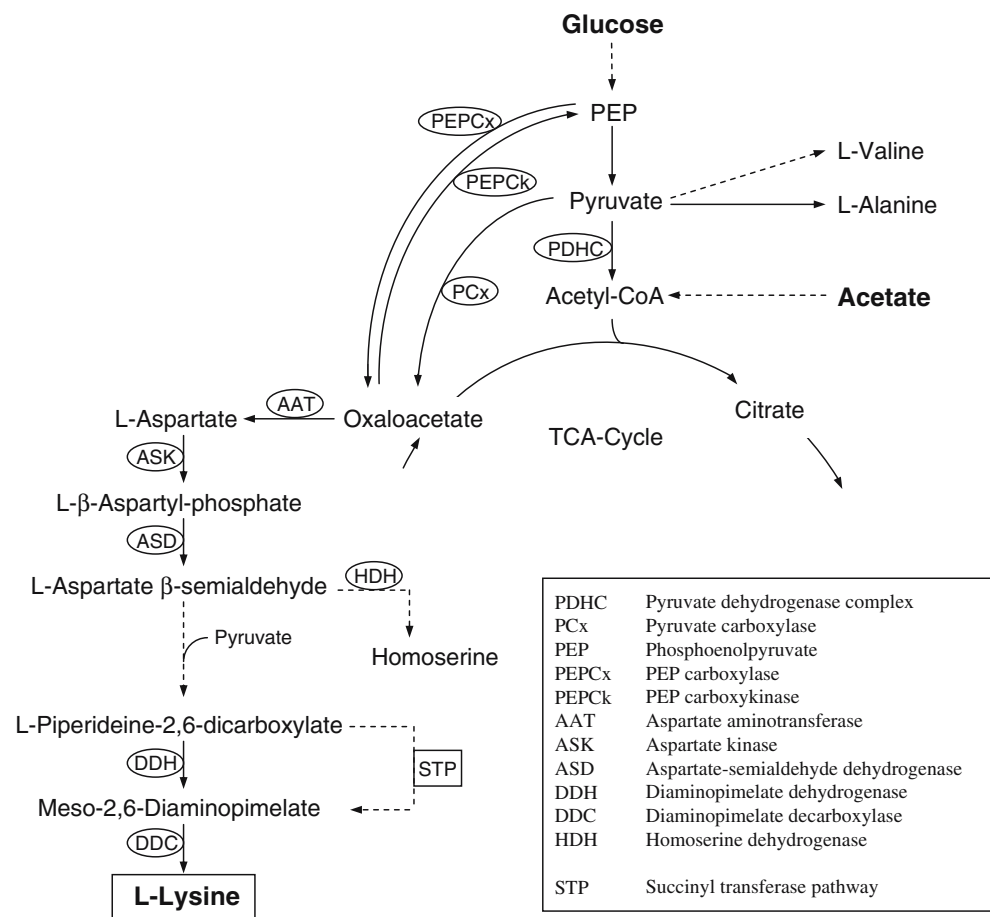
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**Fig. 1** The PEP–pyruvate–oxaloacetate node in *C. glutamicum* and the biosynthetic pathway of L-lysine via the diaminopimelate dehydrogenase (DDH)



operative at low ammonium concentrations (Sonntag et al. 1993; reviewed in Sahn et al. 2000). Four moles of reduced form nicotinamide adenine dinucleotide phosphate (NADPH) are required for the synthesis of 1 mol L-lysine from oxaloacetate. Key enzymes of the L-lysine biosynthetic pathway in *C. glutamicum* are aspartate kinase (ASK), which in the wild-type background of *C. glutamicum*, shows concerted feedback inhibition by L-lysine and L-threonine (Shiio and Miyajima 1969; Nakayama et al. 1966; Kalinowski et al. 1991), and dihydrodipicolinate synthase (DPS), which condenses pyruvate with L-aspartate semialdehyde and competes with homoserine dehydrogenase for this latter substrate (Cremer et al. 1988, 1991). Overexpression of the respective genes, *lysC* and *dapA*, and especially, (over)expression of *lysC* alleles coding for feedback-resistant aspartate kinases (ASKs<sup>FBR</sup>) improved L-lysine production significantly (Eggeling et al. 1998; Cremer et al. 1991; Schruppf et al. 1992). Furthermore, the limitation of the intracellular C flux towards L-threonine (Ohnishi et al. 2002) and the augmentation of the NADPH supply led to an improvement of L-lysine production. The latter has been accomplished by expression of the membrane-bound transhydrogenase genes from *Escherichia coli* in *C. glutamicum* (Kabus et al. 2007) and by redirection of

the C flux from glycolysis to the pentose phosphate pathway by disruption of the phosphoglucose isomerase gene *pgi* (Marx et al. 2003) or by introduction of a mutant allele encoding a feedback-resistant 6-phosphogluconate dehydrogenase (Ohnishi et al. 2005).

