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Deregulation of Aspartokinase by Single Nucleotide Exchange Leads to Global Flux Rearrangement in the Central Metabolism of *Corynebacterium glutamicum*

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Abstract The wild-type *Corynebacterium glutamicum* ATTC 13032 and *Corynebacterium glutamicum* ATTC 13032 *lysC* S301Y, exhibiting a deregulated aspartokinase, were compared concerning growth, lysine production, and intracellular carbon fluxes. Both strains differ by only one single nucleotide over the whole genome. In comparison to the wild-type, the mutant showed significant production of lysine with a molar yield of 0.087 mol (mol glucose^{-1}) whereas the biomass yield was reduced. The deregulation of aspartokinase further led to a global rearrangement of carbon flux throughout the whole central metabolism. This involved an increased flux through the pentose phosphate pathway (PPP) and an increased flux through anaplerosis. Because of this, the mutant revealed an enhanced supply of NADPH and oxaloacetate required for lysine biosynthesis. Additionally, the lumped flux through phosphoenolpyruvate carboxykinase and malic enzyme, withdrawing oxaloacetate back to the glycolysis and therefore detrimental for lysine production, was increased. The reason for this might be a contribution of malic enzyme to NADPH supply in the mutant in the mutant. The observed complex changes are remarkable, because they are due to the minimum genetic modification possible, the exchange of only one single nucleotide.

Key words: ^{13}C labeling, lysine, *lysC*, metabolic flux analysis, mass spectrometry, isotopomer modeling

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 700,000 tons, which are produced worldwide with this microorganism per annum [13]. The key enzyme for regulation of lysine biosynthesis is aspartokinase, which catalyzes the conversion of aspartate to aspartate semialdehyde in the lysine biosynthetic pathway. In *C. glutamicum*, the only aspartokinase isoform present [16] is feedback inhibited by concerted action of threonine and lysine, the two end products of the emerging pathways [10, 15]. The deregulation of aspartokinase has been proposed as the most important step in the development of lysine overproducing strains [13]. Screening and sequencing of feedback-resistant strains of *C. glutamicum* revealed that mutations in the regulatory β-subunit of aspartokinase are responsible for the deregulated lysine production. Subsequently, different nucleotide substitutions were identified in the *lysC* gene that led to a feedback-resistant aspartokinase [17]. Plasmid expression of a feedback-resistant *lysC* gene in the *C. glutamicum* wild-type results in increased accumulation of lysine [3]. Hereby, the plasmid copy number influences the extent of lysine production [5]. Further studies elucidating the metabolic consequences of a deregulated aspartokinase in *C. glutamicum* have so far not been carried out. Accordingly, our knowledge concerning the overall effects of this key mutation in *C. glutamicum* is still very poor. A detailed investigation of the physiological effects of such modifications has been proposed as crucial for future improvement of lysine-producing strains [14]. In this regard, the present work describes expression of a feedback-resistant aspartokinase in the wild-type *C. glutamicum* ATCC 13032 and investigates its impact on growth, lysine production, and intracellular carbon fluxes.

Materials and Methods

Microorganisms

The wild-type *C. glutamicum* ATCC 13032 was obtained from the American Type and Culture Collection (Manassas, VA, U.S.A.). The lysine overproducing mutant *C. glutamicum* ATCC 13032 lysC^{fbr} was kindly donated by Axaron (Heidelberg, Germany). It differs from its precursor by only

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one single nucleotide substitution leading to the replacement S301Y in the *lysC* gene encoding aspartokinase. This replacement has been previously described for release of aspartokinase from feedback inhibition [6].

