Amplified Expression of Fructose 1,6-Bisphosphatase in Corynebacterium glutamicum Increases In Vivo Flux through the Pentose Phosphate Pathway and Lysine Production on Different Carbon Sources

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Corynebacterium glutamicum has been successfully used for the industrial production of lysine for more than 40 years, leading to a current market volume of about 600,000 tons, which are produced worldwide with this microorganism per annum (28). The classically derived producer strains developed and currently used in industry, however, have uncharacterized secondary mutations that are detrimental to their performance and lead to decreased sugar uptake rates, growth rates, or stress tolerance (22). This raises the question of superior strains, which exhibit a limited set of exclusively beneficial mutations.

In order to rationally create such cell factories, comparative sequencing of the C. glutamicum wild-type and lysine-producing strains has recently been introduced as a powerful strategy (22). By this approach, mutations in key reactions such as pathways involved in product synthesis or supply of precursor metabolites can be identified and subsequently introduced into the wild type (21, 22). This approach can be efficiently complemented by comparative metabolic profiling of the organism, which generates a detailed understanding on the quantitative physiology of the organism and, based on the knowledge obtained, also identifies promising genetic targets.

In this regard, metabolic flux analysis provides detailed insight into the central metabolism of lysine producing C. glutamicum (11, 16, 33, 35). The biosynthesis of lysine has a high requirement for NADPH, which has to be provided by the reactions of the central metabolism. The major pathway for NADPH formation in C. glutamicum is the pentose phosphate pathway (PPP) with the two NADPH-generating enzymes glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

The importance of the PPP for lysine production becomes obvious from comparative flux studies of different C. glutamicum mutants, where improvement of the lysine yield is accompanied by an increase of the PPP flux (33). Further evidence for the crucial role of the PPP is provided by the observation that the relatively low lysine yield for fructose-grown cells of C. glutamicum is accompanied by a drastically reduced PPP flux on this carbon source (11). The increase of the flux through the PPP is therefore of high relevance in order to improve lysine production by C. glutamicum (15).

In this regard deregulated expression of fructose 1,6-bisphosphatase (FBPase) was recently suggested as a promising target (11, 35). This enzyme is part of the gluconeogenic pathway and essential for C. glutamicum to grow on noncarbohydrates such as acetate, citrate, and glutamate (23). During growth on sugars, however, fructose 1,6-bisphosphatase is not required and is typically inhibited or repressed by different
C. glutamicum regulation mechanisms (2, 5, 20). lysCfbr......................................................... Exchange T311I in the phatase in

describes deregulated expression of fructose 1,6-bisphosphatase activity (4). During lysine production of glucose or on fructose completely lacks fructose 1,6-bisphosphatase, by the promoter of the tuf gene, encoding elongation factor TU, and the sod gene, encoding superoxide dismutase (Table 1). All genetic modifications were integrated in the genomic DNA as described below.

As the vector for introducing the modified genes, plasmid pClik, which carried a kanamycin resistance cassette and the sacB gene as selection markers, was used. The mutation on pClik was introduced via two recombination events. Since pClik cannot replicate in C. glutamicum, transformation of the organism with the plasmid and subsequent selection for the plasmid kanamycin resistance marker yielded transformants which had integrated the plasmid DNA into the genome via a single-crossover homologous recombination event. Subsequently, each kanamycin-resistant integrant was grown for 1 day without kanamycin to allow a second recombination event to take place. An appropriate dilution of the grown cell culture was spread on an agar plate containing CM medium with 10% sucrose, and were of analytical grade.

Yeast extract and tryptone were obtained from Difco Laboratories. Phosphoglucose isomerase was purchased from Roche Diagnostics (Mannheim, Germany); glucose 6-phosphate dehydrogenase was obtained from Fluka (Buchs, Switzerland) and glucose 6-phosphate dehydrogenase was obtained from Fluka (Buchs, Switzerland) and glucose 6-phosphate dehydrogenase was obtained from Fluka (Buchs, Switzerland) and glucose 6-phosphate dehydrogenase was obtained from Fluka (Buchs, Switzerland).

The second precultivation and main cultivation were carried out on a minimal medium containing 15 g liter−1 carbon source, i.e., glucose, fructose, or sucrose. The minimal medium contained, per liter, (A) 0.055 g CaCl2 · 2 H2O, 0.2 g MgSO4 · 7H2O, and 1 g NaCl in 679 ml deionized water; (B) 15 g glucose in 100 ml deionized water, adjusted to pH 5.0 with HCl; (C) 16 g K2HPO4 and 2 g KH2PO4 in 80 ml deionized water; (D) 5 g (NH4)2SO4 in 100 ml deionized water; (E) 0.5 mg biotin and 1 mg thiamine · HCl in 20 ml deionized water; (F) 20 mg FeSO4 · 7 H2O in 10 ml deionized water, adjusted to pH 1.0 with HCl; (G) 10 ml of 100% trace elements (29); and (H) 30 mg 3,4-dihydroxybenzoic acid and 50 µl 4 M NaOH in 1 ml deionized water. Solutions A to D were autoclaved separately and combined after cooling to room temperature; solutions E to H were sterilized by filtration and subsequently added.

**Materials and Methods**

**Microorganisms.** The present work involved the construction of different mutants of *C. glutamicum* with deregulated lysine biosynthesis on the basis of the wild-type *C. glutamicum* ATCC 13032 (American Type and Culture Collection). The modifications comprised the deregulation of lysine biosynthesis by allelic replacement of the lysC gene encoding aspartokinase with a lysC T311I gene and subsequent exchange of the natural promoter upstream of the fbp gene, encoding fructose 1,6-bisphosphatase, by the promoter of the tuf gene, encoding elongation factor TU, and the sod gene, encoding superoxide dismutase (Table 1). All genetic modifications were integrated in the genomic DNA as described below.