Aside from engineering the biosynthetic L-lysine pathway starting from oxaloacetate and aside from improving NADPH regeneration, optimisation of precursor availability has been found to be crucial for efficient production of L-lysine. Both oxaloacetate and pyruvate are central metabolic precursors for L-lysine formation (see Fig. 1), and their supply should be balanced for optimal L-lysine production. First indications for a positive effect of improved oxaloacetate and pyruvate supply on L-lysine production can be deduced from the generation and analysis of undefined L-lysine-producing mutants of *Brevibacterium flavum* with low citrate synthase activity or low pyruvate dehydrogenase complex (PDHC) activity (Shiio et al. 1982, 1984). Menkel et al. (1989) showed that addition of fumarate to the growth medium and thus increasing the oxaloacetate availability led to a higher L-lysine yield with a producer strain. Later, it has been shown that increasing the carbon (C) flux from pyruvate to oxaloacetate by either overexpression of the pyruvate carboxylase (PCx) gene

(*pyc*) or inactivation of the phosphoenolpyruvate (PEP) carboxykinase gene (*pck*) is highly beneficial for the production of L-lysine by *C. glutamicum* (Peters-Wendisch et al. 2001; Riedel et al. 2001; Petersen et al. 2001). In accordance with these results, Ohnishi et al. (2002) obtained a significant increase in L-lysine accumulation when they introduced a *pyc* allele (*pyc*<sup>P458S</sup>), probably encoding a deregulated enzyme, into a L-lysine-producing *C. glutamicum* strain. These findings made it conceivable that improvement of the availability of the precursor pyruvate might also prove beneficial for efficient production of L-lysine and, therefore, decreasing or abolishing the PDHC activity by genetic engineering represented an attractive approach for further optimisation of L-lysine production by *C. glutamicum*.

Recently, we identified and functionally characterised the E1p subunit of the PDHC in *C. glutamicum* and showed that activity of this complex is essential for growth of this organism on glucose, unless supplemented with acetate (Schreiner et al. 2005). Moreover, we engineered *C. glutamicum* for the production of L-valine from glucose by inactivation of the PDHC and additional overexpression of genes encoding the L-valine biosynthetic enzymes

(Blombach et al. 2007). In the present study, we investigate the effect of PDHC inactivation in the genetically defined lysine-producer *C. glutamicum* DM1729 on growth and L-lysine formation.

## Materials and methods

### Bacterial strains, plasmids, oligonucleotides and culture conditions

All bacterial strains and plasmids and their relevant characteristics and sources, and the oligonucleotides used in this study are listed in Table 1.

*E. coli* was grown aerobically in a 2xTY complex medium (Sambrook et al. 2001) at 37°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Pre-cultures of the different *C. glutamicum* strains were grown in 3.7% (w/v) brain heart infusion (BHI) medium (Merck) containing 0.5% (w/v) acetate. For growth and amino acid fermentations in shake flasks, the cells of an overnight pre-culture were washed with 0.9% (w/v) NaCl and inoculated into CGXII minimal medium (Eikmanns et

**Table 1** Strains, plasmids and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Relevant characteristic(s) or sequence	Source/reference or purpose
<b>Strains</b>		
<i>E. coli</i> DH5 $\alpha$	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan 1985
<i>C. glutamicum</i> WT	WT strain ATCC13032, biotin-auxotrophic	American Type Culture Collection
<i>C. glutamicum</i> DM1729	<i>pyc</i> <sup>P458S</sup> ; <i>hom</i> <sup>V59A</sup> ; <i>lysC</i> <sup>T311I</sup> , derived from <i>C. glutamicum</i> WT	B. Bathe, Degussa AG; Georgi et al. 2005
<i>C. glutamicum</i> DM1729-BB1	strain DM1729 with deletion of <i>aceE</i> encoding the E1p enzyme of the pyruvate dehydrogenase complex	This work
<b>Plasmids</b>		
pK19mutaceE	pK19 <i>mobsacB</i> carrying a 1,155-bp insert with a truncated <i>aceE</i> gene, shortened by 2,077 bp	Schreiner et al. 2005
pVWEx1- <i>pyc</i>	Overexpression of the <i>pyc</i> gene encoding the pyruvate carboxylase	Peters-Wendisch et al. 2001
pJC33	Overexpression of the <i>lysC</i> <sup>S381F</sup> gene encoding the feedback-resistant aspartate kinase	Cremer et al. 1991
pJC40	Overexpression of the <i>ddh</i> gene encoding the diaminopimelate dehydrogenase	Cremer et al. 1991
pJC50	Overexpression of the <i>lysC</i> <sup>S381F</sup> and the <i>dapA</i> genes encoding the feedback-resistant aspartate kinase and the dihydrodipicolinate synthase	Cremer et al. 1991
<b>Oligonucleotides</b>		
delaceE1	5'-ACGCGTCGACCACCAAAAAG GACATCAGACC-3'	Primer to verify deletion of <i>aceE</i> (Schreiner et al. 2005)
delaceE3	5'-GGATAGGTGATTGGAAGTTGGGC AAACGAA GCATGAGGTAACG-3'	Primer to verify deletion of <i>aceE</i> (Schreiner et al. 2005)
delaceE5.3	5'-CGCGGATCCGCGGGATTATCTGTCCC-3'	Primer to verify deletion of <i>aceE</i> (Schreiner et al. 2005)

