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Metabolic flux engineering of L-lysine production in *Corynebacterium glutamicum*—over expression and modification of G6P dehydrogenase

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Abstract

In the present work, metabolic flux engineering of *Corynebacterium glutamicum* was carried out to increase lysine production. The strategy focused on engineering of the pentose phosphate pathway (PPP) flux by different genetic modifications. Over expression of the *zwf* gene, encoding G6P dehydrogenase, in the feedback-deregulated lysine-producing strain *C. glutamicum* ATCC 13032 lysC^{fbr} resulted in increased lysine production on different carbon sources including the two major industrial sugars, glucose and sucrose. The additional introduction of the A243T mutation into the *zwf* gene and the over expression of fructose 1,6-bisphosphatase resulted in a further successive improvement of lysine production. Hereby the point mutation resulted in higher affinity of G6P dehydrogenase towards NADP and reduced sensitivity against inhibition by ATP, PEP and FBP. Overall, the lysine yield increased up to 70% through the combination of the different genetic modifications. Through strain engineering formation of trehalose was reduced by up to 70% due to reduced availability of its precursor G6P. Metabolic flux analysis revealed a 15% increase of PPP flux in response to over expression of the *zwf* gene. Overall a strong apparent NADPH excess resulted. Redox balancing indicated that this excess is completely oxidized by malic enzyme.

Keywords: NADPH; Pentose phosphate pathway; ^{13}C metabolic flux; zwf; G6P dehydrogenase; Redox balance

1. Introduction

Corynebacterium glutamicum is used for industrial production of L-lysine for about 50 years. The lysine market has increased successively to a current annual market volume of about 750,000 tonnes explaining the high interest in superior production strains of *C. glutamicum* (Wittmann and Becker, 2007). During the recent years the classical way of strain optimization by random mutagenesis and selection has been complemented by novel approaches aiming at a rational engineering of the cell. As example genome breeding, based on the introduction of beneficial mutations identified by comparative sequence analysis between wild type and classical producer strains has generated efficient lysine producers (Ohnishi et al., 2002, 2005). Additionally, systems oriented analysis involving, e.g. fluxome, metabolome or transcriptome analysis, has proven useful to gain understanding of the metabolism and to identify promising targets (Becker et al., 2005; Krömer et al., 2004; Wendisch et al., 2006). Especially for the optimization of lysine production in *C. glutamicum* the consideration of the close connection between central metabolism and lysine biosynthetic pathway, has turned out to be crucial. As example, previous metabolic flux studies of different *C. glutamicum* mutants revealed a correlation between lysine production and carbon flux through the pentose phosphate pathway (PPP) (Kiefer et al., 2004; Wittmann and Heinzle, 2002). Hereby, the importance of the PPP arises from the fact that it supplies NADPH required as cofactor in high amounts for the biosynthesis of lysine (Fig. 1). The flux studies suggested an amplification of the PPP flux as promising target to improve lysine formation through increased availability of NADPH (Wittmann and Heinzle, 2002). The potential of this strategy was recently shown by over expression of the *fbp* gene, encoding fructose 1,6-bisphosphatase, in *C. glutamicum* which resulted in a significant increase of PPP flux

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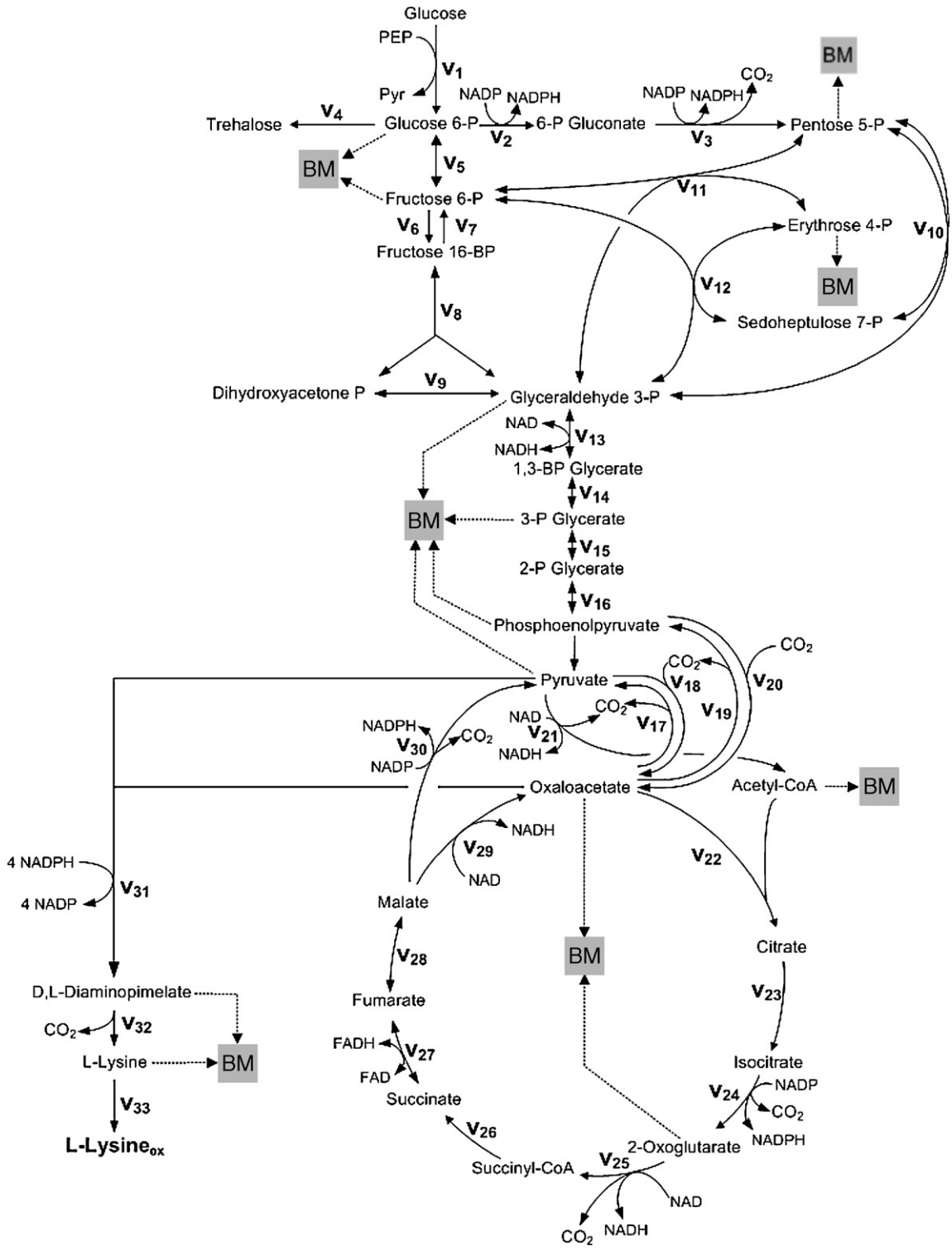

Fig. 1. Pathways of central carbon metabolism and lysine production in *C. glutamicum*. The reactions engineered in the present work comprise the entry step into the PPP, catalyzed by G6P dehydrogenase (v_2), and the gluconeogenetic reaction catalyzed by fructose 1,6-bisphosphate (v_7).

and NADPH formation (Becker et al., 2005). As a consequence, an increase of the lysine yield of up to 40% could be achieved.