Media

Complex medium containing 5 g/l glucose, 5 g/l yeast extract, 10 g/l tryptone, 5 g/l NaCl and 18 g/l agar was used for agar plates. First, precultures were grown in the same medium without agar. Second, precultivation and tracer cultivation were carried out on a minimal medium containing 80 mM glucose. The minimal medium contained per liter: (A) 0.055 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl in 679 ml deionized water; (B) 80 mmol glucose in 100 ml deionized water and adjusted to pH 5.0 with HCl; (C) 16 g K_2HPO_4, 2 g KH_2PO_4 in 80 ml deionized water; (D) 5 g $(NH_4)_2SO_4$ in 100 ml deionized water; (E) 0.5 mg biotin, 1 mg thiamine·HCl in 20 ml deionized water; (F) 20 mg $FeSO_4 \cdot 7H_2O$ in 10 ml deionized water, adjusted to pH 1.0 with HCl; (G) 10 ml of 100× trace elements [18], and (H) 30 mg 3,4-dihydroxybenzoic acid, 50 µl 4 M NaOH in 1 ml deionized water. The solutions (A)–(D) were autoclaved separately and combined after cooling to room temperature; solutions (E)–(H) were sterilized by filtration and subsequently added. For metabolic flux analysis, cultivations were carried out with 99% [1-^{13}C] glucose as substrate.

Cultivation

Cells from frozen glycerol stock cultures were grown on agar plates for 48 h at 30°C. Single colonies from the plate were used to inoculate the first preculture with 50 ml medium in a 500-ml baffled shake flask, which was incubated for 8 h. Cells were harvested by centrifugation (8,800 ×*g*, 2 min, 30°C), washed twice with sterile 0.9% NaCl, and used as inoculum for the second preculture, which was grown in 250-ml baffled shake flasks with 25 ml minimal medium for 8 h. The preparation of the inoculum for the tracer studies was performed as described above. Tracer studies were carried out in 50-ml flasks with 5 ml medium. All shake flask cultivations were carried out at 30°C and 230 rpm on a rotary shaker (5 cm shaking diameter, Multitron, Infors AG, Bottmingen, Switzerland).

Chemicals

Yeast extract and tryptone were obtained from Difco Laboratories (Detroit, U.S.A.). The tracer substrate 99% [1-^{13}C] glucose was purchased from Campro Scientific (Veenendaal, The Netherlands). All other chemicals were from Sigma (St. Louis, MO, U.S.A.), Merck (Darmstadt, Germany), or Fluka (Buchs, Switzerland) and of analytical grade.

Analytics

The quantification of glucose and organic acids was carried out by HPLC [7]. Glycerol and dihydroxyacetone were quantified enzymatically (Boehringer-Mannheim, Darmstadt, Germany). Quantification of amino acids was performed by HPLC with precolumn OPA derivatization [8]. Cell concentration was determined by a photometer at 660 nm or by gravimetric analysis [7]. Mass isotopomer fractions of amino acids from the cell protein and of trehalose from the culture medium were determined by GC-MS, as described previously [2, 23]. The mean experimental error for the mass isotopomer fractions measured in triplicate 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., Nattick, MA, U.S.A.). Details on the applied computational tools are given elsewhere [7, 20–23]. The metabolic network for growth and lysine production of *C. glutamicum* comprised all central metabolic pathways; *i.e.*, glycolysis, PPP, tricarboxylic acid (TCA) cycle, and the enzymes interconverting phosphoenolpyruvate/pyruvate and oxaloacetate/malate. In *C. glutamicum* pyruvate carboxylase, PEP carboxylase, malic enzyme, and phosphoenolpyruvate carboxykinase can catalyze the interconversion of C_3 metabolites of glycolysis and C_4 metabolites of the TCA cycle [12]. The tracer experiments of the present work cause identical labeling patterns for the two alternative anaplerotic reactions and for the three possible decarboxylating reactions, respectively. Therefore, the pools of pyruvate and phosphoenolpyruvate and the pools of oxaloacetate and malate are lumped and the flux of carboxylation and decarboxylation, as regarded in the networks in Figs. 1A and 1B, each represents the overall flux by the concerted action of all potential enzymes involved. Additionally, the pathways for the biosynthesis of lysine and byproducts (Table 2) and the anabolic pathways from intermediary precursors into biomass were implemented (Table 3). Calculation of the anabolic demand for the different precursors was based on data on the biomass composition of *C. glutamicum* [19]. Labeling data of proteinogenic amino acids and of trehalose and stoichiometric data from the cultivations were combined for calculation of metabolic flux. The set of fluxes that gave minimum deviation between experimental and simulated mass isotopomer fractions was taken as the best estimate for the intracellular flux distribution. As error criterion, a weighted sum of least squares was used [21]. Statistical analysis of the obtained fluxes was carried out by a Monte-Carlo approach [21]. From the obtained data, 90% confidence limits for the single parameters were calculated.