al. 1991) to give an initial optical density at 600 nm ( $OD_{600}$ ) of about 1. The medium contained 111 and 222 mM glucose [i.e. 2 or 4% (w/v), respectively] and 170 mM potassium acetate [corresponds to 1% (w/v) acetate]. When indicated in “Results”, 0.5% (w/v) BHI powder (Merck) was added to the medium. The plasmid-carrying strains were grown in the presence of kanamycin ( $50 \mu\text{g ml}^{-1}$ ). *C. glutamicum* was grown aerobically at  $30^\circ\text{C}$  as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. The batch fermentations were performed at  $30^\circ\text{C}$  as 250-ml cultures in a Fedbatch-fermentation system from DASGIP (Jülich, Germany). The fermentation conditions for aeration and pH control were described in Blombach et al. (2007).

#### DNA preparation and transformation

The isolation of plasmids from *E. coli* and *C. glutamicum* was performed as described before (Eikmanns et al. 1994). Plasmid transfer into *C. glutamicum* was carried out by electroporation, recombinant strains were selected on Luria-Bertani/BHI supplemented broth agar plates containing kanamycin ( $50 \mu\text{g ml}^{-1}$ ; van der Rest et al. 1999).

#### Construction of *C. glutamicum* DM1729-BB1

Inactivation of the chromosomal *aceE* gene in the defined L-lysine producer *C. glutamicum* DM1729 was performed as described previously for *aceE* inactivation of *C. glutamicum* WT (Schreiner et al. 2005). The deletion at the chromosomal locus was verified by polymerase chain reaction (PCR) using primers delaceE1/delaceE5.3 and by Southern blot analysis. For the latter, an *aceE*-specific 580-bp DNA probe was generated from chromosomal DNA of *C. glutamicum* WT by PCR with the primers delaceE1 and delaceE3 and hybridised to *SalI*-restricted and size-fractionated chromosomal DNA from *C. glutamicum* DM1729 and the  $\Delta aceE$ -derivative DM1729-BB1. Labelling, hybridisation, washing and detection were conducted using the nonradioactive DNA labelling and detection kit and the instructions from Roche Diagnostics (Penzberg, Germany). As expected, the hybridisation resulted in a signal of 8.3 kb with DNA from strain DM1729 and in a signal of 6.2 kb in the *C. glutamicum* DM1729-BB1.

#### Determination of PDHC enzyme activity

To determine the specific PDHC activities in cell extracts, *C. glutamicum* DM1729 and DM1729-BB1 were grown in BHI medium containing 1% (w/v) glucose and 1% (w/v) acetate and harvested in the exponential growth phase ( $OD_{600}$  of about 7), washed twice in 100 mM Tris-HCl, pH 7.2, 3 mM L-cysteine, 10 mM  $\text{MgCl}_2$  and resuspended

in 0.5 ml of the same buffer. Cell disruption was performed as described by Schreiner et al. (2005) with a RiboLysert<sup>TM</sup> at  $4^\circ\text{C}$ . The protein concentrations of the cell extracts were determined by the Bradford method using bovine serum albumin as standard.

PDHC enzyme activities were determined photometrically according to the method described by Guest and Creaghan (1974). One unit of activity is defined as 1  $\mu\text{mol}$  reduced form nicotinamide adenine dinucleotide formed per minute at  $30^\circ\text{C}$ .

#### Analytatics

For quantification of substrates and products, 1-ml samples were taken from the cultures, centrifuged at  $13,600\times g$  (10 min), and the supernatant was used for determination of amino acid, glucose and/or organic acid concentrations in the culture fluid. The amino acid concentrations were determined by reversed-phase high-pressure liquid chromatography as described before (Blombach et al. 2007). Glucose, acetate and L-lactate concentrations were determined by enzymatic tests from Roche Diagnostics. The pyruvate concentrations were determined enzymatically according to Bergmeyer (1983).

Online analysis of the oxygen and carbon dioxide content of the exhaust gas was performed using the GA4 gas analyser from DASGIP (Jülich, Germany).