**TABLE 1. Site-specific primer sequences used to verify allelic replacements in *C. glutamicum***

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>lysC&lt;sup&gt;Cre&lt;/sup&gt;</td>
<td>lysC T311I-1: 5′-GGAGAGAGAGAGAGTTGCCACATGCTGACGAGCATC-3′&lt;br&gt;lysC T311I-2: 5′-CTTCTCTCTGTCGACGAATTCAATCTTACGGCCTG-3′</td>
</tr>
<tr>
<td>P&lt;sub&gt;E&lt;/sub&gt;FTULfbp</td>
<td>Pefu-F: 5′-GGTTAGCTGGTAGTTGAAA-3′&lt;br&gt;FBP-R: 5′-GGGAGAATGGAAAATTCGT-3′</td>
</tr>
<tr>
<td>P&lt;sub&gt;S&lt;/sub&gt;ODfbp</td>
<td>Psod-F: 5′-TATCCGGCATTATCCGGG-3′&lt;br&gt;FBP-R: 5′-GGGAGAATGGAAAATTCGT-3′</td>
</tr>
</tbody>
</table>
Mass-spectrometric 13C labeling analysis. Mass isotopomer fractions of amino acids from the cell protein were determined by gas chromatography-mass spectroscopy (GC-MS) (11, 35). For this purpose, cells (about 1 mg dry cell mass) were harvested from the culture and washed twice with deionized water. The pellet was then incubated with 50 μl 6 M HCl for 24 h at 105°C, subsequently neutralized with 6 M NaOH, and separated from insoluble matter by centrifugation (5 min; Ultrafree-MC filter units, 0.22-μm-pore-size Durapore membrane; Millipore). The remaining clear solution was lyophilized. Analysis of the amino acids was performed after derivatization into the t-butyl-dimethylsilyl derivative (13, 31). All samples were first measured in scan mode to check for potential isobaric interference between analytes and other sample components.

The labeling patterns of the amino acids were then determined in triplicate via selective ion monitoring of selected ion clusters, representing [M-57] fragments with the complete carbon skeletons of the amino acids. The labeling pattern of trehalose from the culture supernatant was determined in selective ion monitoring mode from its trimethylsilyl derivative via the ion cluster at m/z 361 to 367 corresponding to a fragment ion that contains an entire monomer unit of trehalose and thus a carbon skeleton equal to that of glucose 6-phosphate as described previously (11, 35). The trehalose measurement was also carried out in triplicate. The mean experimental error for the mass isotopomer fractions was about 0.15%.

Metabolic modeling and parameter estimation. All metabolic simulations were carried out on a personal computer using Matlab 7.0 (Mathworks Inc.). Details of the applied computational tools are given elsewhere (32, 33, 35). The network for growth of and lysine production by C. glutamicum grown on glucose comprised all central metabolic pathways, i.e., glycolysis, PPP, tricarboxylic acid cycle, and anaerobic carbon fixation. Additionally, the pathways for the glyoxylate shunt and different by-products thereof and for anaerobic pathways from intermediary precursors to biomass were implemented. For glycine synthesis, two possible routes were considered, i.e., via serine and via threonine aldolase (24). Based on previous results, the glyoxylate pathway was assumed to be inactive (33).

Calculation of the anabolic demand for the different precursors was based on data on the biomass composition of C. glutamicum which considered the specific anabolic demand for cell wall synthesis based on the diaminopimelate content of the cell (30).

Determination of the in vitro activity of fructose 1,6-bisphosphatase in cell extracts of C. glutamicum was based on the protocol by Sugimoto and Shiio (27) with slight modifications. First, 50 μl of substrate solution (100 mM fructose 1,6-bisphosphate, 100 mM Tris/Cl, pH 7.8, 4°C), subsequent resuspension in disruption buffer to a concentration of 0.25 g cell dry weight ml⁻¹, and then disruption on ice using ultrasound (5 1-s pulses, 20 μm) (Q50; Branson, USA). Cell debris was recovered by centrifugation (twice for 30 min, 9,800 × g, 4°C). The remaining cell extract was used for determination of protein content and enzyme activity. The protein content was quantified using the method of Bradford (3) and a reagent solution from Sigma.

Analysis of fructose 1,6-bisphosphatase activity. Determination of the in vitro activity of fructose 1,6-bisphosphatase in cell extracts of C. glutamicum was based on the protocol by Sugimoto and Shiio (27) with slight modifications. First, 50 μl of substrate solution (100 mM fructose 1,6-bisphosphatase, 100 mM Tris/Cl, pH 7.8, 30°C) was pipetted into a 1.5-ml polystyrene cuvette. The reaction was started by adding 950 μl of reaction mix, which contained 900 μl of reaction buffer (100 mM Tris/Cl, 10 mM MgCl₂, 0.5 mM NADP, 2 U of phosphoglucoisomerase, 1 U of glucose 6-phosphate dehydrogenase, pH 7.8, 30°C) and 50 μl of cell extract. The final protein concentration in the assay was in the range of 0.3 to 0.4 mg ml⁻¹. The activity of fructose 1,6-bisphosphatase was determined by monitoring the formation of NADPH₂ via measurement of absorbance at 340 nm.