In the present work, we embarked on other strategies to enhance the PPP flux in order to increase the production of lysine in *C. glutamicum*. This included the over expression of the *zwf* gene, encoding G6P dehydrogenase. This enzyme displays the key enzyme with regard to control of the PPP flux, but has not been directly engineered to increase lysine production (Moritz et al., 2000). Here, the over expression was realized through genomic replacement of the natural promoter of the *zwf* gene by the strong promoter of the *sod* gene, encoding superoxide dismutase. Moreover, a A243T point mutation

Table 1
Strains of *C. glutamicum* investigated in the present study

Strain	Modification(s)	Reference
Corynebacterium glutamicum ATCC 13032	Wild type	ATCC
C. glutamicum ATCC 13032 lysC^{fbr}	Exchange T311I in the *lysC* gene (NCgl0247)	Becker et al. (2005)
C. glutamicum ATCC 13032 P$_{sod}$zwf	*lysC* T311I + replacement of the natural promoter of the *zwf* gene (NCgl1514) by the promoter of the *sod* gene (NCgl2826)	This work
C. glutamicum ATCC 13032 zwf243	lysC^{fbr} + exchange A243T in the *zwf* gene (NCgl1514)	This work
C. glutamicum ATCC 13032 P$_{sod}$zwf243	P$_{sod}$zwf + exchange A243T in the *zwf* gene (NCgl1514)	This work
C. glutamicum ATCC 13032 P$_{sod}$fbp-zwf243	P$_{sod}$zwf243 + replacement of the natural promoter of the *fbp* gene (NCgl0976) by the promoter of the *sod* gene (NCgl2826)	This work

All genetic modifications were introduced in the genomic DNA by allelic replacement. Strains with feedback-deregulated aspartokinase (lysC^{fbr}) exhibit lysine overproduction.

was introduced into the *zwf* gene. This mutation has been previously identified via comparative sequencing between the wild type and a classically derived production strain and was shown to result in increased lysine production, despite the exact metabolic consequences of this mutation have not been elucidated so far (Ohnishi et al., 2002; Zelder et al., 2005). Subsequently, superior lysine producers were generated by combination of the two mutations and additional implementation of an over expressed fructose 1,6-bisphosphatase gene (Becker et al., 2005). All strains were characterized concerning cell growth and lysine production. The studies were complemented by characterization of the wild type and the mutant G6P dehydrogenase to investigate the consequences of the introduced A243T mutation. Additionally, intracellular metabolite levels and metabolic fluxes through the central carbon metabolism were elucidated, in order to unravel the metabolic consequence of the genetic modifications and understand the overall cellular behavior.

2. Materials and methods

2.1. Microorganisms

In the present work, the wild type *C. glutamicum* ATCC 13032 (American Type and Culture Collection, Manassas, USA) and different lysine producing *C. glutamicum* mutants were investigated (Table 1). The lysine-producing strains were constructed on the basis of *C. glutamicum* ATCC 13032 lysC^{fbr} which carries a T311I mutation in the *lysC* gene (NCgl0247) and encodes a feedback-resistant aspartokinase (Becker et al., 2005). All modifications were integrated in the genomic DNA by allelic replacement as described previously (Becker et al., 2005). The parent strain was modified by replacing the natural promoter upstream of the *zwf* gene, encoding glucose 6-phosphate dehydrogenase (NCgl1514) by the promoter of the *sod* gene, encoding superoxide dismutase (NCgl2826). A further modification included the introduction of a point mutation in the *zwf* gene, resulting in the amino acid exchange A243T. The corresponding nucleotide exchange in the *zwf*-gene results in a loss of the restriction site for *Nco*I. For verification of the nucleotide exchange, the relevant part of the *zwf*-gene was amplified using the primer pair zwf-F and zwf-R (Table 2) and subsequently the PCR-product was digested with *Nco*I. Additionally the *fbp* gene encoding fructose 1,6-bisphosphatase (NCgl0480) was over expressed, by replacing the natural promoter with that of the *sod* gene. The primer sequences used for verification of the promoter exchange upstream of the *zwf*- or *fbp*-gene are listed in Table 2.

2.2. Media

First pre-cultures were grown in complex medium containing 5 g L^{-1} glucose, 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone and 5 g L^{-1} NaCl. Agar plates were prepared by adding 18 g L^{-1} agar. Second pre-cultivations and main cultivations were performed in minimal medium containing 80 mM glucose, 80 mM fructose or 40 mM sucrose, respectively. The minimal medium additionally contained per liter: 0.055 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl, 16 g K_2HPO_4, 2 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 0.5 mg biotin, 1 mg Ca-panthothenic acid, 1 mg thiamine·HCl, 20 mg $FeSO_4 \cdot 7H_2O$, 30 mg 3,4-dihydroxybenzoic acid and 10 mL of a 100× trace element solution (Vallino and Stephanopoulos, 1993). In tracer experiments for metabolic flux analysis, the naturally glucose was replaced by 99% [1-^{13}C] glucose.