The carbon evolution rate [CER; given in  $\text{mol}/(\text{l}\times\text{h})$ ] was determined by the following equation:

$$\text{CER} = \frac{v_{g,\text{in}}}{V_R V_n} \left( Y_{\text{CO}_2}^{\text{out}} \frac{(1 - Y_{\text{O}_2}^{\text{in}} - Y_{\text{CO}_2}^{\text{in}})}{(1 - Y_{\text{O}_2}^{\text{out}} - Y_{\text{CO}_2}^{\text{out}})} - Y_{\text{CO}_2}^{\text{in}} \right)$$

$V_N$ , the mol volume of the ideal gas (l/mol) at standard conditions;  $V_R$ , the working volume of the bioreactor (l);  $v_{g,\text{in}}$ , the volumetric inlet air flow (l/h) at standard conditions; and  $Y_{\text{O}_2}^{\text{in}}$ ,  $Y_{\text{CO}_2}^{\text{in}}$  and  $Y_{\text{O}_2}^{\text{out}}$ ,  $Y_{\text{CO}_2}^{\text{out}}$ , the molecular fractions of oxygen and carbon dioxide in the inlet and outlet air, respectively.

#### Determination of the ratio CDW per $OD_{600}$

For determination of the cell dry weight (CDW) per  $OD_{600}$ , cells of a BHI pre-culture of *C. glutamicum* DM1729 and DM1729-BB1 were washed with 0.9% (w/v) NaCl and inoculated into CGXII medium containing 4% (w/v) glucose, 1% (w/v) acetate and 0.5% (w/v) BHI powder to give an initial  $OD_{600}$  of about 1. Cells of both strains were harvested by centrifugation (5,000 rpm,  $4^\circ\text{C}$ , 20 min) in the exponential phase ( $OD_{600}$  of about 9) and in the stationary phase ( $OD_{600}$  for DM1729 of about 60 and for DM1729-BB1 of about 14). After two washing steps with water, the

cells were dried for 24 h at 105°C, and the CDW was determined. For both DM1729 and DM1729-BB1, an  $OD_{600}$  of 1 corresponded to 0.3 g CDW  $\Gamma^{-1}$ . According to Liebl (2005), the C content of the *C. glutamicum* CDW amounts to 41% (w/w).

#### Substrate-specific biomass and product yields in the parallel fermentation system

For analysis of the substrate-specific biomass and product yields, *C. glutamicum* DM1729 and DM1729-BB1 were grown in CGXII medium containing 4% (w/v) glucose, 1% (w/v) acetate and 0.5% (w/v) BHI. The yields were calculated by relating the total initial C content of the culture (i.e. C in initial CDW, glucose and acetate) to the total C recovered in CDW, amino acids and organic acids after complete consumption of all C sources. Before calculating the final biomass and product yields, we subtracted (from the recovered C) the amount of biomass and product C derived from BHI, amounting to 1.7 and 0.5% of the total recovered C for *C. glutamicum* DM1729 and DM1729-BB1, respectively. The final biomass and product yields are given as mol C/mol C.

## Results

### Construction and analysis of a PDHC-deficient derivative of *C. glutamicum* DM1729

To study the influence of inactivation of the PDHC on L-lysine production, we deleted the *aceE* gene encoding the E1p subunit of the PDHC in the lysine-producer *C. glutamicum* DM1729, resulting in *C. glutamicum* DM1729-BB1. The parental strain DM1729 originates from *C. glutamicum* WT and has undergone three allelic exchanges (*pyc*<sup>P458S</sup>; *hom*<sup>V59A</sup>; *lysC*<sup>T311I</sup>), which previously have been described to transform *C. glutamicum* WT to a L-lysine producer (Ohnishi et al. 2002; Georgi et al. 2005). Successful deletion of *aceE* in *C. glutamicum* DM1729-BB1 was verified (see “Materials and methods”), and the parental strain *C. glutamicum* DM1729 and the deletion mutant DM1729-BB1 were tested for PDHC activity and growth.

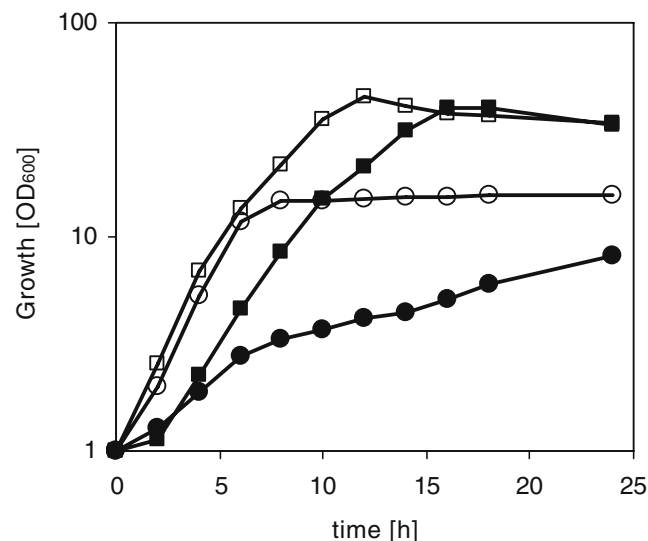
PDHC activities were determined in extracts of cells grown in BHI medium containing glucose and acetate. *C. glutamicum* DM1729 showed specific PDHC activities of  $64 \pm 6$  mU mg protein<sup>-1</sup>, whereas *C. glutamicum* DM1729-BB1 mutant was devoid of any detectable PDHC activity ( $<1$  mU mg protein<sup>-1</sup>). These results show that deletion of the *aceE* gene in *C. glutamicum* DM1729 led to complete inactivation of the PDHC.

