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Review

Pathway Analysis and Metabolic Engineering in *Corynebacterium glutamicum*

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The Gram-positive bacterium *Corynebacterium glutamicum* is used for the industrial production of amino acids, *e.g.* of L-glutamate and L-lysine. During the last 15 years, genetic engineering and amplification of genes have become fascinating methods for studying metabolic pathways in greater detail and for the construction of strains with the desired genotypes. In order to obtain a better understanding of the central metabolism and to quantify the *in vivo* fluxes in *C. glutamicum*, the [^{13}C]-labelling technique was combined with metabolite balancing to achieve a unifying comprehensive pathway analysis. These methods can determine the flux distribution at the branch point between glycolysis and the pentose phosphate pathway. The *in vivo* fluxes in the oxidative part of the pentose phosphate pathway calculated on the basis of intracellular metabolite concentrations and the kinetic constants of the purified glucose-6-phosphate and 6-phosphogluconate dehydrogenases determined *in vitro* were in full accordance with the fluxes measured by the [^{13}C]-labelling technique. These data indicate that the oxidative pentose phosphate pathway in *C. glutamicum* is mainly regulated by the ratio of NADPH/NADP concentrations and the specific activity of glucose-6-phosphate dehydrogenase. The carbon flux *via* the oxidative pentose phosphate pathway correlated with the NADPH demand for L-lysine synthesis.

Although it has generally been accepted that phosphoenolpyruvate carboxylase fulfills a main anaplerotic function in *C. glutamicum*, we recently detected that a biotin-dependent pyruvate carboxylase exists as a further anaplerotic enzyme in this bacterium. In addition to the activities of these two carboxylases three enzymes catalysing the decarboxylation of the C_4 metabolites oxaloacetate or malate are also present in this bacterium. The individual flux rates at this complex anaplerotic node were investigated by using [^{13}C]-labelled substrates. The results indicate that both carboxylation and decarboxylation occur simultaneously in *C. glutamicum* so that a high cyclic flux of oxaloacetate *via* phosphoenolpyruvate to pyruvate was found.

Furthermore, we detected that in *C. glutamicum* two biosynthetic pathways exist for the synthesis of DL-diaminopimelate and L-lysine. As shown by NMR spectroscopy the relative use of both pathways *in vivo* is dependent on the ammonium concentration in the culture medium. Mutants defective in one pathway are still able to synthesise enough L-lysine for growth, but the L-lysine yields with overproducers were reduced. The luxury of having these two pathways gives *C. glutamicum* an increased flexibility in response to changing environmental conditions and is also related to the essential need for DL-diaminopimelate as a building block for the synthesis of the murein sacculus.

Key words: Anaplerotic enzymes / *Corynebacterium glutamicum* / Lysine synthesis / Metabolic engineering / Pathway analysis / Pentose phoshate pathway.

Introduction

In the mid-1950s, Kinoshita and coworkers in Japan isolated a bacterium which excretes large quantities of L-glutamic acid into the culture medium (Kinoshita *et al.*, 1957). This bacterium, *Corynebacterium glutamicum*, is a short, aerobic, Gram-positive rod capable of growing on a variety of sugars or organic acids. Under optimal conditions this organism converts glucose into high yields of L-glutamic acid within a few days. Currently about 1×10^6 tons of this amino acid are produced annually as a flavouring agent with this microorganism (Leuchtenberger, 1996). During the past 40 years various mutants of *C. glutamicum* have been isolated which are also able to produce significant amounts of other L-amino acids. For example, nowadays L-lysine is produced with mutants deregulated in the biosynthetic pathway on a scale of 4.5×10^5 tons/year. This amino acid is mainly used as a feed additive.

The common practice of developing amino-acid-overproducing strains by mutagenesis and selection is a very well established technique (Rowlands, 1984). Mutagenic procedures can be optimised in terms of type of mutagen and dose. Screens can be designed to allow maximum expression and detection of the desirable mutant types. So far the improvement of amino acid-producing strains has mainly been carried out by an iterative procedure of mutagenesis and selection. However, the precise genetic and physiological changes resulting in increased overproduc-

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tion of amino acids in various *C. glutamicum* strains have remained unknown. Success in attempts to further increase the productivities and yields of already highly productive strains will depend on the availability of detailed information on the metabolic pathways, their regulations, and mutations.