RESULTS

In vitro activity of fructose 1,6-bisphosphatase in the different strains. Fructose 1,6-bisphosphatase exhibited a low basal activity in vitro activity in the parent strain C. glutamicum lysCₚₑᵗ (“Fig.” (1)). The natural promoter of the fbp gene obviously leads to a weak constitutive expression of the gluconeogenic enzyme fructose 1,6-bisphosphatase also during growth on sugars. Hereby the activity observed was relatively similar for glucose, fructose, and sucrose. For amplification of fructose 1,6-bisphosphatase expression, the natural promoter of the gene in the chromosome of C. glutamicum was replaced by two other C. glutamicum promoters, Pₕₑᵗᵤ from the gene for elongation factor Tu and Pₕₑₕₑ from the gene for superoxide dismutase. The corresponding mutants C. glutamicum lysCₚₑᵗ Pₕₑᵗᵤ and C. glutamicum lysCₚₑᵗ Pₕₑₕₑ show increased levels of fructose 1,6-bisphosphatase activity (Fig. 1). Using the tuf promoter, the increase of fructose 1,6-bisphosphatase activity was up to 10-fold, whereas a weaker amplification was achieved using the sod promoter. Interestingly, the activity of fructose-1,6-bisphosphatase in the two mutants also depended on the carbon source applied. It was almost twice as high in cells grown on fructose as on cells grown on glucose or sucrose. Overall, the enzyme data clearly showed that the construction of the mutants was successful, and the use of a strong promoter instead of the natural one upstream of the chromosomal fbp gene allowed its targeted overexpression in C. glutamicum. In a next step, the different strains were compared with regard to growth and product formation in order to investigate the metabolic consequences of fructose 1,6-bisphosphatase overexpression in C. glutamicum.

Influence of fructose 1,6-bisphosphatase overexpression on lysine production. C. glutamicum lysCₚₑᵗ showed significant lysine production when grown on any of the substrates (Table 3). This is the consequence of a feedback-resistant aspartokinase contained in this strain, which causes deregulated lysine biosynthesis. Similar lysine yields of about 85 C-mmol C⁻¹mol⁻¹ (1 C-mol = 1 mol C) resulted from growth on glucose or sucrose, whereas production by fructose-grown cells was about 25% lower. The additional amplification of fructose 1,6-

FIG. 1. In vitro activity of fructose 1,6-bisphosphatase in different strains of Corynebacterium glutamicum. The data are given for Corynebacterium glutamicum ATCC 13032 lysCₚₑᵗ and two mutants, Corynebacterium glutamicum ATCC 13032 lysCₚₑᵗ Pₕₑₕₑ and Corynebacterium glutamicum ATCC 13032 lysCₚₑᵗ Pₕₑᵗᵤ. The strains were grown on minimal medium containing glucose, fructose, or sucrose as the sole carbon source. All measurements were carried out in triplicate; corresponding deviations are given.
biphosphatase in \textit{C. glutamicum} lysC\textsubscript{bhr} P\textsubscript{EFPT\textsubscript{bfr}} led to a strong increase in the lysine yield on all three carbon sources. The highest increase (40%) was observed for cells grown on glucose, but the improvement was also significant for cells grown on sucrose (30%) or fructose (30%). The lysine yield in \textit{C. glutamicum} lysC\textsubscript{bhr} P\textsubscript{SOD\textsubscript{bfr}} was, however, not significantly higher on any of the carbon sources.

\textbf{Influence of fructose 1,6-biphosphatase overexpression on growth.} In addition to a strongly increased lysine production, \textit{C. glutamicum} lysC\textsubscript{bhr} P\textsubscript{EFPT\textsubscript{bfr}} also differed from \textit{C. glutamicum} lysC\textsubscript{bhr} with respect to growth. For growth on glucose, the specific growth rate $\mu$ (0.31 h$^{-1}$) was lower for \textit{C. glutamicum} lysC\textsubscript{bhr} P\textsubscript{EFPT\textsubscript{bfr}} than for lysC\textsubscript{bhr} (0.39 h$^{-1}$). This was, however, not the consequence of a reduced uptake of substrate. In fact, the specific substrate uptake rate was not affected. Both strains showed relatively similar values ($q_S = 4.9$ mmol g$^{-1}$ h$^{-1}$ for \textit{C. glutamicum} lysC\textsubscript{bhr} and $q_S = 4.8$ mmol g$^{-1}$ h$^{-1}$ for \textit{C. glutamicum} lysC\textsubscript{bhr} P\textsubscript{EFPT\textsubscript{bfr}}). The reduced growth rate was the consequence of a reduced biomass formation related to FBPase overexpression (Table 3). Thus, overexpression of FBPase did not affect the uptake of substrate into the cells but rather influenced the intracellular distribution of the substrate carbon, with a shift from biomass toward lysine production.

Interesting effects were observed for fructose-grown cells. \textit{C. glutamicum} lysC\textsubscript{bhr} P\textsubscript{EFPT\textsubscript{bfr}} grew faster ($\mu = 0.35$ h$^{-1}$) than the parent strain ($\mu = 0.27$ h$^{-1}$) and had a higher biomass yield (Table 3). A beneficial effect was also observed concerning the specific fructose uptake rate, which was higher for \textit{C. glutamicum} lysC\textsubscript{bhr} P\textsubscript{EFPT\textsubscript{bfr}} ($q_S = 4.7$ mmol g$^{-1}$ h$^{-1}$) than for \textit{C. glutamicum} lysC\textsubscript{bhr} ($q_S = 4.4$ mmol g$^{-1}$ h$^{-1}$). The metabolic effects were less pronounced in the other FBPase mutant. Obviously, the reason for the similar phenotypes of \textit{C. glutamicum} lysC\textsubscript{bhr} and \textit{C. glutamicum} lysC\textsubscript{bhr} P\textsubscript{SOD\textsubscript{bfr}} is the relatively weak amplification of fructose 1,6-biphosphatase using the sod promoter (Fig. 1).