For comparative analysis of the growth of *C. glutamicum* DM1729 and DM1729-BB1, shake flask cultivations were

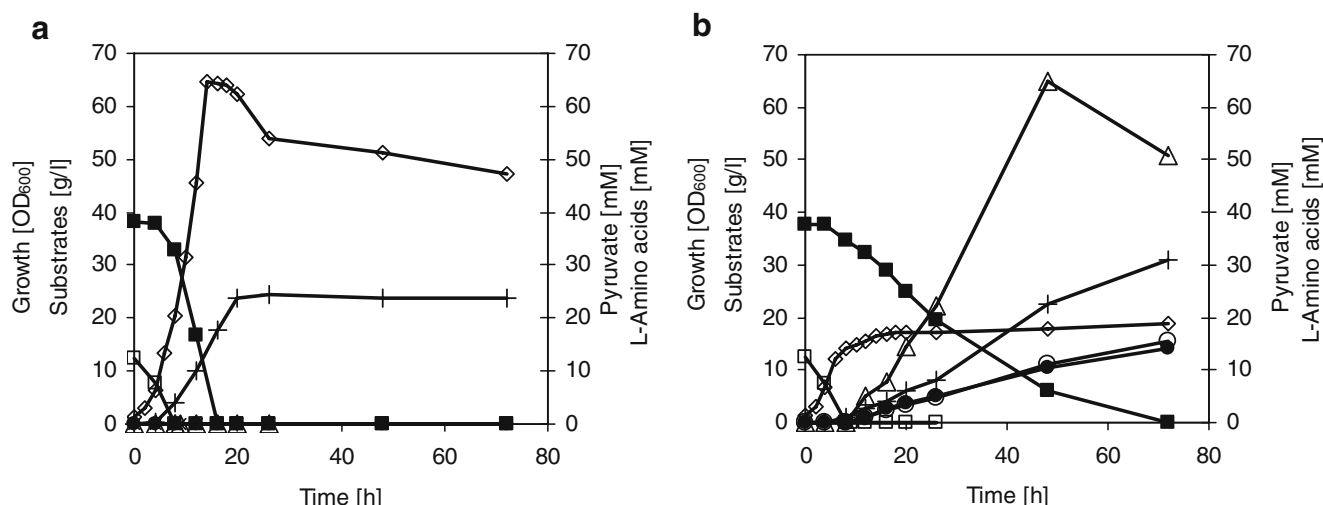
performed with minimal medium containing different substrates. *C. glutamicum* DM1729-BB1 was unable to grow in minimal medium containing glucose, unless supplemented with acetate (data not shown). In minimal medium containing 2% (w/v) glucose plus 1% (w/v) acetate, *C. glutamicum* DM1729 had a short lag-phase and then grew exponentially with a maximal growth rate of  $0.34$  h<sup>-1</sup> to an  $OD_{600}$  of 34 (10.2 g CDW/l) within 16 h. *C. glutamicum* DM1729-BB1 showed a maximal growth rate of  $0.19$  h<sup>-1</sup> and an  $OD_{600}$  of about 8 (2.4 g CDW/l) after 24 h (Fig. 2). When the medium containing glucose and acetate additionally was supplemented with 0.5% (w/v) of BHI powder, both *C. glutamicum* DM1729 and DM1729-BB1 showed no lag-phase and grew exponentially with growth rates of  $0.44$  h<sup>-1</sup> to maximal  $OD_{600}$  of about 36 (10.8 g CDW/l) and 16 (4.8 g CDW/l), respectively (Fig. 2).