2.3. Cultivation

Cultivation was carried out at 30 °C and 230 rpm on a rotary shaker (shaking diameter 5 cm, Multitron, Infors AG, Bottmingen, Switzerland). First pre-cultures (50 mL medium in 500 mL baffled flask) were inoculated with single colonies from agar plates and incubated for 8 h. Cells were harvested by centrifugation (8800 × *g*, 2 min, 4 °C), washed with sterile 0.9% NaCl, and used as inoculum for the second pre-cultivation. This was then

Table 2
Site-specific forward and reverse primer sequences used for verification of the mutant strains of *C. glutamicum* by PCR

Target gene	Primer sequence
P$_{sod}$zwf	Psod-F: 5′-CTACGAAAGGATTTTTTACCC-3′ Zwf-R: 5′-ATCAACGCGTTTGCCCAAATAGTGGTCGATG-3′
P$_{sod}$fbp	Psod -F: 5′-TAGCTGCCAATTATTCCGGG-3′ FBP-R: 5′-GCGAGAATGGAAATTGCGTT-3′
zwf	Zwf-F: 5′-GGCTCGAAAGAAGCTGCTCC-3′ Zwf-R: 5′-GCCATCTTCTTCGCGAAGTCC-3′

carried out in 25 mL minimal medium in 250 mL baffled flasks, containing the carbon source of the subsequent main culture. Main cultures were performed in triplicate (25 mL in 250 mL baffled flasks) using cells from the second pre-culture as inoculum. For metabolic flux analysis, tracer cultivations with 5 mL minimal medium containing 80 mM of [1-^{13}C] glucose as substrate were carried out in 50 mL baffled flasks. Dissolved oxygen in the flasks was monitored via immobilized sensor spots containing a fluorophore with an O_2-dependent luminescent decay time (Wittmann et al., 2003). The dissolved oxygen level was above 20% of saturation during the cultivation, so that sufficient oxygen supply of the cells was ensured. The pH was in the range of 7.0 ± 0.2 during the whole cultivation.

2.4. Respiration analysis

For quantification of the respiratory quotient (RQ), i.e. the ratio between the CO_2 formed to the O_2 consumed, cells were grown in a 250 mL bioreactor (Meredos, Bovenden, Germany) with 100 mL working volume at 30 ± 0.1 °C, pH 7.0 ± 0.1 and 800 rpm. The dissolved oxygen level was above 20% of saturation during the whole cultivation. The aeration rate was maintained at 100 mL min^{-1} by a mass flow controller (Brooks Instruments, Veenendaal, The Netherlands). The composition of aeration and exhaust gas was measured on-line by a quadrupole mass spectrometer (Omnistar, Balzers, Vaduz, Liechtenstein) with 2 min intervals.

2.5. Chemicals

Yeast extract and tryptone were obtained from Difco Laboratories (Detroit, USA). Ninety-nine percent [1-^{13}C] glucose was purchased from Campro Scientific (Veenendaal, The Netherlands). All other chemicals were purchased from Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland) and of analytical grade.

2.6. Substrate and product analysis

Concentration of glucose, fructose, trehalose and organic acids was quantified in 1:10-diluted cultivation supernatant by HPLC (Kontron Instruments, Neufahrn, Germany). Separation was carried out on an Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad, Hercules, USA) at 55 °C with 7 mM H_2SO_4 as mobile phase and a flow rate of 1 mL min^{-1}. Refraction index (sugars) and UV absorption at 210 nm (organic acids) was used for detection. For quantification of sucrose the column temperature was set to 15 °C and the concentration of the mobile phase was 10 mM H_2SO_4. Amino acid quantification and determination of cell concentration were performed as described previously (Krömer et al., 2004, 2005).

2.7. Quantification of intracellular metabolites

From exponentially growing cell cultures, 10 mL samples were transferred into glass vials incubated in liquid nitrogen. In parallel, supernatant samples were taken involving separation of the cells through filtration (0.2 μm pore size, Sartorius, Göttingen, Germany) to account for metabolites occurring in the medium (Bolten et al., 2007). Similarly to previous data obtained for the wild type of *C. glutamicum*, the fraction of the metabolites of interest in the medium was below 5% so that the amount measured could be almost exclusively attributed the intracellular pool. For metabolite extraction, 10 M HCl was added to a final pH of 1.2. Subsequently cells were thawed and extracted for 8 min in a 50 °C water bath followed by neutralization through addition of 10 M KOH (pH 6.5–7) and removal of cell debris by centrifugation (15 min, 10,000 × *g*, 4 °C; Biofuge stratos, Kendro, Langenselbold, Germany). Quantification of G6P and F6P was performed by enzymatic analysis (Bergmeyer et al., 1974).

2.8. Enzyme assays

Crude cell extracts were prepared from cells grown in minimal medium as described above. Cells were harvested during the exponential phase by centrifugation (9800 × *g*, 5 min, 4 °C), washed with disruption buffer (100 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$ and 0.75 mM DTT) and disrupted by sonication. Cell debris was removed by centrifugation (9800 × *g*, 2 × 30 min, 4 °C). The obtained supernatant was used for determination of the activity of G6P dehydrogenase. Protein concentration was determined by the method of Bradford (1976). The reaction mix for quantification of the G6P dehydrogenase activity contained 100 mM Tris/HCl (pH 7.8), 200 mM KCl, 1 mM NADP, 10 mM $MgCl_2$, 5 mM G6P and 50 μL cell extract in a total volume of 1 mL (Moritz et al., 2000). The enzyme activity was determined by monitoring the formation of NADPH at 340 nm. Negative controls were carried out without G6P or without cell extract, respectively. Michaelis–Menten affinity constants were determined by varying the concentrations of the substrates, G6P or NADP, respectively. To test the influence of effectors on activity, the reaction mix additionally contained ATP, PEP, FBP or NADPH in varied concentrations as given in Section 3.

2.9. Mass spectrometric ^{13}C labeling analysis

Mass isotopomer fractions of amino acids from hydrolyzed and lyophilized cell protein and of trehalose from lyophilized culture supernatant were determined by gas chromatography–mass spectrometry (GC–MS) (Kiefer et al., 2004; Wittmann et al., 2004). Sample preparation and measurement was performed as described previously (Becker et al., 2005).

2.10. Metabolic modeling, metabolic network and biomass requirements

For metabolic flux analysis of *C. glutamicum* a mathematical model of the central carbon metabolism of *C. glutamicum* was used as recently described (Becker et al., 2005). As growth and lysine production of the cultivation experiments with ^{13}C-labeled and naturally labeled glucose agreed very well, mean

values for biomass and product-formation and the corresponding precursor demand from the three replicate cultivations with naturally labeled glucose were used for flux calculation. Simulations were carried out on a personal computer using Matlab 7.0 (Mathworks Inc., Nattick, USA). A detailed description of model and parameter estimation is given elsewhere (Wittmann and Heinzle, 2001, 2002). Since the growth rate of the strains slightly differed, additional flux calculations were carried out including variation of anabolic precursor demand to account for possible effects of a growth rate dependent change in cell composition. These, however, did not reveal a significant influence on the flux distribution presented.