\textbf{Influence of fructose 1,6-biphosphatase overexpression on by-product formation.} Glycerol and dihydroxyacetone were the most prominent by-products for fructose-grown cells of \textit{C. glutamicum} lysC\textsubscript{bhr} (Table 4). At the end of the cultivation, these two compounds together represented a fraction of 26 C-mmol liter$^{-1}$ and thus about 80% of the carbon, which was obtained in the form of the desired product lysine with a final concentration of about 33 C-mmol liter$^{-1}$. The comparative analysis for the other mutants revealed that overexpression of FBPase also had an effect on the formation of these by-products (Table 4). In the P\textsubscript{SOD\textsubscript{bfr}} mutant, the sum of the levels of glycerol and dihydroxyacetone was decreased by about 60%, whereas an even stronger decrease of more than 80% was observed for the P\textsubscript{EFPT\textsubscript{bfr}} mutant. This shows that overexpression of FBPase was also beneficial in terms of the decrease in by-product formation.

\textbf{Influence of fructose 1,6-biphosphatase overexpression on metabolic carbon fluxes.} The data from the enzyme studies and from the comparative cultivations clearly showed that overexpression of fructose 1,6-biphosphatase in \textit{C. glutamicum} leads to a strong increase of the in vitro activity, i.e., the capacity of the enzyme, and as a second and important consequence significantly improves lysine production in that organism. It was now interesting to see how the genetic modification in detail affects the metabolic pathways of \textit{C. glutamicum}. To this end, \textsuperscript{13}C metabolic flux analysis was performed for the two strains \textit{C. glutamicum} lysC\textsubscript{bhr} and \textit{C. glutamicum} lysC\textsubscript{bhr} P\textsubscript{EFPT\textsubscript{bfr}}. This technique combines cultivations on \textsuperscript{13}C tracer substrates, here $[^1\text{C}]$glucose, with the analysis of kinetic and stoichiometric parameters from the culture and mass-spectrometric \textsuperscript{13}C labeling analysis of metabolites formed during cultivation (11, 32, 33, 35). The latter reflect, like a fingerprint, the intracellular flux distribution.

Growth of and lysine production by the tracer cultivations, at 80 mg biomass (mmol glucose)$^{-1}$ and 78 mmol lysine (mol glucose)$^{-1}$ for \textit{C. glutamicum} lysC\textsubscript{bhr} and 58 mg biomass (mmol glucose)$^{-1}$ and 116 mmol lysine (mol glucose)$^{-1}$ for \textit{C. glutamicum} lysC\textsubscript{bhr} P\textsubscript{EFPT\textsubscript{bfr}}, agreed very well with the values for three parallel incubations with naturally labeled glucose. This allowed using mean values and standard deviations for biomass and product yield for the three replicates (Table 5) and the corresponding precursor demand for anabolism (Table 6) for the error-weighted flux calculation. In the mid-exponential phase, the tracer cultivations were harvested; this was followed by GC-MS analysis of \textsuperscript{13}C labeling patterns, i.e., mass isotope distributions, of amino acids from the cell protein and of trehalose from the cultivation supernatant (Table 7). Together with the stoichiometric data, this provided an extended data set for the flux calculation.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{C. glutamicum strain} & \textbf{Carbon source} & \textbf{$Y_{\text{lysC}}$} & \textbf{$Y_{\text{X/S}}$} \\
\hline
lysC\textsubscript{bhr} & Glucose & 84.7 & 13.0 \\
 & Fructose & 73.6 & 8.2 \\
 & Sucrose & 78.8 & 10.9 \\
\hline
lysC\textsubscript{bhr} P\textsubscript{EFPT\textsubscript{bfr}} & Glucose & 120.6 & 10.4 \\
 & Fructose & 95.0 & 9.0 \\
 & Sucrose & 101.2 & 11.5 \\
\hline
lysC\textsubscript{bhr} P\textsubscript{SOD\textsubscript{bfr}} & Glucose & 87.3 & 12.7 \\
 & Fructose & 76.9 & 9.1 \\
 & Sucrose & 79.4 & 11.6 \\
\hline
\end{tabular}
\caption{Growth and production characteristics of lysine-producing \textit{C. glutamicum} ATCC 13032 lysC\textsubscript{bhr}, \textit{C. glutamicum} ATCC 13032 lysC\textsubscript{bhr} P\textsubscript{EFPT\textsubscript{bfr}}, and \textit{C. glutamicum} ATCC 13032 lysC\textsubscript{bhr} P\textsubscript{SOD\textsubscript{bfr}} on glucose, fructose, and sucrose as the carbon source.}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{C. glutamicum strain} & \textbf{Glycerol} & \textbf{Dihydroxyacetone} \\
\hline
lysC\textsubscript{bhr} & 2.1 & 6.4 \\
lysC\textsubscript{bhr} P\textsubscript{SOD\textsubscript{bfr}} & 1.1 & 2.5 \\
P\textsubscript{EFPT\textsubscript{bfr}} & 0.6 & 1.0 \\
\hline
\end{tabular}
\caption{Formation of glycerol and dihydroxyacetone during cultivation of lysine-producing \textit{C. glutamicum} ATCC 13032 lysC\textsubscript{bhr}, \textit{C. glutamicum} ATCC 13032 lysC\textsubscript{bhr} P\textsubscript{EFPT\textsubscript{bfr}}, and \textit{C. glutamicum} ATCC 13032 lysC\textsubscript{bhr} P\textsubscript{SOD\textsubscript{bfr}} on fructose.}
\end{table}
Qualitative inspection of the labeling data directly showed that the degree of \(^{13}\text{C}\) labeling was significantly lower for \(C.\ glutamicum\) lysC\textsuperscript{cbr} PEFTU\textsuperscript{fbr}, as visualized, e.g., by the relatively large fractions of nonlabeled mass isotopomers (M\(_0\)) for the different amino acids (Table 7). Previous simulations for \(C.\ glutamicum\) have shown that, using \([^{1-13}\text{C}]\text{glucose}\) as the tracer substrate, high PPP flux relates to low labeling enrichment for various metabolites (34). The data provided a first hint of a possibly higher PPP flux in \(C.\ glutamicum\) due to overexpression of fructose 1,6-bisphosphatase. The larger fraction of nonlabeled glucose 6-phosphate, measured as the M\(_0\) fraction of the corresponding glucose monomer of trehalose (Table 7), indicated a higher reversibility of glucose 6-phosphate isomerase in \(C.\ glutamicum\) lysC\textsuperscript{cbr} PEFTU\textsuperscript{fbr}.