RESULTS AND DISCUSSION

In contrast to the wild-type of *C. glutamicum*, the feedback-resistant mutant revealed significant secretion of lysine

Table 1. Biomass and metabolites of *C. glutamicum* ATCC 13032 and *C. glutamicum* ATCC 13032 lysC^{fbr}. Experimental yields are mean values of parallel incubations with corresponding deviations. All yields are given in (mmol product)/(mol), except for the yield for biomass, which is given in (mg of dry biomass)/(mmol).

Yield	Wild-type	lysC^{fbr}
Biomass	92.78±0.65	80.35±0.21
Lysine	0.08±0.08	86.80±1.01
Valine	0.55±0.01	1.19±0.01
Alanine	0.46±0.03	0.52±0.03
Glycine	7.78±0.02	1.32±0.17
Glycerol+DHA	1.24±0.20	1.72±0.09
Trehalose	3.58±0.03	9.60±1.61
2-Oxoglutarate	1.54±0.01	2.05±0.09
Lactate	3.99±0.33	0.00±0.00

(Table 1). The additional carbon required for the overproduction of lysine was mainly withdrawn from anabolism, as indicated by the decreased biomass yield in the mutant strain. Slight differences were also observed in the spectrum of byproducts formed. Figs. 1A and 1B show the carbon fluxes through the central metabolic pathways of the *C. glutamicum* wild-type and the lysC^{fbr} mutant. The fluxes are given as relative numbers normalized to the specific glucose uptake rate. Both strains showed significantly different flux distributions. *C. glutamicum* lysC^{fbr} exhibited an increased flux through the lysine pathway. Moreover, it showed an enhanced PPP flux. A similar correlation between lysine production and PPP flux was previously observed for different classically derived mutant strains of *C. glutamicum*. Because of the undefined genetic background