2.11. Redox balancing

To obtain a closer insight into the cofactor metabolism of the PPP engineered mutants, a complete redox balance (Eq. (1)) was set up on basis of the flux data considering all possible redox reactions during the oxidative phosphorylation (Yang et al., 2006).

$$v_{O_2} - (\tfrac{1}{2} v_{XADH} - \tfrac{1}{2} v_{NADPH}) = 0 \quad (1)$$

Here v_{O_2} denotes the oxygen consumption flux and v_{XADH} and v_{NADPH} are the net production of NADH + FADH and NADPH, respectively. Taking all relevant reactions into account v_{XADH} and v_{NADPH} can be calculated from the fluxes of the metabolic network of *C. glutamicum*:

$$v_{XADH} = v_{13} + v_{21} + v_{25} + v_{27} + v_{29} + Y_{NADH/X} Y_{X/S} \quad (2)$$

$$v_{NADPH} = v_2 + v_3 + v_{24} - Y_{NADH/X} Y_{X/S} - 4v_{31} \quad (3)$$

with an anabolic NADPH consumption of $Y_{NADPH/X} = 16.4\ \text{mmol g}^{-1}$ (Wittmann and de Graaf, 2005) and an anabolic NADH production of $Y_{NADH/X} = 3.2\ \text{mmol g}^{-1}$ (Yang et al., 2006). G6P dehydrogenase (v_2), 6-phosphogluconate dehydrogenase (v_3) and isocitrate dehydrogenase (v_{24}) are considered as major NADPH supplying enzymes (Wittmann and de Graaf, 2005). The oxygen consumption flux was calculated via the experimentally determined respiratory quotient (RQ) and the carbon dioxide production flux (v_{CO_2}):

$$v_{O_2} = \frac{v_{CO_2}}{RQ} \quad (4)$$

For calculation of v_{CO_2} all CO_2 producing and consuming reactions from central carbon metabolism and from anabolism were considered, whereby $v_{anaplerosis} = v_{18} + v_{20} - v_{17} - v_{19} - v_{30}$ displays the anaplerotic net flux and the anabolic CO_2 production for *C. glutamicum* is $Y_{CO_2/X} = 1025\ \mu\text{mol g}^{-1}$ (Yang et al., 2005).

$$v_{CO_2} = v_3 + v_{21} + v_{24} + v_{25} + v_{33} - v_{anaplerosis} + Y_{CO_2/X} Y_{X/S} \quad (5)$$

3. Results

3.1. In vitro activity of G6P dehydrogenase in different strains of C. glutamicum

The specific *in vitro* activity of G6P dehydrogenase was clearly affected by over expression of the *zwf* gene (Fig. 2A). Whereas the wild type and the strain lysC^{fbr} showed a similar activity, the replacement of the natural promoter of the *zwf* gene by the *sod* promoter resulted in a four-fold increased activity of the enzyme. This indicates that the use of the *sod* promoter leads to an increased amount of G6P dehydrogenase in the cell. The point mutation A243T did not significantly influence the specific activity of G6P dehydrogenase as indicated by the similar values observed for P_{sod}zwf and P_{sod}zwf243.

3.2. Kinetic characterization of the wild type and the A243T-variant of G6P dehydrogenase

To understand the consequence of the point mutation in the *zwf* gene, the two enzyme variants were compared concerning their kinetic behavior. In different aspects the mutated enzyme showed superior properties. This included a higher affinity towards NADP, one of its substrates (Table 3). Moreover, a stronger resistance against inhibition by metabolites of

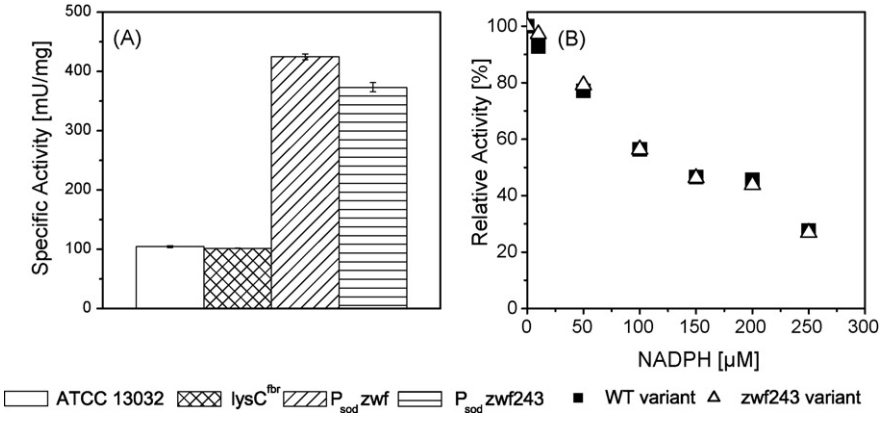

Fig. 2. Specific *in vitro* activity of G6P dehydrogenase in the strains *C. glutamicum* ATCC 13032, lysC^{fbr}, P_{sod}zwf and P_{sod}zwf243 (A); specific *in vitro* activity [%] of the wild type and the A243T variant by addition of NADPH as inhibitor (B). The data represent mean values from three different measurements of cell extracts from cells grown on minimal medium with glucose as carbon source. The corresponding deviations are given.

Table 3
Kinetic characterization of the wild type and the A243T-variant of G6P dehydrogenase from *C. glutamicum*: Michaelis–Menten affinity constants for G6P ($K_{M,G6P}$) and NADP ($K_{M,NADP}$) and inhibition of enzyme activity through concerted action of ATP, phosphoenolpyruvate (PEP) and fructose 1,6-bisphosphate added in equimolar amounts

Enzyme variant	$K_{M,G6P}$ [μM]	$K_{M,NADP}$ [μM]	Inhibition by metabolites (remaining activity, %)[a]					
			5 mM ATP	5 mM PEP	5 mM FBP	1 mM ATP, PEP, FBP[b]	2 mM ATP, PEP, FBP[b]	3 mM ATP, PEP, FBP[b]
Wild type	500 ± 31	100 ± 7	68.8 ± 0.1	62.6 ± 0.1	84.4 ± 0.2	84.8 ± 0.1	79.4 ± 0.1	71.3 ± 0.2
A243T mutant	481 ± 31	75 ± 4	86.0 ± 0.2	72.5 ± 0.1	87.0 ± 0.1	89.9 ± 0.1	88.8 ± 0.1	85.8 ± 0.2

The data represent mean values from three different measurements with cell extracts from cells grown on minimal medium with glucose as carbon source.
[a] Given as relative value related to the activity without addition (100%).
[b] [ATP] = [FBP] = [PEP].

the central metabolism could be observed. Upon addition of 5 mM ATP or PEP a much higher activity was retained for the mutant enzyme, whereas a somewhat weaker influence resulted for FBP addition. Combined addition of all three metabolites in a physiological concentration range between 1 and 2 mM also revealed that the A243T point mutation diminishes the regulatory control of G6P dehydrogenase on the metabolic level. No significant difference was observed concerning the affinity of the two enzymes towards the substrate G6P (Table 3) and the inhibition by NADPH (Fig. 2B).