In recent years genetic engineering has become a fascinating alternative to mutagenesis and random screening procedures (Sahm *et al.*, 1995). Introduction of genes into microorganisms *via* recombinant DNA techniques is a most powerful method for the construction of strains with the desired genotypes. The opportunity of introducing heterologous genes and regulatory elements permits the construction of metabolic configurations with novel and beneficial characteristics. Furthermore, this approach avoids the complication of uncharacterised mutations that are often obtained with classical whole cell mutagenesis. The improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the application of recombinant DNA technology is called metabolic engineering (Bailey, 1991).

A quantitative description of how a metabolite flux is controlled by individual pathway reactions and how this control changes in response to environmental and genetic changes will provide a rational basis for metabolic engineering. Recent research has led to astonishing progress with respect to the *in vivo* quantification of carbon fluxes and flux control (Eggeling *et al.*, 1996). In this review we present results on the analysis of metabolic fluxes at branching points in the central metabolism and the pathway of L-lysine biosynthesis of *C. glutamicum*. Furthermore, it is shown how metabolic flux analysis can be used in the directed design of the metabolism by using recombinant DNA techniques.

Methods for Pathway Analysis

Knowledge of the pathways and the regulation of their fluxes is a prerequisite for the purposeful metabolic engineering of desired traits in biotechnological organisms. Therefore, for many years, methods for the quantitative assessment of metabolic fluxes have been developed and applied. As shown in Table 1, several intrinsically different approaches can be distinguished. All these methods have their specific advantages and limitations. No single method on its own permits a comprehensive pathway analysis. Methods based on kinetic models (Hayashi and Sakamoto, 1986; Vallino and Stephanopoulos, 1993), control theory (Kacser and Burns, 1981; Heinrich *et al.*, 1977; Joshi and Palsson, 1990) and enzyme analysis have the severe drawback that their applicability for *in vivo* flux determination is questionable since their *in vivo* experimental validation is extremely difficult. Genetic analysis is of little use for quantitative pathway analysis, but represents a very good tool for introducing large defined changes in metabolism thereby allowing the regulatory responses of the metabolic network to be studied. Magnetisation transfer measured by *in vivo* NMR (Brindle, 1988; Schoberth *et al.*, 1996) is a powerful technique especially because it can quantify fast reaction rates *in vivo*. However, due to the low sensitivity of NMR bacteria must be cultivated at high cell densities, which often leads to problems of inadequate oxygen supply (Weuster-Botz and de

Table 1 Various Approaches for Pathway Analysis.

Type of approach	Characteristics	Limitation
Kinetic models	Describes subsection of metabolism based on *in vitro* rate constants of individual enzymes	Many rate constants unknown; extrapolation to *in vivo* situation may not be valid
Control theory	Assigns flux control coefficients to individual reactions based on flux measurements at slightly different enzyme activities	Experimental validation extremely difficult; only valid for infenitesimally small enzyme activity changes
Isotopic tracer experiments	Identification of pathway structure and flux ratios at branchpoints based on isotopic enrichment measurements after application of labelled substrates	Requires costly specifically labelled substrates and special detection equipment
In vivo NMR magnetisation transfer	Direct quantification of reaction rate constants *in vivo* from NMR spin transfer measurements on intact cells	Limited to reactions with highly concentrated metabolites ($> \approx 1$ mM) and rate constants between 0.05 and 5 s^{-1}
Metabolite balancing	Absolute flux quantitation based on extracellular flux measurements and metabolite balances	Underdetermined network forces to apply severe simplifications in the metabolic model
Enzyme analysis	Determination of enzyme properties (kinetic constants) and identification of enzyme effectors from enzyme assay measurements in crude extracts	No information on the activity *in vivo*
Genetic analysis	Obtains qualitative data on pathway use by studying effects of gene deletion or overexpression	No quantitative information on *in vivo* activity; changing gene expression disturbs metabolism

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Graaf, 1996). Therefore, the application of *in vivo* NMR is limited to special cases where *e. g.* only a limited number of highly concentrated metabolites is to be studied, thus obviating the need to highly concentrate the cells, or cases where special systems for in situ cultivation of microorganisms in the NMR magnet can be used (de Graaf *et al.*, 1992; Hartbrich *et al.*, 1996).