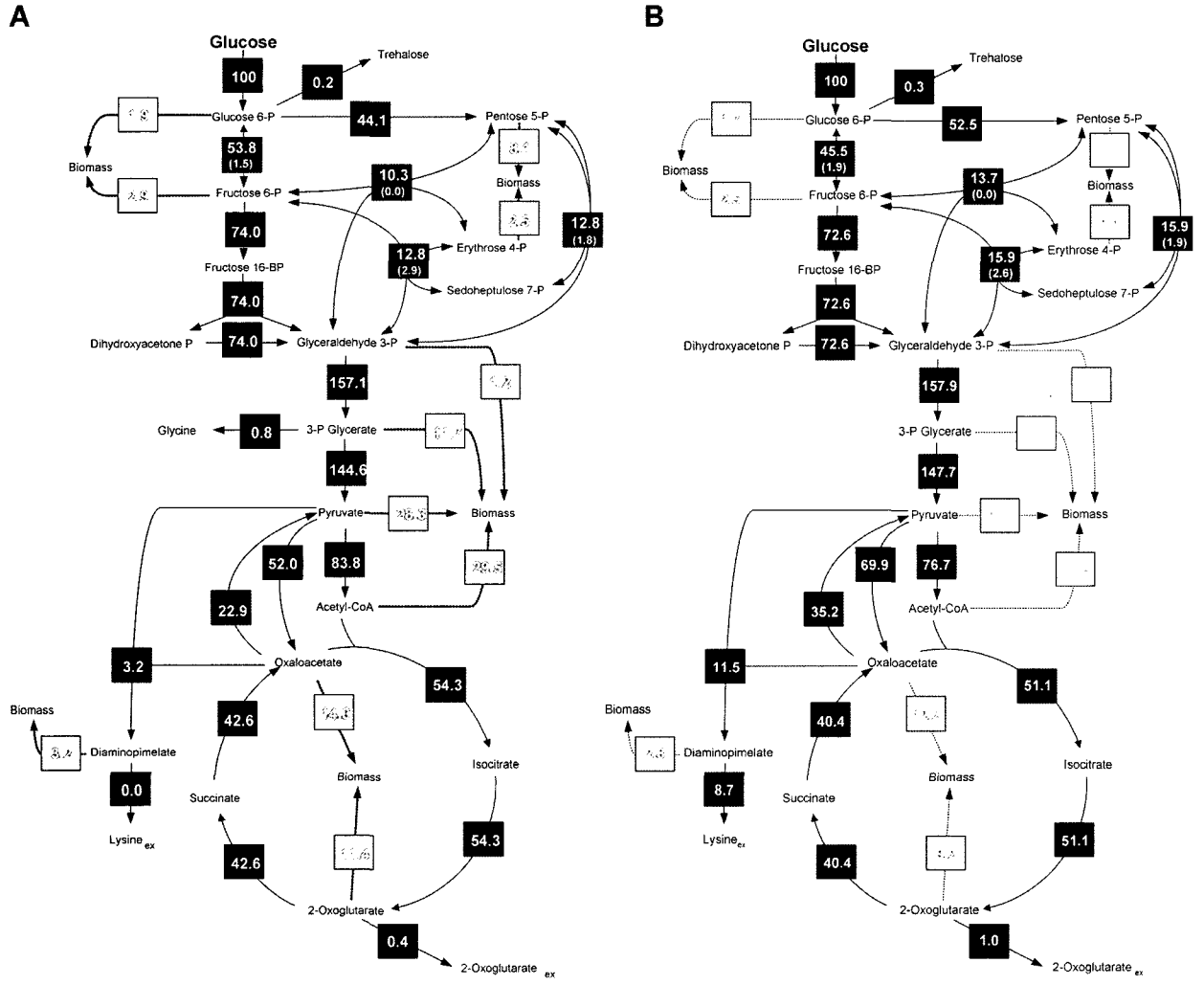

Fig. 1. *In vivo* carbon flux distribution in the central metabolism of *Corynebacterium glutamicum* ATCC 13032 (**A**) and *Corynebacterium glutamicum* ATCC 13032 lysCbfr (S301Y) (**B**).

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 ^{13}C tracer experiment on 99% [1-^{13}C] glucose and labeling measurement of amino acids from the cell protein and of trehalose from culture supernatant by GC-MS, respectively. Net fluxes are given in square symbols. Numbers in brackets below the fluxes of transaldolase, transketolase, and glucose-6-phosphate isomerase indicate flux reversibility.

Table 2. Anabolic demand of the wild-type *C. glutamicum* ATCC 13032 and lysine overproducing *C. glutamicum* ATCC 13032 lysC^{fbr} on glucose.

Precursor demand[a] mmol/(mol glucose)	Wild-type	lysC^{fbr}
Glucose-6-phosphate	19.0	16.5
Fructose-6-phosphate	28.6	24.7
Pentose-5-phosphate	81.6	70.6
Erythrose-4-phosphate	24.9	21.5
Glyceraldehyde-3-phosphate	12.0	10.4
3-Phosphoglycerate	120.2	104.1
Pyruvate/Phosphoenolpyruvate	301.9	261.5
α-Ketoglutarate	289.2	250.5
Oxaloacetate	137.1	118.8
Acetyl CoA	113.6	98.3
Diaminopimelate+Lysine[b]	32.3	28.0

[a]The estimation of precursor demand was based on the experimental biomass yield obtained for each strain (Table 1) and the biomass composition previously measured for *C. glutamicum*.

[b]Diaminopimelate and lysine are regarded as separate anabolic precursors. This is because of the fact that anabolic fluxes from pyruvate and oxaloacetate into diaminopimelate (cell wall) and lysine (protein) contribute, in addition to the flux of lysine secretion, to the overall flux through the lysine biosynthetic pathway.

of these strains, it could however not be precisely concluded in the earlier study whether this flux increase into the PPP is the response to the increased demand for NADPH or the result of other mutations, such as in PPP enzymes. The present study, however, clearly proves that the amplification of the lysine pathway alone can activate the PPP. The linking mechanism between the lysine flux and the PPP flux is probably the intracellular NADPH level. It has been previously shown that the PPP enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in *C. glutamicum* are inhibited by NADPH [9]. We conclude that the increased flux through the lysine pathway and the resulting increased demand for NADPH cause a reduced intracellular level of NAPDH, which then partially releases the PPP enzymes mentioned above from the inhibition by NADPH and leads to an increased PPP flux. The increased activity of the PPP resulted in a higher NADPH supply (156%) in the lysine-producing mutant as compared with the wild-type (142%).