### Fermentation pattern and yields of *C. glutamicum* DM1729 and DM1729-BB1

For characterisation of the product pattern of *C. glutamicum* DM1729 and DM1729-BB1, shake flask fermentations in minimal medium containing 4% (w/v) glucose, 1% (w/v) acetate and 0.5% (w/v) BHI were performed, and growth, glucose and acetate utilisation and pyruvate and amino acid accumulation were monitored in the course of the experiment. *C. glutamicum* DM1729 grew exponentially within 20 h to an  $OD_{600}$  of about 62 (18.6 g CDW/l), metabolised glucose and acetate simultaneously and produced 23 mM L-lysine with a resulting biomass-specific yield ( $Y_{P/X}$ ) of 1.23 mmol/(g CDW) (Fig. 3a and Table 2). Apart from



**Fig. 2** Growth of *C. glutamicum* DM1729 and DM1729-BB1 in minimal medium containing 2% glucose and 1% acetate (filled square DM1729; filled circle DM1729-BB1) or 2% glucose, 1% acetate and 0.5% BHI (open square DM1729; open circle DM1729-BB1)



**Fig. 3** Growth, substrate consumption and product (pyruvate and L-amino acid) accumulation during a representative shake flask batch cultivation of **a** *C. glutamicum* DM1729 and **b** DM1729-BB1 in minimal medium initially containing glucose (4%), acetate (1%) and

BHI (0.5%). *Diamond* growth; *filled square* glucose; *open square* acetate; *triangle* pyruvate; *open circle* L-alanine; *filled circle* L-valine; *plus sign* L-lysine. Three independent fermentations were performed, all three showing comparable results

L-lysine, no other amino acids and no acetate, pyruvate or L-lactate were observed in the culture fluid of *C. glutamicum* DM1729. The PDHC-deficient mutant DM1729-BB1 also showed exponential growth in the first 6 to 8 h of fermentation to an OD<sub>600</sub> of about 14 (4.2 g CDW/l) and metabolised glucose and acetate simultaneously (Fig. 3b). With depletion of acetate, *C. glutamicum* DM1729-BB1 stopped growth but continued to metabolise glucose and to form amino acids and pyruvate. After complete consumption of glucose, *C. glutamicum* DM1729-BB1 reached a final OD<sub>600</sub> of about 19 (5.7 g CDW/l) and produced 30 mM L-lysine with a  $Y_{P/X}$  of 5.27 mmol/(g CDW) (Fig. 3b and Table 2). In addition to L-lysine, *C. glutamicum* DM1729-BB1 excreted about 14 mM L-valine, 16 mM L-alanine and up to 65 mM pyruvate into the culture broth (Fig. 3b).

We also studied the fermentation patterns, the biomass-specific L-lysine yields ( $Y_{P/X}$ ), the substrate-specific biomass yields ( $Y_{X/S}$ ) and product yields ( $Y_{P/S}$ ) of *C. glutamicum* DM1729 and DM1729-BB1 in minimal medium containing glucose, acetate and BHI in a controlled parallel fermentation system providing a constant pH of 7.0 and a constant oxygen concentration of 30% saturation. Both strains started growth with nearly identical growth rates of 0.4 h<sup>-1</sup> and reached ODS<sub>600</sub> of about 51 (15.3 g CDW/l; DM1729) and 16 (4.8 g CDW/l; DM1729-BB1) after 17 and 8 h, respectively. After depletion of acetate in the medium (at  $t=8$  h), *C. glutamicum* DM1729-BB1 but not *C. glutamicum* DM1729 showed a severe drop in the CER from about 41 to 7.5 mmol/(l×h). L-Lysine production continued until both acetate and glucose were exhausted and the final L-lysine concentrations in the *C. glutamicum*

**Table 2** Final optical densities, L-lysine and pyruvate concentrations and biomass-specific L-lysine yields ( $Y_{P/X}$ ) of *C. glutamicum* DM1729 and recombinant derivatives grown in shake flasks in minimal medium

<i>C. glutamicum</i> strain	Plasmid-bound overproduced enzyme(s)	OD <sub>600</sub>	L-Lysine <sup>a</sup> (mM)	Pyruvate (mM)	$Y_{P/X}$ [mmol L-lysine/(g CDW)]
DM1729		62	23	<1	1.23
DM1729-BB1		19	30	65	5.27
DM1729-BB1 (pVWEx1-pyc)	PCx	21	35	55	5.57
DM1729-BB1 (pJC33)	ASK <sup>FBR</sup>	20	7	80	1.17
DM1729-BB1 (pJC40)	DDH	19	47	55	8.23
DM1729-BB1 (pJC50)	ASK <sup>FBR</sup> , DPS	14	11	110	2.63

<sup>a</sup> Values are means from at least two independent cultivations. Standard deviations were in all experiments below 5%.

PCx Pyruvate carboxylase; ASK<sup>FBR</sup> feedback-resistant aspartate kinase; DDH diaminopimelate dehydrogenase; DPS dihydrodipicolinate synthase; CDW cell dry weight

**Table 3** Substrate-specific biomass and product yields and productivities of *C. glutamicum* DM1729 and DM1729-BB1 grown in batch culture in minimal medium containing 4% glucose, 1% acetate and 0.5% BHI

<i>C. glutamicum</i> strain	Yield (mol C/mol C) <sup>a</sup>					Productivity [mmol L-lysine/ (g CDW×h)]
	CDW	L-Lysine	L-Valine	L-Alanine	Pyruvate	
DM1729	0.33	0.09	<0.001	<0.001	<0.001	0.100
DM1729-BB1	0.14	0.13	0.08	0.06	0.03	0.133

<sup>a</sup>CDW and product concentrations were determined after complete consumption of glucose and acetate. The molar yields are given as mol C in biomass or mol C in the products per mol C in the substrates (glucose and acetate) consumed. Values are means from three independent cultivations; the standard deviations were in all experiments below 10%.