Based on all experimental data, the intracellular carbon fluxes were calculated using a \(^{13}\text{C}\) flux model that involves scrambling in symmetric molecules, \(^{13}\text{C}\) incorporation from metabolite and isotopomer balancing (35). This model fully describes a mathematical model corresponding to the optimized set of fluxes (Calc). M\(_0\) denotes the relative amount of nonlabeled mass isotopomer fraction, M\(_1\) the relative amount of the single-labeled mass isotopomer fraction, and corresponding terms stand for higher labeling. Amino acids were analyzed by GC-MS as trimethyl-dimethylsilyl derivatization of the global minimum. Concerning the obtained fit, excellent agreement between experimentally determined and calculated mass isotopomer ratios was achieved (Table 7). All conclusions given below for the relative fluxes also hold for absolute fluxes, since the specific glucose uptake rates for the two strains were nearly identical, as shown above.

The obtained metabolic flux distributions of \(C.\ glutamicum\) lysC\textsuperscript{cbr} and \(C.\ glutamicum\) lysC\textsuperscript{cbr} PEFTU\textsuperscript{fbr} are displayed in Fig. 2 and 3. Obviously, overexpression of fructose 1,6-bisphosphatase affected different pathways in the central metabolism of \(C.\ glutamicum\). Most importantly, carbon flux was redirected from glycolysis toward the PPP. The PPP flux was 10% higher in the FBPase mutant than in the parent strain, so the supply of NADPH by the PPP, accordingly, was about 20% higher in the mutant. The net flux through glucose 6-phosphate isomerase was lower in \(C.\ glutamicum\) lysC\textsuperscript{cbr} PEFTU\textsuperscript{fbr}. Interestingly, this enzyme exhibited a slightly higher reversibility when the fructose 1,6-bisphosphatase was overexpressed. It seems likely that the overexpressed fructose 1,6-bisphosphatase increased the intracellular pool of fructose 6-phosphate and thus favored the reverse reaction of glucose 6-phosphate isomerase.

In the FBPase mutant, a slightly higher flux entered the pyruvate pool from the lower glycolytic chain. This was partly due to the reduced formation of glycerol and dihydroxyacetone. Overexpression of fructose 1,6-bisphosphatase further led to a flux redirection from anaplerotic carboxylation toward the lysine pathway and, via pyruvate dehydrogenase, toward the tricarboxylic acid cycle. As an example, the flux through pyruvate dehydrogenase was about 10% higher in the FBPase mutant. This results in a significantly higher NADPH supply via isocitrate dehydrogenase in the FBPase mutant, wherein

### Table 5. Biomass and metabolites of \(C.\ glutamicum\) ATCC 13032 lysC\textsuperscript{cbr} and \(C.\ glutamicum\) ATCC 13032 lysC\textsuperscript{cbr} PEFTU\textsuperscript{fbr} from cultivation on 99% \([^{1-13}\text{C}]\text{glucose}\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>lysC\textsuperscript{cbr}</th>
<th>lysC\textsuperscript{cbr} PEFTU\textsuperscript{fbr}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass (mg)</td>
<td>78.7 ± 1.5</td>
<td>62.4 ± 1.8</td>
</tr>
<tr>
<td>Lysine (mg)</td>
<td>84.7 ± 2.9</td>
<td>120.6 ± 3.0</td>
</tr>
<tr>
<td>Glycine (mg)</td>
<td>1.8 ± 0.1</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>Trehalose (mg)</td>
<td>8.8 ± 0.4</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>α-Ketoglutarate (mg)</td>
<td>17.4 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Lactate (mg)</td>
<td>0.8 ± 0.0</td>
<td>3.7 ± 0.1</td>
</tr>
</tbody>
</table>

\(\text{M}^0\) Experimental yields are mean values of parallel incubations with corresponding deviations. All yields are given in mmol of product mol \(^{-1}\) of glucose.

### Table 6. Anabolic demand of \(C.\ glutamicum\) ATCC 13032 lysC\textsuperscript{cbr} and \(C.\ glutamicum\) ATCC 13032 lysC\textsuperscript{cbr} PEFTU\textsuperscript{fbr} on glucose