Responding to the increased demand for pyruvate and oxaloacetate, the net flux through anaplerotic carboxylation increased about 5%. Accordingly, the anaplerotic net flux displays the lumped activity of all enzymes involved. A closer inspection revealed that the forward flux increased by almost 20% and thus was much stronger then indicated by the net flux. Thus, only a small fraction of the additionally supplied oxaloacetate was utilized for lysine biosynthesis

Table 3. Relative mass isotopomer fractions of amino acids from the cell protein and of secreted trehalose of *C. glutamicum* ATCC 13032 and lysine overproducing *C. glutamicum* ATCC 13032 lysC^{fbr} cultivated on 99% [1-^{13}C] glucose. 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 t-butyl-dimethylsilyl derivates and trehalose as trimethylsilyl derivate, respectively.

Analyte		*C. glutamicum* ATCC 13032				*C. glutamicum* ATCC 13032 *lysC^{fbr}*		
		M_0	M_1	M_2		M_0	M_1	M_2
Alanine (m/z 260)	exp	0.494	0.368	0.107	exp	0.517	0.350	0.103
	calc	0.493	0.368	0.107	calc	0.516	0.351	0.104
Valine (m/z 288)	exp	0.321	0.406	0.197	exp	0.350	0.398	0.182
	calc	0.323	0.406	0.196	calc	0.354	0.397	0.180
Threonine (m/z 404)	exp	0.320	0.385	0.199	exp	0.344	0.378	0.188
	calc	0.323	0.379	0.200	calc	0.345	0.374	0.190
Aspartate (m/z 418)	exp	0.321	0.381	0.201	exp	0.345	0.376	0.190
	calc	0.322	0.378	0.201	calc	0.345	0.373	0.191
Glutamate (m/z 432)	exp	0.244	0.372	0.244	exp	0.255	0.369	0.237
	calc	0.234	0.365	0.247	calc	0.258	0.367	0.235
Serine (m/z 390)	exp	0.439	0.369	0.142	exp	0.458	0.356	0.138
	calc	0.435	0.369	0.145	calc	0.454	0.356	0.141
Phenylalanine (m/z 336)	exp	0.256	0.388	0.236	exp	0.279	0.384	0.225
	calc	0.259	0.386	0.236	calc	0.285	0.385	0.222
Glycine (m/z 246)	exp	0.745	0.181		exp	0.742	0.182	
	calc	0.745	0.182		calc	0.744	0.184	
Tyrosine (m/z 466)	exp	0.223	0.353	0.253	exp	0.246	0.354	0.236
	calc	0.223	0.359	0.252	calc	0.245	0.361	0.240
Trehalose (m/z 361)	exp	0.057	0.605	0.210	exp	0.072	0.596	0.208
	calc	0.058	0.607	0.210	calc	0.073	0.597	0.207