3.3. Influence of the genetic modifications on lysine production and biomass formation

The different mutants were first compared concerning growth and production characteristics (Tables 4 and 5). For the feedback-resistant parent strain *C. glutamicum* lysC^{fbr} the lysine yield ranged between 74 c mmol c mol^{-1} for fructose-grown cells and 85 c mmol c mol^{-1} for glucose-grown cells (Becker et al., 2005). Over expression of the *zwf* gene via the *sod* promoter markedly increased the lysine production. A positive effect in the corresponding strain *C. glutamicum* P$_{sod}$zwf was observed on all tested carbon sources, whereby the highest increase of more than 30% resulted for glucose-grown cells (Table 4). *C. glutamicum* P$_{sod}$zwf243, in which the *zwf* gene was additionally modified by the A243T amino acid exchange, showed an even higher lysine production. This was most pronounced on fructose and sucrose as carbon source. By the additional over expression of the *fbp* gene the lysine production was further enhanced on all carbon sources. The significant impact of the introduced modifications becomes obvious from the comparison of the lysine yield between *C. glutamicum* P$_{sod}$fbp-zwf243, carrying all three genetic changes, and the original parent strain *C. glutamicum lysC*^{fbr}. The combined introduction of the modifications specified led to an overall increase of the lysine yield of about 50% on glucose and on fructose, whereas on sucrose the increase was about 70%. With an increase of the lysine yield, the biomass yield successively decreased on glucose and on sucrose whereas it remained almost constant on fructose (Table 4). Concerning the specific rates, glucose uptake remained almost unaffected by the genetic modifications (Table 5) so that the observed differences between the mutants display a redistribution of carbon

Table 4
Growth and production characteristics of lysine-producing *C. glutamicum* ATCC 13032 lysC^{fbr}, *C. glutamicum* P$_{sod}$zwf, *C. glutamicum* P$_{sod}$zwf243 and *C. glutamicum* P$_{sod}$fbp-zwf243 on glucose, fructose and sucrose

Strain	Carbon source	$Y_{Lys/S}$, c mmol (c mol)$^{-1}$	$Y_{X/S}$, g (c mol)$^{-1}$
lysC^{fbr}	Glucose	84.7 ± 2.9[a]	13.0 ± 0.3[a]
	Fructose	73.6 ± 1.2[a]	8.2 ± 0.1[a]
	Sucrose	78.8 ± 3.5[a]	10.9 ± 0.4[a]
P$_{sod}$zwf	Glucose	112.6 ± 2.1	10.4 ± 0.8
	Fructose	88.4 ± 0.1	7.8 ± 0.5
	Sucrose	101.6 ± 0.2	10.4 ± 0.3
P$_{sod}$zwf243	Glucose	117.1 ± 2.7	11.1 ± 1.0
	Fructose	97.3 ± 0.3	7.6 ± 0.6
	Sucrose	112.0 ± 3.0	10.7 ± 0.3
P$_{sod}$fbp-zwf243	Glucose	130.0 ± 5.5	9.9 ± 0.6
	Fructose	111.9 ± 7.1	8.4 ± 0.2
	Sucrose	133.5 ± 6.3	8.4 ± 0.3

The data given are lysine yield ($Y_{Lys/S}$) and biomass yield ($Y_{X/S}$) and represent mean values from three parallel cultivation experiments.
[a] Data taken from Becker et al. (2005).

Table 5
Specific rates for growth (μ), glucose uptake (q_{Glc}) and lysine production (q_{Lys}) and respiratory quotient (RQ) of *C. glutamicum* ATCC 13032 lysC^{fbr}, *C. glutamicum* P$_{sod}$zwf, *C. glutamicum* P$_{sod}$zwf243 and *C. glutamicum* P$_{sod}$fbp-zwf243 grown on glucose

Strain	μ [h^{-1}]	q_{Glc} [mmol g^{-1} h^{-1}]	q_{Lys} [mmol g^{-1} h^{-1}]	RQ [mol mol^{-1}]
lysC^{fbr}	0.38 ± 0.00[a]	4.9 ± 0.1[a]	0.42 ± 0.01[a]	n.d.[b]
P$_{sod}$zwf	0.32 ± 0.01	5.2 ± 0.2	0.59 ± 0.01	1.16 ± 0.04
P$_{sod}$zwf243	0.33 ± 0.02	5.0 ± 0.3	0.59 ± 0.01	1.15 ± 0.06
P$_{sod}$fbp-zwf243	0.29 ± 0.01	4.9 ± 0.2	0.64 ± 0.03	1.19 ± 0.08

The data for the reference strain *C. glutamicum* lysC^{fbr} are taken from Becker et al. (2005).
[a] Data taken from Becker et al. (2005).
[b] n.d.: Not determined.

Table 6
Trehalose formation and intracellular level of G6P and F6P during cultivation of lysine producing *C. glutamicum* lysC^{fbr}, *C. glutamicum* P$_{sod}$zwf, *C. glutamicum* P$_{sod}$zwf243 and *C. glutamicum* P$_{sod}$fbp-zwf243 on glucose

C. glutamicum ATCC 13032	Trehalose yield [c mmol c mol^{-1}]	G6P$_{intracellular}$ [μmol g^{-1}]	F6P$_{intracellular}$ [μmol g^{-1}]
lysC^{fbr}	17.6 ± 2.2[a]	23.5 ± 0.3	2.6 ± 0.6
P$_{sod}$zwf	8.1 ± 0.8	12.7 ± 0.3	2.8 ± 0.4
P$_{sod}$zwf243	5.8 ± 0.5	12.6 ± 0.7	1.8 ± 0.2
P$_{sod}$fbp-zwf243	12.6 ± 0.3	36.0 ± 2.2	4.5 ± 1.7

The trehalose yield was determined from three parallel cultivations. The intracellular G6P and F6P level was measured in two independent cell extracts each analyzed in duplicate.
[a] Value taken from Becker et al. (2005).

flux. In response to that specific growth rate decreased with increasing lysine production.