In past years it has become apparent that the integration of the metabolite balancing and isotopic tracer methods leads to an extremely powerful approach for quantitative pathway analysis (Wiechert and de Graaf, 1996, 1997; Wiechert *et al.*, 1997; Marx *et al.*, 1996, 1997). Metabolite balancing is a generally applicable technique based on the precise and comprehensive absolute quantitation of extracellular fluxes (*i. e.* between cells and surrounding medium: substrate uptake and product excretion rates) as well as of the quantities of intracellular metabolites needed for the synthesis of macromolecular biomass constituents (protein, DNA, RNA, lipids; Vallino and Stephanopoulos, 1990, 1993). Thus, once the precise biomass composition and the growth rate of a specific microorganism are known, the withdrawal flux of each precursor metabolite (ribose-5-phosphate, PEP, pyruvate, oxaloacetate etc.) from the central metabolism for biomass synthesis can be calculated. In this way, all fluxes to all products and by-products (including biomass) can be determined. However, the method cannot discriminate between carbon dioxide formation in the pentose phosphate pathway and the citric acid cycle, so that these two fluxes remain undetermined. Also, while the total anaplerotic flux can be calculated, metabolite balancing in principle cannot discriminate between anaplerotic carboxylation and the glyoxylate pathway as a source of oxaloacetate/malate. Moreover, in cases where anaplerotic C3-carboxylating and gluconeogenic C4-decarboxylating reactions are active simultaneously, these cannot be resolved using metabolite balances because an overall cycling does not influence the carbon balance. Of course, balances of other metabolites esp. ATP and/or NADP may be included in order to resolve the remaining unknown fluxes. However, the fact that many pathways influencing the balance of these compounds are either unknown or extremely difficult to quantititate severely limits this approach in practice.

In contrast, isotopic tracer methods such as [^{13}C]-labelling and NMR are ideally suited to provide the missing flux information because they allow the split ratios of flux distributions to be determined at important metabolic branch points including glucose-6-phosphate, pyruvate and oxaloacetate (Marx *et al.*, 1996). Figure 1 illustrates this by a simplified example of the determination of the flux split ratio of glycolysis *vs*. the pentose phosphate pathway. In recent years, very efficient approaches have been developed for obtaining comprehensive labelling information (Marx *et al.*, 1996, 1997). In a typical experiment, microorganisms are incubated at a metabolic steady state with a [^{13}C]-labelled substrate until at least 95% of the biomass in the fermenter has been synthesised from the

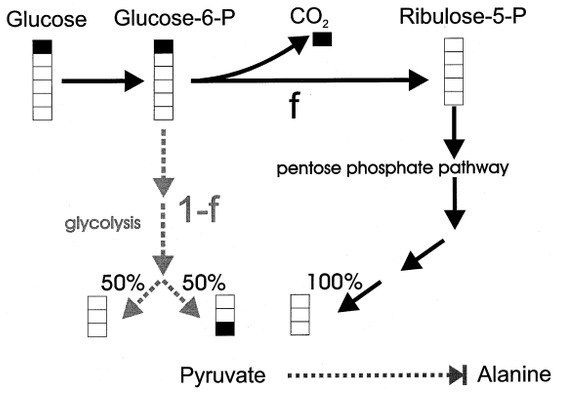

Fig. 1 Scheme Illustrating the Determination of the Flux Split Ratio of the Pentose Phosphate Pathway (f) *versus* Glycolysis (1-f) by Using [1-^{13}C]Glucose.
In the absence of any pentose phosphate pathway activity, two molecules of pyruvate, one unlabelled, the other labelled in C-3, are formed from each glucose. Activity of the oxidative pentose phosphate pathway leads to a release of the label in carbon dioxide, so that only unlabelled pyruvate results. With both pathways active, the labelling of pyruvate C-3 is proportional to 1-f.

[^{13}C]-labelled substrate. The cells are then harvested and hydrolysed, and the amino acids from the hydrolysate are purified for NMR analysis (Marx *et al.*, 1996). Since the carbon skeletons of the precursor metabolites from the central metabolism are preserved in the amino acids in a predifined and known way (Gottschalk, 1986), the [^{13}C]-labelling patterns of the latter represent the labelling *in vivo* of the precursor metabolites (Bacher *et al.*, 1999). Thus, oxaloacetate can be accessed from aspartate, methionine and threonine, 2-oxoglutarate from proline, glutamate and arginine, pyruvate from alanine, valine, leucine and isoleucine, 3-phosphoglycerate from serine and glycine, PEP as well as erythrose-4-phosphate from phenylalanine and tyrosine, and ribose-5-phosphate from histidine. The integration of this NMR-detected, comprehensive [^{13}C]-labelling data set with metabolite balancing measurements in a unifying approach, for which very versatile software tools have been developed (Wiechert and de Graaf, 1997; Wiechert *et al.*, 1997), allows the comprehensive quantitative pathway analysis of the central metabolism, including glycolysis, pentose phosphate pathway, citric acid cycle, glyoxylate cycle, C3-carboxylating anaplerotic reactions as well as C4-decarboxylating gluconeogenic reactions.