CDW cell dry weight

DM1729 and DM1729-BB1 cultures were 26 and 36 mM, respectively, with a  $Y_{P/X}$  of 1.7 and 6.3 mmol/g CDW. Additionally and as observed before in the shake flasks, *C. glutamicum* DM1729-BB1 excreted significant amounts of L-valine, L-alanine and pyruvate. As shown in Table 3, the L-lysine  $Y_{X/S}$  of *C. glutamicum* DM1729-BB1 was more than twofold lower, whereas all  $Y_{P/SS}$  (given as mol C/mol C) and the L-lysine productivity [mmol L-lysine/(g CDW×h)] were significantly higher than those of *C. glutamicum* DM1729.

L-Lysine production with *C. glutamicum* DM1729 and DM1729-BB1 derivatives overexpressing L-lysine biosynthetic genes

As shown above, *C. glutamicum* DM1729-BB1 excreted aside from L-lysine also pyruvate, L-alanine and L-valine into the medium. This observation prompted us to try to direct the C flux more efficiently towards L-lysine by additional overexpression of the PCx gene and of L-lysine biosynthetic genes. For this purpose, we transformed *C. glutamicum* DM1729-BB1 with plasmids pVWEx1-pyc, pJC33, pJC40 and pJC50. These plasmids carry the genes encoding PCx, ASK<sup>FBR</sup>, DDH and ASK<sup>FBR</sup> plus DPS, respectively (see Fig. 1). L-Lysine shake flask fermentations with the recombinant *C. glutamicum* DM1729-BB1 strains were carried out, and Table 2 shows the final ODS<sub>600</sub>, the extracellular L-lysine and the pyruvate concentrations after complete consumption of glucose and acetate and the overall L-lysine yields ( $Y_{P/X}$ ). Independent of the plasmid, all *C. glutamicum* DM1729-BB1 derivatives showed about the same or slightly lower final ODS<sub>600</sub> when compared to the parental strain. The *C. glutamicum* DM1729-BB1 strains with plasmids pJC33 and pJC50, both carrying the lys<sup>C<sup>FBR</sup></sup> gene encoding the ASK<sup>FBR</sup>, showed severely reduced L-lysine and severely enhanced pyruvate accumulation when compared to the host strain. In contrast to the former two DM1729-BB1 derivatives, the *C. glutamicum* DM1729-BB1 strains with plasmids pVWEx1-pyc and pJC40 produced significantly more L-lysine (17 and 57%,

respectively) and less pyruvate (15% both) than the plasmid-free *C. glutamicum* DM1729-BB1. However, the two strains still excreted about 55 mM pyruvate and 15 to 20 mM L-valine and L-alanine into the medium.

## Discussion

As previously described for the *aceE* deletion derivative of *C. glutamicum* WT (Schreiner et al. 2005) and in contrast to the parental strain DM1729, *C. glutamicum* DM1729-BB1 showed no PDHC activity and was unable to grow in minimal medium containing glucose as sole C and energy source. In minimal medium containing glucose plus acetate, *C. glutamicum* DM1729-BB1 showed slower growth and a lower final OD<sub>600</sub> than the parental strain DM1729. Slower growth on glucose and acetate was not observed with *C. glutamicum* WT  $\Delta aceE$  when compared to the WT strain (Schreiner et al. 2005), and therefore, the *aceE* deletion obviously is unfavourable for the growth when combined with the allelic exchanges introduced into *C. glutamicum* DM1729. It might well be that the acetylcoenzyme A (acetyl-CoA) provision from acetate (via acetate kinase and phosphotransacetylase, see Fig. 1) together with an oxaloacetate drain off towards L-lysine limits the citrate synthase reaction and, in consequence, limits the synthesis of amino acids derived from glutamate. In favour of this hypothesis, we could partly relieve the slower growth of *C. glutamicum* DM1729-BB1 by adding 3 mM of glutamate to the medium (data not shown). However, although the slower growth rate could be completely relieved by addition of small amounts of BHI, the differences in the final ODS<sub>600</sub> of *C. glutamicum* DM1729 and DM1729-BB1 remained. The stop of growth and, thus, the lower final biomass of strain DM1729-BB1 at a given substrate (glucose+acetate) concentration can be attributed to the exhaustion of acetate. Upon depletion of acetate in the growth medium, acetyl-CoA formation within the PDHC-negative cells drops down, and therefore, biosynthetic pathways starting from acetyl-CoA (e.g. fatty acid biosynthesis) and also the TCA

cycle become almost completely inactive. The latter conclusion is indicated by the observations that CO<sub>2</sub> formation and O<sub>2</sub> uptake rates of the *C. glutamicum* DM1729-BB1 culture (but not those of the *C. glutamicum* DM1729 culture) severely dropped down after consumption of acetate.