3.4. Influence of the genetic modifications on by-product formation

Additionally to the improvement of lysine production, metabolic engineering also affected the formation of trehalose, the dominating by-product of *C. glutamicum* when grown on glucose. As shown in Table 6, over expression of the *zwf* gene resulted in a 53% reduced trehalose formation in the strain P$_{sod}$zwf compared to the parent strain lysC^{fbr}. In the strain P$_{sod}$zwf243 the trehalose production was even reduced by 67%. Intracellular metabolite analysis revealed that the reduced trehalose flux in the strains P$_{sod}$zwf and P$_{sod}$zwf243 was linked to a decreased availability of G6P, the metabolic precursor of trehalose (Table 6). The concomitant over expression of the *fbp* gene impaired the effect of the *zwf* modifications so that the reduction of trehalose formation was obvious, but somewhat smaller in the triple-mutant strain (28%). This was also reflected by an increased intracellular pool of G6P. In response to that also the F6P pool was significantly higher in the P$_{sod}$fbp-zwf243 strain (Table 6).

3.5. Metabolic flux response to the genetic changes

As shown over expression of the *zwf* gene leads to a strong increase of the *in vitro* activity of G6P dehydrogenase and of lysine production. To investigate, how the genetic modifications in detail affected the *in vivo* flux through the PPP and other metabolic pathways of *C. glutamicum* ^{13}C metabolic flux analysis was performed for the different strains. For this purpose tracer studies were carried out on [1-^{13}C] glucose. In the mid exponential phase the tracer cultivations were harvested followed by GC–MS analysis of ^{13}C labelling patterns, i.e. mass isotopomer distributions, of amino acids from the cell protein and of trehalose from the cultivation supernatant. Together with stoichiometric data on growth and production this provided an extended data set for the flux calculation, which was based on minimization of the deviation between the experimentally determined and simulated mass isotopomer fractions. The obtained key fluxes of central metabolic reactions in the different mutants grown on glucose are summarized in Table 7, whereby the deviations given for each flux parameter represent the 90% confidence intervals. An excellent fit between simulated and measured labelling patterns was obtained (Table 8). Twenty-fold repetition of the parameter estimation with statistically varied starting values for the free flux parameters led to identical solutions for each of the strains, which ensured the identification of the global minimum.

The strains P$_{sod}$zwf and P$_{sod}$zwf243 revealed a strongly enhanced PPP flux in comparison to the parent strain, whereas the glycolytic flux was substantially reduced. Thus, the over expression of the *zwf* gene caused a clear flux redirection at the G6P node. The additional over expression of fructose 1,6-bisphosphatase contributed to a further flux increase into the PPP. Beside the PPP, also the flux through the TCA cycle,

Table 7
Intracellular fluxes and corresponding 90% confidence intervals for *C. glutamicum* lysC^{fbr}, *C. glutamicum* P$_{sod}$zwf, *C. glutamicum* P$_{sod}$zwf243 and *C. glutamicum* P$_{sod}$fbp-zwf243 cultivated on [1-^{13}C] glucose

Flux parameter	lysC^{fbr}	P$_{sod}$zwf	P$_{sod}$zwf243	P$_{sod}$fbp-zwf243
G6P dehydrogenase	46.8 ± 0.6	62.1 ± 0.2	60.9 ± 0.2	66.1 ± 0.2
G6P isomerase	49.8 ± 0.5	35.8 ± 0.2	37.1 ± 0.2	32.4 ± 0.2
Transaldolase, transketolase 1	14.0 ± 0.2	19.4 ± 0.2	19.0 ± 0.1	20.9 ± 0.1
Transketolase 2	11.9 ± 0.3	17.8 ± 0.4	17.0 ± 0.1	19.3 ± 0.1
F16BP aldolase	73.3 ± 0.2	71.1 ± 0.9	71.2 ± 0.2	70.7 ± 0.2
Glyceraldehyde 3-P dehydrogenase	157.5 ± 0.8	159.2 ± 2.2	158.6 ± 0.5	160.1 ± 0.6
Pyruvate carboxylase[a]	34.2 ± 1.0	30.5 ± 1.2	32.4 ± 0.5	31.3 ± 0.9
Pyruvate kinase	147.3 ± 0.9	150.6 ± 3.3	149.5 ± 0.7	152.8 ± 0.6
Pyruvate dehydrogenase	77.5 ± 3.0	87.4 ± 8.3	82.4 ± 1.9	88.3 ± 2.2
Citrate synthase	52.5 ± 4.3	67.7 ± 10.6	61.2 ± 2.3	69.9 ± 2.3
2-Oxoglutarate dehydrogenase	41.2 ± 4.6	59.7 ± 11.7	52.8 ± 2.5	62.6 ± 2.9
Lysine secretion	8.5 ± 0.3	11.3 ± 0.2	11.7 ± 0.3	13.0 ± 0.5
Trehalose secretion	0.9 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.6 ± 0.0

The data are given as relative values (in %) normalized to the glucose uptake rate which was 4.9 mmol g^{-1} h^{-1} (for lysC^{fbr}), 5.2 mmol g^{-1} h^{-1} (for P$_{sod}$zwf), 5.0 mmol g^{-1} h^{-1} (for P$_{sod}$zwf243) and 4.9 mmol g^{-1} h^{-1} (for P$_{sod}$fbp-zwf243) and thus did not significantly differ between the strains. The flux data for the reference strain *C. glutamicum* lysC^{fbr} are taken from Becker et al. (2005).
[a] Given as the lumped net flux through anaplerotic carboxylation.