In addition, the degree of reversibility of several reactions, including transaldolase, transketolase, phosphoglucose isomerase and the phosphoglycerate mutase and enolase can be estimated as these give rise to specific transfer of [^{13}C]-labelling (Wiechert *et al.*, 1997). Knowledge about the reversibility of reactions *in vivo* yields important information as to the capacity of enzymes for further flux increase. Since, however, the consideration of reversibility of reactions in the flux model increases the number of degrees of freedom, even more labelling information is generally needed. This can be provided by the analysis of [^{13}C] isotopic distributions, because a molecule with N

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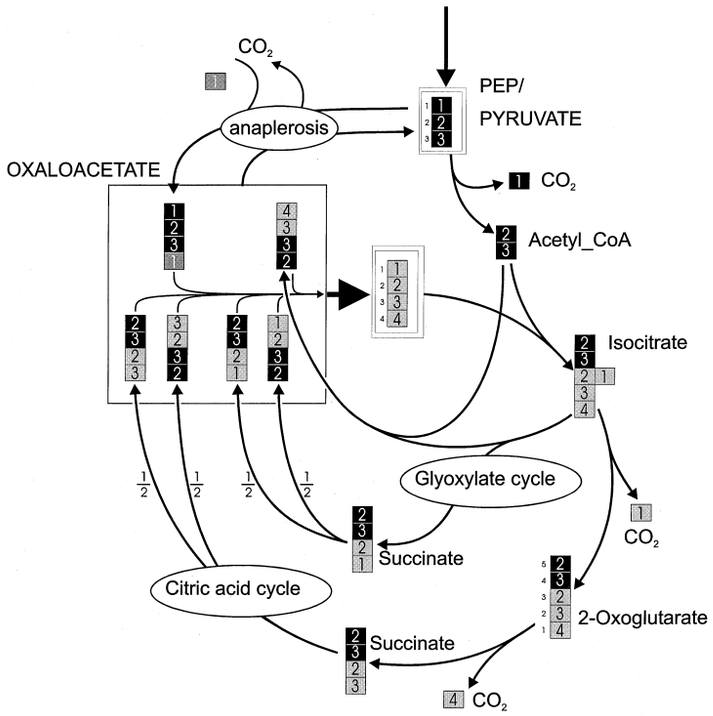

Fig. 2 Scheme Illustrating the Use of the Isotopomeric Composition of the Oxaloacetate Pool to Determine Flux Split Ratios for the C3-Carboxylating, C4-Decarboxylating, Citric Acid Cycle and Glyoxylate Pathway Reactions *in Vivo*.
In the experiment, [U-^{13}C]glucose is applied together with a much larger amount of unlabelled glucose. The black boxes symbolise [^{13}C] isotopes.

carbon atoms can yield as much as 2^N isotopomer information (Wiechert and de Graaf, 1996). Figure 2 shows a simplified illustration of how flux split ratios for the C3-carboxylating, C4-decarboxylating, citric acid cycle and glyoxylate pathway reactions can be inferred from isotopomers in oxaloacetate. In typical applications of this technique, the substrate consists of a mixture of 90% unlabelled and 10% uniformly [^{13}C]-labelled glucose (Szyperski, 1995). The fragments of the original [U-^{13}C]glucose backbone can then be traced by NMR isotopomer analysis of the amino acids in biomass hydrolysates (Figure 3), thus revealing the position and intensity of C-C bond cleavage throughout the metabolism. The use of two-dimensional heteronuclear single quantum correlation NMR spectroscopy even permits the direct analysis of the biomass hydrolysate, *i.e.* no amino acid purification is necessary (Szyperski, 1995). A very efficient isotopomer modelling approach has recently been developed (Wiechert *et al.*, 1999; Möllney *et al.*, 1999). Integrated with metabolite balancing, an extremely powerful and efficient tool for pathway analysis is now available.