<table>
<thead>
<tr>
<th>Precursor demand [mmol (mol glucose) (^{-1})]</th>
<th>lysC\textsuperscript{cbr}</th>
<th>lysC\textsuperscript{cbr} PEFTU\textsuperscript{fbr}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose 6-phosphate</td>
<td>16.1 ± 0.3</td>
<td>12.8 ± 0.2</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>24.2 ± 0.5</td>
<td>19.2 ± 0.4</td>
</tr>
<tr>
<td>Pentose 5-phosphate</td>
<td>69.2 ± 1.3</td>
<td>54.8 ± 1.0</td>
</tr>
<tr>
<td>Erythrose 4-phosphate</td>
<td>21.1 ± 0.4</td>
<td>16.7 ± 0.3</td>
</tr>
<tr>
<td>Glyceroldehyde 3-phosphate</td>
<td>10.2 ± 0.2</td>
<td>8.0 ± 0.2</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>101.9 ± 1.9</td>
<td>80.8 ± 1.5</td>
</tr>
<tr>
<td>Pyruvate/phosphoenolpyruvate</td>
<td>240.4 ± 4.6</td>
<td>190.5 ± 3.6</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>96.3 ± 1.8</td>
<td>76.3 ± 1.5</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>116.3 ± 2.2</td>
<td>92.2 ± 1.8</td>
</tr>
<tr>
<td>Acetate carboxylase A</td>
<td>250.1 ± 4.8</td>
<td>198.1 ± 3.8</td>
</tr>
<tr>
<td>Diaminopimelate + lysine</td>
<td>27.4 ± 0.5</td>
<td>21.7 ± 0.4</td>
</tr>
</tbody>
</table>

\(\text{M}^0\) All yields are given in mmol product mol \(^{-1}\) except the yield for biomass, which is given in mg of dry biomass mol \(^{-1}\).

\(\text{M}^1\) The estimation of precursor demand was based on the experimental biomass yield obtained for each strain (Table 3) and the biomass composition previously measured for \(C.\ glutamicum\) (9).

### Table 7. Relative mass isotopomer fractions of amino acids from cell protein and of secreted trehalose of lysine-producing \(C.\ glutamicum\) ATCC 13032 lysC\textsuperscript{cbr} and \(C.\ glutamicum\) ATCC 13032 lysC\textsuperscript{cbr} PEFTU\textsuperscript{fbr}

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Type</th>
<th>(C.\ glutamicum) lysC\textsuperscript{cbr}</th>
<th>(C.\ glutamicum) lysC\textsuperscript{cbr} PEFTU\textsuperscript{fbr}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>M(_2)</td>
<td>0.509 ± 0.354</td>
<td>0.106</td>
</tr>
<tr>
<td>Valine</td>
<td>M(_2)</td>
<td>0.348 ± 0.389</td>
<td>0.184</td>
</tr>
<tr>
<td>Threonine</td>
<td>M(_2)</td>
<td>0.334 ± 0.376</td>
<td>0.196</td>
</tr>
<tr>
<td>Aspartate</td>
<td>M(_2)</td>
<td>0.333 ± 0.375</td>
<td>0.196</td>
</tr>
<tr>
<td>Glutamate</td>
<td>M(_2)</td>
<td>0.250 ± 0.366</td>
<td>0.239</td>
</tr>
<tr>
<td>Serine</td>
<td>M(_2)</td>
<td>0.247 ± 0.365</td>
<td>0.240</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>M(_2)</td>
<td>0.274 ± 0.381</td>
<td>0.228</td>
</tr>
<tr>
<td>Glycine</td>
<td>M(_2)</td>
<td>0.271 ± 0.382</td>
<td>0.224</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>M(_2)</td>
<td>0.741 ± 0.185</td>
<td>0.741</td>
</tr>
<tr>
<td>Trehalose</td>
<td>M(_2)</td>
<td>0.062 ± 0.601</td>
<td>0.207</td>
</tr>
</tbody>
</table>

\(\text{M}^1\) 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 nonlabeled mass isotopomer fraction, M\(_1\) the relative amount of the single-labeled mass isotopomer fraction, and corresponding terms stand for higher labeling. Amino acids were analyzed by GC-MS as trimethyl-dimethylsilyl derivatization and trehalose as the trimethylsilyl derivative.
FIG. 2. In vivo carbon flux distribution in the central metabolism of glucose-grown *Corynebacterium glutamicum* ATCC 13032 lysC^ATCC^ during lysine production. The strain contains a feedback-resistant aspartokinase. Fluxes were estimated from the best fit to the experimental results using a comprehensive approach of combined metabolite balancing and isotopomer modeling for a ^13^C tracer experiment during growth on [1-^13^C]glucose and measurement of labeling of amino acids from the cell protein and of trehalose from the culture supernatant by GC-MS. Net fluxes are given in square symbols; for reversible reactions the direction of the net flux is indicated by an arrow beside the corresponding black box. Numbers in brackets below the fluxes of transaldolase, transketolase, and glucose 6-phosphate isomerase indicate flux reversibility. Fluxes toward anabolism are displayed in gray boxes. All fluxes are expressed as a molar percentage of the mean specific glucose uptake rate (4.9 mmol g^-1 h^-1), which was set to 100%.
FIG. 3. In vivo carbon flux distribution in the central metabolism of glucose-grown *Corynebacterium glutamicum* ATCC 13032 lysC<sup>Δ</sup> P<sub>EPTU</sub>fbp during lysine production. The strain contains a feedback-resistant aspartokinase and exhibits amplified expression of fructose 1,6-bisphosphatase. The fluxes were estimated from the best fit to the experimental results using a comprehensive approach of combined metabolite balancing and isotopomer modeling for a <sup>13</sup>C tracer experiment during growth on [1-<sup>13</sup>C]glucose and measurement of labeling of amino acids from the cell protein and of trehalose from the culture supernatant by GC-MS. Net fluxes are given in square symbols; for reversible reactions the direction of the net flux is indicated by an arrow beside the corresponding black box. Numbers in brackets below the fluxes of transaldolase, transketolase, and glucose 6-phosphate isomerase indicate flux reversibility. Fluxes toward anabolism are displayed in gray boxes. All fluxes are expressed as a molar percentage of the mean specific glucose uptake rate (4.8 mmol g<sup>−1</sup> h<sup>−1</sup>), which was set to 100%.
the 15% increase observed for this enzyme was almost as high as that of the PPP. The increased tricarboxylic acid cycle flux was probably the consequence of the reduced anabolic demand in *C. glutamicum* lysC<sup>thr</sup> P<sub>EFTUfbp</sub>.