Table 8
Relative mass isotopomer fractions of amino acids from the cell protein and of secreted trehalose of lysine producing *C. glutamicum* P_{sod}zwf, *C. glutamicum* P_{sod}zwf243 and *C. glutamicum* P_{sod}fbp-zwf243 cultivated on 99% [1-^{13}C] glucose

Analyte	P_{sod}zwf		P_{sod}zwf243		P_{sod}fbp-zwf243	
	Calc	Exp	Calc	Exp	Calc	Exp
Alanine (*m/z* 260)						
M_0	0.530	0.533	0.529	0.532	0.544	0.550
M_1	0.338	0.337	0.339	0.337	0.330	0.323
M_2	0.103	0.102	0.104	0.103	0.099	0.098
Valine (*m/z* 288)						
M_0	0.375	0.378	0.376	0.377	0.387	0.388
M_1	0.388	0.391	0.388	0.391	0.387	0.386
M_2	0.171	0.170	0.171	0.170	0.165	0.165
Threonine (*m/z* 404)						
M_0	0.347	0.348	0.349	0.350	0.353	0.353
M_1	0.371	0.373	0.371	0.372	0.368	0.372
M_2	0.191	0.190	0.189	0.189	0.189	0.188
Aspartate (*m/z* 418)						
M_0	0.347	0.347	0.349	0.350	0.352	0.352
M_1	0.370	0.373	0.370	0.372	0.367	0.371
M_2	0.191	0.190	0.190	0.189	0.189	0.188
Glutamate (*m/z* 432)						
M_0	0.268	0.265	0.269	0.267	0.274	0.272
M_1	0.365	0.365	0.365	0.365	0.365	0.365
M_2	0.230	0.232	0.230	0.232	0.227	0.229
Trehalose (*m/z* 361)						
M_0	0.096	0.096	0.091	0.092	0.104	0.104
M_1	0.582	0.582	0.586	0.587	0.576	0.576
M_2	0.203	0.203	0.203	0.203	0.202	0.202
Serine (*m/z* 390)						
M_0	0.471	0.473	0.470	0.471	0.479	0.477
M_1	0.345	0.345	0.345	0.346	0.340	0.341
M_2	0.138	0.137	0.138	0.137	0.136	0.136
Phenylalanine (*m/z* 336)						
M_0	0.308	0.305	0.305	0.302	0.355	0.356
M_1	0.382	0.384	0.382	0.382	0.404	0.399
M_2	0.210	0.210	0.212	0.211	0.184	0.186
Glycine (*m/z* 246)						
M_0	0.749	0.749	0.751	0.750	0.746	0.745
M_1	0.179	0.178	0.178	0.178	0.181	0.181

The data given comprise experimental GC–MS data (exp) and values predicted by the solution of the mathematical model corresponding to the optimized set of fluxes (calc). M_0 denotes the relative amount of non-labelled mass isotopomer fraction, M_1 the relative amount of the single labelled mass isotopomer fraction and corresponding terms stand for higher labelling. Amino acids were analyzed by GC–MS as *t*-butyl-dimethylsilyl derivate and trehalose as trimethylsilyl derivate, respectively.

the other important NADPH-supplying pathway in *C. glutamicum*, was strongly enhanced. The generally high activity of the NADPH-providing pathways PPP and TCA cycle in the different *zwf* mutants resulted in a strong NADPH supply, which was even higher than the actual demand for anabolism and lysine production, and caused a high apparent NADPH excess in all *zwf* mutants (Table 9). A closer inspection of the cofactor metabolism for the PPP engineered mutants was carried out by an extended redox balance (Table 9). This considered all possible reactions during oxidative phosphorylation leading to oxidation of reduction equivalents (NADH, FADH and NADPH) which are formed in the catabolic and anabolic pathways of carbon metabolism. All mutants revealed a significant surplus of both, NADH/FADH and of NADPH. The overall oxygen consumption was not sufficient to completely account for oxidation of all reduction equivalents formed, i.e. the redox balance did not close as one would expect. This is a clear indication of additional metabolic reactions contributing to oxidation of cofactors, which is discussed in more detail below. Interestingly, the higher demand for oxaloacetate as precursor for lysine in the optimized strains was not reflected by an increased flux through anaplerotic carboxylation (Table 7). This is probably related to the reduced oxaloacetate demand for anabolism, reflected by the reduced biomass yield (Table 4).

4. Discussion

4.1. Strain engineering for improved lysine production

In the present work, metabolic flux engineering of *C. glutamicum* was carried out by a combination of targeted genetic modifications and flux analysis in order to increase lysine production. The strategy focused on engineering of the PPP flux by different genetic modifications. Over expression of the *zwf* gene by the strong promoter of the *sod* gene resulted in significant improvement of lysine production on different carbon sources, including the two major industrial sugars sucrose and glucose. The four-fold increase of the *in vitro* activity of the enzyme was, however, reflected only by a 1.4-fold increase of the corresponding *in vivo* activity, i.e. the flux, which reveals the strong metabolic regulation of G6P dehydrogenase *in vivo* (Moritz et al., 2000). In addition to the *sod* promoter, also the *eftu* (elongation factor TU) promoter was tested to over express the *zwf* gene. The impact on specific enzyme activity and lysine production, however, was much smaller as compared to the result obtained with the *sod* promoter (data not shown). Comparing lysine production of the different strains of the obtained genealogy on glucose, one would guess on a first glance that the over expression via the *sod* promoter was most beneficial, since this resulted in the largest increase of production, whereas the additional introduction of the point mutation A243T did not give a further improvement (Table 4). Exchanging the order of the introduced mutations, however, revealed a completely different picture. The strain zwf243, additionally constructed, only containing the mutated enzyme with its natural promoter, exhibited a high lysine yield of 120 c mmol c mol^{-1}, which was not further improved when the *zwf* gene was additionally over expressed by the *sod* promoter. This clearly shows that the metabolic response to a genetic perturbation not only depends on the introduced modification, but to a large extent on the genetic background of the host strain. The benefit of the A243T mutation in the *zwf* gene is due to an improved kinetic behavior of the encoded G6P dehydrogenase as reflected, e.g. by an increased affinity towards NADP and a decreased sensitivity against inhibition by ATP, PEP and FBP, respectively (Table 3). A significantly weaker inhibition of the mutant enzyme was already observed

Table 9
Redox balance of PPP engineered *C. glutamicum* ATCC 13032 P_{sod}zwf, *C. glutamicum* ATCC 13032 P_{sod}zwf243 and *C. glutamicum* ATCC 13032 P_{sod}fbp-zwf243 calculated from the estimated fluxes (Table 7), anabolic CO_2 and NADH production, anabolic NADPH demand and the respiratory quotient (Table 5)