An important drawback of the methods described so far is that only a single, stationary metabolic state can be analysed and described per experiment, *i.e.* no information on transient metabolic states during batch cultivations can be obtained. Since, however, most industrial fermentation processes are run in (fed)batch mode, method development now aims at time-resolved pathway analysis. The problem for the isotopic tracer methods in that case is that the macromolecular biomass constituents can

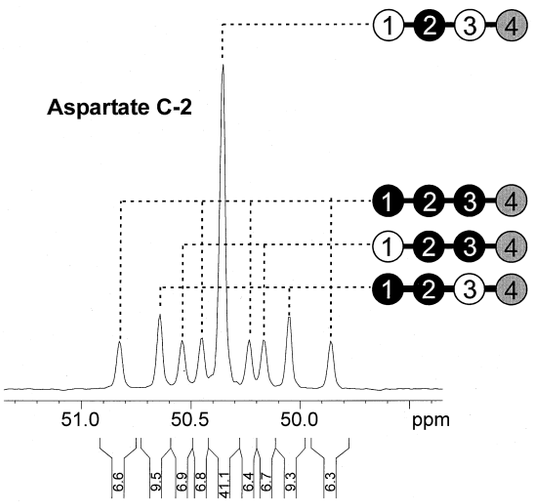

Fig. 3 ^{13}C NMR Spectrum of C-2 of Aspartate from *C. glutamicum* Grown on a Mixture of [U-^{13}C] and Unlabelled Glucose.
Due to different scalar couplings to C-1 and C-3, the various isotopomer species can directly be quantitated from the spectrum. Black circles denote ^{13}C carbons.

no longer be used as a source of labelling data, as their isotopic enrichment progresses only at a slow rate. Thus, cytoplasmic metabolites which are 10- to more than 10 000-fold less concentrated than *e.g.* the proteinogenic amino acids have to be used. While highly concentrated amino acids like glutamate, glutamine, aspartate and alanine generally allow flux analysis of specific pathways using

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Table 2 *In Vivo* Activities of the Oxidative Pentose Phosphate Pathway in Various Chemostat-Cultivated Isogenic Strains of *C. glutamicum* MH20-22B as Determined by ^{13}C NMR Isotope Analysis.

Strain	Condition	Lysine production	PPP Activity	Reference
LE4	growth	–	36	Marx *et al.*, 1997
LE4	glutamate production	–	25	Marx *et al.*, 1997
MH20-22B (with feedback-resistant aspartate kinase)	lysine production	19	66	Marx *et al.*, 1996
MH20-22B ΔGDH with overexpressed homologous (NADP-dependent) glutamate dehydrogenase	lysine production	30	76	Marx *et al.*, 1999
MH20-22B ΔGDH with overexpressed heterologous (NAD-dependent) glutamate dehydrogenase	lysine production	18	26	Marx *et al.*, 1999

Strain LE4 is an isogenic strain of MH20-22B with a feedback-sensitive aspartate kinase. Values are given as percentage of the molar glucose uptake rate.

NMR (Tesch *et al.*, 1999), it is to be expected that more sensitive detection methods like GC-MS (Christensen and Nielsen, 1999) which are capable of stable isotope quantification will play an increasingly important role in the near future. Furthermore, the combination of pathway analysis with yet another facility, namely metabolite pool determination, will represent a key development in the study of metabolic regulation and gene functional analysis. The integration of enzyme analysis (Table 1) in such an approach will result in the establishment of computer models of metabolism which have great predictive power for metabolic engineering.