Carbon channeled through the central catabolic pathways is withdrawn to a lesser extent for anabolic purposes, so that a relatively high fraction remains to be oxidized in the tricarboxylic acid cycle. Overall, the NADPH flux supplied in *C. glutamicum* lysC<sup>thr</sup> P<sub>EFTUfbp</sub> by the PPP and by isocitrate dehydrogenase was 180%, and thus markedly higher than the corresponding flux of 146% for *C. glutamicum* lysC<sup>thr</sup>. The relatively large amount of NADPH formed then functions as a driving force for the deregulated lysine pathway and probably is the reason for the observed significant improvement in lysine production. The flux calculation further revealed that glycine used in anabolism and also secreted into the medium is mainly derived from serine. In *C. glutamicum* lysC<sup>thr</sup> P<sub>EFTUfbp</sub>, 90.8% of the glycine stems from serine whereas only 9.2% is supplied via threonine. In *C. glutamicum* lysC<sup>thr</sup>, the relative supplies of glycine from serine and from threonine were 93.4% and 6.6%, respectively, and thus similar. Note that for simplicity reasons the glycine fluxes are not explicitly shown in Fig. 2 and 3 but rather are lumped into the anabolic demand for oxaloacetate and 3-phosphoglycerate.

**DISCUSSION**

Overexpression of fructose 1,6-bisphosphatase allows a significant improvement of lysine production in *C. glutamicum* grown on glucose, sucrose, or fructose. Therefore, this genetic modification seems promising for application to industrial production processes which are based on starch, molasses, or raw sugar. The narrow confidence intervals obtained from the Monte Carlo analysis for the fluxes underline the fact that carbon fluxes were estimated with high precision (Table 8).

As shown here, amplification of fructose 1,6-bisphosphatase leads to a redirection of carbon from glycolysis toward the PPP. Interestingly, this was not reflected by an increased formation of trehalose, which one might have expected. In fact, the trehalose level was even lower in the P<sub>EFTUfbp</sub> mutant. In addition, formation of the by-products glycerol and dihydroxyacetone was significantly decreased. These compounds are also secreted in large amounts by other strains of *C. glutamicum* during growth (4) and lysine production (11). The reduced formation of glycerol and dihydroxyacetone has a positive side effect, because these compounds are not reutilized during the cultivation of *C. glutamicum* and thus result in wasted carbon with respect to product synthesis (2).

So far, the genes involved in the formation of glycerol and dihydroxyacetone in *C. glutamicum* have not been identified (11). The levels of glycerol and dihydroxyacetone were directly related to the in vitro activity of fructose 1,6-bisphosphatase. Obviously, the formation of these products decreased with increasing flux from fructose 1,6-bisphosphate toward fructose 6-phosphate. Thus, we conclude that, as previously speculated, their synthesis is an overflow phenomenon in *C. glutamicum* caused by limited capacity of an enzyme downstream of the fructose 1,6-bisphosphatase pool, such as glyceraldehyde 3-phosphate dehydrogenase (4).

Fructose 1,6-bisphosphatase is known to be effectively regulated at the metabolic level by, e.g., phosphoenolpyruvate or AMP (23). *C. glutamicum* lysC<sup>thr</sup> revealed a low basal in vitro fructose 1,6-bisphosphatase activity during growth. Comparable results were previously obtained for the wild-type strain *C. glutamicum* ATCC 13032 grown on glucose, gluconate, ribose, citrate, pyruvate, or lactate (23). Due to a strong metabolic regulation, this enzyme is supposed to exhibit negligible in vivo activity during growth on sugars (10, 32). The metabolic regulation of FBPase was also observed in mutants with an overexpressed *fbp* gene, since the expression level, but not the properties, of the enzyme were changed. It therefore appears likely that the actual in vivo activity of FBPase in the mutants is lower than the in vitro levels measured.

Considering a protein content of about 55% (27), the in vitro activity of FBPase of 125 μM<sup>-1</sup> observed for glucose-grown *C. glutamicum* lysC<sup>thr</sup> P<sub>EFTUfbp</sub> can be expressed as a potential flux of 4.3 mmol g<sup>-1</sup> h<sup>-1</sup>. This is almost as high as the total influx of substrate into the cell. The actual in vivo flux of FBPase is probably below that level, but surely it is significant. Otherwise, all the metabolic changes such as improved lysine production, reduced by-product formation, or alteration of intracellular carbon flux would not have been observed. The data clearly show that FBPase was active in vivo in *C. glutamicum* lysC<sup>thr</sup> P<sub>EFTUfbp</sub> and in *C. glutamicum* lysC<sup>thr</sup> P<sub>SOGfbp</sub>. The exact in vivo flux through fructose 1,6-bisphosphatase in the mutants could not be assessed in the present work, since this additional reaction does not influence stoichiometry or labeling patterns of the metabolites analyzed.