Strain	P_{sod}zwf	P_{sod}zwf243	P_{sod}fbp-zwf243
CO_2 production			
Glucose 6-P dehydrogenase	62.1	60.9	66.1
Pyruvate dehydrogenase	87.4	82.4	88.3
Anaplerotic carboxylation	−30.5	−32.4	−31.3
Isocitrate dehydrogenase	67.7	61.2	69.9
α-Ketoglutarate dehydrogenase	59.7	52.8	62.6
Lysine secretion	11.3	11.7	13.0
Anabolism	6.2	7.6	6.0
Total	263.9	244.2	274.6
O_2 consumption			
Total	227.5	212.3	230.8
NADH production			
Glyceraldehyde 3-P dehydrogenase	159.2	158.6	160.1
Pyruvate dehydrogenase	87.4	82.4	88.3
α-Ketoglutarate dehydrogenase	59.7	52.8	62.6
Fumarase	59.7	52.8	62.6
Malate dehydrogenase	59.7	52.8	62.6
Anabolism	20.0	25.0	19.0
Total	445.7	424.4	455.2
NADPH production			
Glucose 6-P dehydrogenase	62.1	60.9	66.1
6-P gluconate dehydrogenase	62.1	60.9	66.1
Isocitrate dehydrogenase	67.7	61.2	69.9
Lysine secretion	−45.0	−46.8	−52.1
Anabolism	−102.6	−109.2	−95.3
Total	44.3	27.0	54.8
Redox balance			
Total	−17.5	−13.3	−24.2
[H] oxidation flux			
Total	35.0	26.8	48.4

at a concentration of 1 mM, which is within the physiological range found for these metabolites in *C. glutamicum* (Moritz et al., 2002). Thus, it seems likely that the mutant enzyme variant displays a higher activity *in vivo*. Also the higher affinity towards NADP might contribute to this effect since actual NADP levels are in the range of 100 μM (Moritz et al., 2002).

4.2. NADPH metabolism

As shown by metabolic flux analysis, the genetic manipulations contributed to extreme changes in the NADPH metabolism of the cell. Increased fluxes through PPP and TCA cycle resulted in a significantly increased NADPH supply. The increased lysine yield associated with these flux changes demonstrates that NADPH supply has definitely a limiting role in lysine production in batch cultures of *C. glutamicum*, although assumptions for chemostat cultures are different (Sahm et al., 2000). Whereas the parent strain $lysC^{fbr}$ exhibits a slight apparent NADPH deficiency (Kim et al., 2006), the NADPH supply in the PPP engineered strains was much higher than the demand (Table 9). The resulting high apparent NADPH excess in these strains indicates that the performed changes could not be fully exploited for lysine production, but leave a remaining potential for further optimization. The extent of this potential is illustrated by the fact that the product yield of the best mutant, *C. glutamicum* P_{sod}fbp-zwf243, could be even doubled, if one could channel all the excess NADPH into the lysine pathway. The NADPH surplus in the *zwf* mutants points at limitations in other parts of the metabolic network which arise in response to the PPP flux engineering. These limits might be associated with oxaloacetate supply, as it has been shown that especially the increase of the anaplerotic flux in *C. glutamicum* leads to an improved lysine production (Koffas et al., 2003; Peters-Wendisch et al., 2001). In fact the anaplerotic net flux remained almost constant in strains investigated here, so only the reduction of the anabolic oxaloacetate demand enabled the higher flux of this precursor into the lysine pathway.

The high apparent NADPH excess in the PPP mutants points at additional reactions that consume NADPH. Possible candidates comprise superoxide-generating NAD(P)H oxidase in the respiratory chain (Matsushita et al., 2001) or malic enzyme operation in the carboxylating direction (de Graaf, 2000; Petersen et al., 2000). Clear experimental evidence supporting the role of either one of these reactions has not been obtained to date.

It has however been assumed that the malic enzyme, operating in the decarboxylating direction, can function as additional NADPH source in strains with a seeming NADPH deficiency (Dominguez et al., 1998). To obtain a closer insight into the cofactor metabolism of the PPP engineered mutants, we set up a complete balance considering all possible redox reactions during the oxidative phosphorylation (Table 9). In none of the strains the cofactor balance was closed. This clearly indicates the presence of an additional, so far unassigned flux of [H] oxidation, required to close the balance. In all three strains this [H] oxidation flux was almost as high as the apparent NADPH excess. A significant contribution of NAD(P)H oxidase in the respiratory chain to the NADPH oxidation can be excluded, since the activity of this enzyme as part of the respiratory chain would consume oxygen and thus result in a closed redox balance. It is therefore very likely that, in each PPP mutant, the apparent NADPH excess is completely oxidized by malic enzyme operating in the carboxylating direction.

4.3. Trehalose formation

Trehalose is a typical by-product of *C. glutamicum*. Trehalose excretion is undesired in lysine production since this compound cannot be taken up by the cells due to lack of a corresponding uptake system. The different *zwf* mutants of *C. glutamicum* all exhibited a significantly reduced trehalose formation which is a positive side effect of metabolic engineering of the PPP flux. The origin of the reduced trehalose formation is a reduced intracellular concentration of G6P, the metabolic precursor of trehalose (Table 6). In *C. glutamicum*, trehalose can be formed via three different pathways, all of which use G6P as substrate (Tzvetkov et al., 2003). An accelerated conversion of G6P by the PPP as a result of the higher G6PDH activity reduces trehalose formation by decreasing the availability of this precursor. A similar finding has been previously reported for reduced lysine production on fructose, linked to substantially lower PPP flux, as compared to glucose (Kiefer et al., 2002). The positive effect of the *zwf* modifications on trehalose formation was diminished by simultaneous over expression of fructose 1,6-bisphosphatase. This should be due to the fact that this modification "pushes" carbon into the PPP via the G6P pool, whereas engineering of G6P dehydrogenase "pulls" carbon from the G6P pool into this pathway. Accordingly the G6P pool in this mutant was substantially higher.

5. Conclusions

Summarizing, metabolic flux analysis provided valuable data to understand the cellular response resulting from genetic engineering and to visualize metabolic imbalances to guide further strain engineering. On basis of these and previous data we regard the analysis of metabolic fluxes as an essential tool in metabolic engineering as previously suggested (Stephanopoulos, 1999). The combination of the over expressed *zwf* gene with the A243T mutation and the amplified expression of fructose 1,6-bisphosphatase resulted in a further stepwise increase of production, showing that the different strategies to increase lysine production via an increased PPP flux nicely complement each other. It, however, becomes obvious from the created imbalance in the NADPH metabolism that in order to finally arrive at the level of an optimal producer and fully exploit all genetic perturbations, further metabolic optimization must include a balanced engineering of the different parts of the metabolic network, i.e. product pathway, cofactor supply and precursor supply.

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