or anabolism. The major fraction was recycled back to glycolysis. The fact that the back flux from oxaloacetate to glycolysis was significantly increased appears interesting. This finding is different from previous flux results showing a typically decreased *in vivo* activity of the corresponding enzymes with increased lysine production [21]. Two possibilities seem likely to explain this increase of back flux from oxaloacetate to glycolysis. The first one involves an enhanced contribution of phosphoenolpyruvate carboxykinase. Together with pyruvate carboxylase and pyruvate kinase, this enzyme can catalyze the cyclic conversion of phosphoenolpyruvate. During each turn of this cycle, one ATP is cleaved into ADP and P_i. *C. glutamicum* lysC^{fbr} still has relatively high fluxes through NADH-generating reactions in glycolysis and the TCA cycle, but a significantly decreased demand for ATP as indicated by the low biomass yield. The enhanced recruitment of such a cycle could therefore be linked to energy metabolism and the waste of excess ATP. Secondly, the increased back flux might be due to increased activity of malic enzyme as an additional source for NADPH to meet the increased demand for this reduction equivalent [4, 7]. In the present work, fluxes were determined with high precision, as indicated by statistical analysis (Table 4). It can therefore be concluded that the flux differences observed are clearly strain specific. In the past, different strategies have been pursued for the development of lysine overproducers. In addition to feedback-resistant strains, as described here, mutants lacking a functional homoserine dehydrogenase (*hsd*$^-$) have been considered. These strains require threonine and methionine for growth. After depletion of threonine, aspartokinase is released from feedback inhibition, which induces lysine overproduction. Therefore, such mutants reveal a biphasic cultivation profile with separated phases for growth and production. Concerning metabolic fluxes, the different types of producers differ greatly. The lysine production phase in *hsd*$^-$ strains is characterized by a comparably high lysine yield and a low biomass yield. This poses a high NADPH demand to the metabolism. Cells compensate for that by a redirection of flux from glycolysis into the PPP. The relative PPP flux observed during the lysine production phase is typically around 60 and 70% and thus much higher as compared with *C. glutamicum* lysC^{fbr}. Additionally, the back flux withdrawing oxaloacetate from the TCA cycle into glycolysis is significantly lower. The overall production yield of the two types of producers in batch culture, however, is not significantly different. *C. glutamicum* ATCC 13287 *hsd*$^-$, obtained through one single round of random mutation and selection from the wild-type, and *C. glutamicum* lysC^{fbr} (this study) have relatively similar values of 0.12 and 0.09 mol/mol [21].

In summary, the local perturbation of the lysine pathway by a single nucleotide exchange in the gene encoding aspartokinase carried out in the present work leads to a global change of carbon flux in *C. glutamicum*. Important pathways previously suggested to be engineered for improvement of lysine production are thus directly or indirectly affected by the feedback release of aspartokinase. The cellular response observed involves the total metabolic network as a functional unit of interconnected pathways and reactions. It is remarkable that the observed change of the phenotype is caused by the exchange of only one single nucleotide in the genome, the minimal genetic modification possible. The comparison of two *C. glutamicum* lysC^{fbr} strains (this work and Ref. [2]) shows that even different single nucleotide exchanges in the same gene can lead to different flux phenotypes. Compared with the strain examined here, *C. glutamicum* lysC^{fbr} T311I showed the same trend with slightly increased lysine yield, decreased biomass yield, and increased PPP flux. However, all changes were less pronounced. With all caution regarding the fact that the differences between the two *lysC* strains are rather small, and perhaps to some extent within the experimental error, this could be related to different properties of the two aspartokinase variants, depending on the nucleotide exchange performed. In this regard, it was recently shown

Table 4. Statistical evaluation of metabolic fluxes of lysine production of *C. glutamicum* ATCC 13032 (wild-type) and lysine overproducing *C. glutamicum* ATCC 13032 lysC^{fbr} on glucose, determined by ^{13}C tracer studies with mass spectrometry and metabolite balancing: 90% confidence intervals 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.

Flux parameter	Wild-type	lysC^{fbr}
Glucose-6-phosphate isomerase	[53.7 53.9]	[41.2 41.9]
Glucose-6-phosphate dehydrogenase	[44.0 44.2]	[55.8 56.6]
Glyceraldehyde-3-phosphate dehydrogenase	[156.8 157.7]	[160.0 161.7]
Pyruvate kinase	[144.2 145.1]	[151.2 153.7]
Pyruvate dehydrogenase	[82.8 84.9]	[84.1 91.3]
Pyruvate carboxylase/phosphoenolpyruvate carboxylase[a]	[51.3 51.7]	[59.6 64.9]
Phosphoenolpyruvate carboxykinase/malic enzyme[a]	[22.2 23.5]	[29.2 33.0]
Citrate synthase	[54.6 55.5]	[63.4 72.5]
Oxoglutarate dehydrogenase	[41.2 44.2]	[63.4 72.5]

[a]Lumped reactions.

for the aspartokinase III from *E. coli* that the strength of feedback resistance depends on the position and the type of the mutated nucleotide in the *lysC* gene [11]. Complex changes of cellular systems as response to single perturbations have been previously described for different biological systems [1]. Clearly, tools such as metabolic flux analysis are very valuable to directly visualize these often complex rearrangements of metabolism and to understand the underlying regulatory mechanisms.

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