Shiio et al. (1984) already showed a positive effect of low PDHC activity on the performance of a L-lysine-producing *B. flavum* mutant. However, this mutant was obtained by several rounds of random mutagenesis, and the conclusion concerning a direct relation between PDHC activity and L-lysine production was questionable. Our results of the comparative shake flask and fermenter experiments with *C. glutamicum* DM1729 and its PDHC-deficient derivative DM1729-BB1 demonstrate that inactivation of PDHC in this strain led to improved L-lysine production. After complete consumption of the C sources, the final L-lysine titers, the  $Y_{P/X}$  and the  $Y_{P/S}$  values of *C. glutamicum* DM1729-BB1 were increased by 30 to 40%, by 200 to 300% and by about 40%, respectively, when compared to *C. glutamicum* DM1729. It should be noted, however, that  $Y_{P/X}$  and  $Y_{P/S}$  in both strains were very similar ( $\pm 10\%$ ) in the first 8 h of the fermentations, i.e. as long as the media contained acetate. The higher overall L-lysine yields were due to the fact that after depletion of acetate the PDHC-deficient mutant stopped to form biomass and to oxidise the substrate to CO<sub>2</sub>, but continued to produce L-lysine as long as glucose was available. In contrast and as indicated by the constant growth and the CERs, the parental strain DM1729 used a significant proportion of the available substrate for biomass formation and respiration. As shown recently for L-valine production with *C. glutamicum* WT  $\Delta aceE$  (Blombach et al. 2007), it can be expected that increasing the initial glucose concentration and/or fed-batch cultivation of *C. glutamicum* DM1729-BB1 further improves L-lysine production.

In addition to L-lysine, *C. glutamicum* DM1729-BB1 excreted pyruvate, L-alanine and L-valine (the latter two derived from pyruvate) into the culture broth, indicating that L-lysine production in this strain is limited by the reactions from pyruvate to L-lysine and not by pyruvate availability. This hypothesis is corroborated by our results obtained with *C. glutamicum* DM1729-BB1 carrying the PCx and the DDH genes on plasmid. Both strains produced more L-lysine and less pyruvate; however, they still excreted pyruvate into the medium. This result shows that, even in the strains with enhanced C flux towards L-lysine, there is still a surplus of pyruvate, which in principle should be available for L-lysine production. However, the results also show that, in *C. glutamicum* DM1729-BB1, the oxaloacetate supply on the one and the DDH reaction on the other hand are bottlenecks for L-lysine-overproduction. Whereas overexpression of the PCx gene and thus

improvement of oxaloacetate supply has previously been shown to be advantageous for L-lysine production by an undefined L-lysine-producing *C. glutamicum* strain (DG52-5; Peters-Wendisch et al. 2001; Petersen et al. 2001), a positive effect of the overexpression of the DDH gene on L-lysine production has not been observed in other L-lysine producers so far (Cremer et al. 1991). The improvement of L-lysine production by *ddh* overexpression might be restricted to the  $\Delta aceE$  background, as a *C. glutamicum* DM1729 derivative carrying pJC40 showed the same accumulation of L-lysine (and pyruvate) as the host strain DM1729 (data not shown). However, the results of the fermentations with plasmid-carrying derivatives of *C. glutamicum* DM1729-BB1 indicate that a balanced supply of pyruvate as well as of oxaloacetate is important for L-lysine production and they indicate a further potential to optimise L-lysine production by engineering the L-lysine biosynthetic pathway from oxaloacetate and pyruvate as precursors.

Surprisingly, the *C. glutamicum* DM1729-BB1 strains with plasmids carrying the gene encoding the ASK<sup>FBR</sup> showed lower L-lysine and higher pyruvate accumulation than the plasmid-free host strain. As elevation of ASK<sup>FBR</sup> in *C. glutamicum* DM1729 (data not shown) and also in other L-lysine-producing strains led to improvement of L-lysine production (see “Introduction”), the shift in the product spectrum was unexpected and can only be explained by, so far, unknown regulatory devices.

Further approaches to improve L-lysine production with *C. glutamicum* DM1729 will aim at the construction of acetate-prototrophic derivatives with low specific PDHC activity and sophisticated sets or combinations of plasmid-bound L-lysine biosynthetic genes. Such defined derivatives of *C. glutamicum* DM1729 should not depend on acetate or BHI for optimal growth, should rapidly grow to high cell concentrations and may prove useful as cost-effective biocatalysts for the production of L-lysine or of pyruvate itself and/or of other products derived from this intermediate.

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