In previous studies with *S. cerevisiae*, overexpression of fruc-

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**TABLE 8. Statistical evaluation of metabolic fluxes of lysine-producing *C. glutamicum* ATCC 13032 lysC<sup>thr</sup> P<sub>EFTUfbp</sub> on glucose**

<table>
<thead>
<tr>
<th>Flux parameter</th>
<th>90% CIs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine-6-phosphate isomerase</td>
<td>49.3, 50.3</td>
</tr>
<tr>
<td>Glycine-6-phosphate dehydrogenase</td>
<td>46.3, 47.3</td>
</tr>
<tr>
<td>Transaldolase</td>
<td>13.8, 14.2</td>
</tr>
<tr>
<td>Transketolase 1</td>
<td>13.8, 14.2</td>
</tr>
<tr>
<td>Transketolase 2</td>
<td>11.6, 12.2</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>156.8, 158.3</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>146.3, 148.5</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase</td>
<td>74.7, 81.1</td>
</tr>
<tr>
<td>Pyruvate carboxylase</td>
<td>67.6, 75.7</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase</td>
<td>33.7, 41.0</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>49.0, 57.1</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>49.0, 57.1</td>
</tr>
<tr>
<td>Oxoglutarate dehydrogenase</td>
<td>37.4, 46.1</td>
</tr>
<tr>
<td>Aspartokinase</td>
<td>10.7, 11.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by 13C tracer studies with mass spectrometry and metabolite balancing; 90% confidence intervals (CI) of key flux parameters were obtained by a Monte Carlo approach, including 100 independent parameter estimation runs for each strain with statistically varied experimental data.

<sup>b</sup> Flux reversibility is defined as ratio of back flux to net flux.
tose 1,6-bisphosphatase did not affect the growth rate or biomass yield, probably due to strong inhibition of the enzyme by fructose 2,6-bisphosphate (19). Thereby, the enzyme was inhibited by about 95% already at a fructose 2,6-bisphosphate concentration of 8 μM. In comparison, the fructose 1,6-bisphosphatase in C. glutamicum is only slightly inhibited by fructose 2,6-bisphosphate (23). This might be a major reason why overexpressed fructose 1,6-bisphosphatase could evolve significant activity in vivo.

Due to the presence of an active fructose 1,6-bisphosphatase, metabolic cycling between the pools of fructose 6-phosphate and fructose 1,6-bisphosphate probably occurred in overexpressing strains. This metabolic cycle, catalyzing the breakdown of ATP into ADP and P_i, did not, however, show any negative effects in C. glutamicum lysCfrb PSODbfbp or C. glutamicum lysCfrb P_EFTEFbfbp. In fact, during growth on fructose and sucrose, the presence of an active fructose 1,6-bisphosphatase was even beneficial, as evidenced by an increased growth rate, substrate uptake rate, or biomass yield. Thus, we conclude that the wasting of energy that is probably linked to the overexpression of fructose 1,6-bisphosphatase does not play a major role in the strains examined. In this regard, it was found for Saccharomyces cerevisiae that high-level overexpression of an unregulated fructose 1,6-bisphosphatase from Escherichia coli had only minor effects but provided a slight competitive advantage during growth in mixed cultures (20).

Overexpression of fructose 1,6-bisphosphatase allows a significant improvement of lysine production in C. glutamicum by the redirection of carbon from glycolysis toward the PPP and an increased NADPH supply. As shown herein, lysine production is increased during growth on glucose as well as on sucrose, so this modification seems useful for both starch- and molasses-based production processes. The use of different promoters provides a means of fine-tuning the fructose 1,6-bisphosphatase level, so that for each strain the enzyme activity could be adjusted to a level that would result in optimal performance. In this regard, the future search for natural C. glutamicum promoters with different expression levels could provide a useful tool for targeted strain engineering.

Since the metabolic regulation of fructose 1,6-bisphosphatase seems similar for different organisms, its overexpression could lead to similar effects in other bacteria, such as E. coli or Bacillus subtilis. In this regard, the redirection of flux toward the PPP resulting from overexpression of fructose 1,6-bisphosphatase should also be of interest for the production of other NADPH-demanding compounds, such as methionine (14), isoleucine (18), and fatty acids (6), as well as for products directly stemming from the PPP, such as vitamin B, (25), nucleotides (1, 10), and aromatic amino acids (7, 8). Due to negative metabolic regulation of fructose 1,6-bisphosphatase, only a certain fraction of the enzyme is probably active in vivo, so that high expression levels are required to achieve significant metabolic effects. An interesting alternative might, therefore, be to release FBPase from metabolic regulation, e.g., by mutation of the enzyme.

In addition to overexpression of fructose 1,6-bisphosphatase, other attempts have been made to redirect carbon flux toward the PPP in C. glutamicum. In this regard, the deletion of the gene for phosphogluconate isomerase, which forces the cell to completely metabolize the substrate glucose via the PPP, has been suggested (15). This deletion indeed leads to improved production of lysine in C. glutamicum but is, however, linked to severe growth defects. Moreover, the lack of phosphogluconate isomerase blocks the recycling of carbon from glycolysis back toward the PPP. Due to this, fructose, as the sole carbon source or as part of sucrose, cannot reenter the PPP, so that the maximum lysine yield possible for such a strain will be significantly below the theoretical optimum of 0.75 mol mol−1, which requires a 150% flux through the PPP (26).

Heterologous expression of fructokinase in C. glutamicum grown on fructose or on sucrose is an interesting alternative method to potentially increase the fructose flux toward the PPP and to increase amino acid production (17). C. glutamicum ATCC 13032 expressing fructokinase, however, exhibits a growth deficiency on sucrose. The metabolic effects involved are not yet clear. As shown here, fructose 1,6-bisphosphate overexpression leads to increased PPP flux in C. glutamicum. It seems promising to further enhance the PPP flux, e.g., by introducing additional modifications such as modified variants of 6-phosphogluconate dehydrogenase (21).

Summarizing the present work shows that overexpression of FBPass is useful for industrial lysine production by C. glutamicum and, generally, also for the production of other NADPH-demanding compounds, as well as for products directly stemming from the PPP. It provides an excellent example of how quantitative physiological studies and genetic engineering can be combined in an effective manner for rational engineering of production strains.

REFERENCES