Regulation of the Pentose Phosphate Pathway in *C. glutamicum*

Corynebacterium glutamicum, being an important amino acid-producing organism, is dependent on an adequate supply of NADPH for the reductive synthesis of amino acids. The main pathway of NADPH regeneration in *C. glutamicum* is the oxidative pentose phosphate pathway (PPP), in addition to the enzyme isocitrate dehydrogenase, which is strictly NADP-specific in *C. glutamicum*. Therefore, the study of the regulation of the PPP has received considerable attention. Unfortunately, the split ratio of glycolysis *vs*. PPP at the glucose-6-phosphate branch point cannot be quantified accurately by metabolite balancing techniques unless it is assumed that all NADPH-requiring and -generating reactions are known and that the organism keeps an exact balance of NADPH. However, several publications question such an assumption. Therefore, [^{13}C] isotopic tracer techniques have been applied to study PPP activity in several organisms, using the principle shown in Figure 1. Upon the use of [1-^{13}C]glucose, oxidative PPP activity causes the [^{13}C]-1 carbon of glucose-6-phosphate to be released as CO_2, thereby leading to a reduced labelling of pyruvate as compared to the theoretical value of 50% which would be observed if only glycolysis were active. Using this principle, PPP activity was determined in a number of different strains of *C. glutamicum* under various fermentation conditions (Sonntag *et al.*, 1995; Marx *et al.*, 1996, 1997, 1999). Selected data for several isogenic strains derived from L-lysine-producing *C. glutamicum* MH20-22B are given in Table 2. The strongly reduced PPP activity during glutamate production is in agreement with the PPP serving mainly to generate NADPH since only 1 mol NADPH per mol glutamate is required. The data for the lysine-producing strains show that increased lysine yields correlate with increased PPP activities. This also seems plausible since 4 mol of NADPH must be regenerated per mol of lysine produced. Thus, NADPH could represent a limiting factor in lysine biosynthesis.

Unexpectedly, an increase of NADPH availability by replacing the native NADP-dependent glutamate dehydrogenase of *C. glutamicum* by an NAD-dependent isoenzyme from *Peptostreptococcus asaccharolyticus* did not increase the lysine yield. Instead, it was found that the activity of the PPP was reduced 3-fold in this strain compared to the strain with plasmid-encoded homologous NADP-dependent dehydrogenase (Marx *et al.*, 1999). This made it even more interesting to study the regulation of the flux over this pathway *in vivo*, so as to be able to predict whether a limiting role of the PPP for lysine synthesis is to be expected. For this purpose, the metabolic balancing/-stable isotope labelling pathway analysis was integrated with classical enzyme determinations (Moritz *et al.*, 2000). Thus, the NADP-dependent glucose-6-phosphate (Glc6P) dehydrogenase and 6-phosphogluconate (6PG) dehydrogenase were purified from crude extracts of *C. glutamicum* and kinetically characterised. Both enzymes were found to operate according to a sequential mechanism, and for both enzymes the product NADPH was the most important inhibitor with K_i in the range of 30 – 40 μM, while the K_m values for NADP were 20 – 40 μM and for the sugar phosphates 50 – 150 μM. For the regulation of the PPP only the glucose-6-phosphate dehydrogenase was considered, because the spontaneous and rapid decay of its unstable product 6-phospho-glucono-δ-lactone makes this enzyme in fact irreversible. For this enzyme, a rate equation was proposed that enables the enzyme flux to be calculated from the determined kinetic constants, measured enzyme activity in the crude extract, and meas-

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ured concentrations of glucose-6-phosphate, NADP and NADPH. Upon investigation of the strains with plasmid-encoded homologous NADP-dependent and heterologous NAD-dependent glutamate dehydrogenases derived from *C. glutamicum* MH20-22B (see Table 2) a very good match between calculated and experimentally determined fluxes was found, *i.e*. the three-fold decrease of PPP flux in the heterologous glutamate dehydrogenase mutant (Table 2) was adequately predicted. An interesting observation in these strains was furthermore that the substrates glucose-6-phosphate and NADP were both present in concentrations well above the respective K_m values, even though the cells were in a carbon-limited chemostat culture. The NADPH concentration was found to be as much as 10 times higher than the respective K_i value. It was shown that due to this special situation the rate equation for glucose-6-phosphate dehydrogenase could be formulated as

$$V \approx C \cdot \frac{V_{max} \cdot R}{K + R}$$

where R = [NADP]/[NADPH], V_{max} is the glucose-6-phosphate dehydrogenase-specific enzyme activity, and C and K are constants whose values depend on K_m for glucose-6-phosphate and NADP as well as on K_i for NADPH and the glucose-6-phosphate concentration. For the studied strains, the values of R were around 0.5 – 1.1, while that of K was about 2 and that of C roughly 0.7 (Moritz *et al.*, 2000). This demonstrates that the intracellular concentrations of NADP and NADPH in the carbon-limited chemostat culture of *C. glutamicum* are such that the PPP operates approximately at 15 – 25 % of saturation, so that no limiting role during lysine production is expected. However, in fed-batch cultures featuring much higher specific lysine production rates the situation may be different. Thus, the *in vivo* regulation of the pentose phosphate pathway activity in *C. glutamicum* was successfully elucidated using an integrated pathway analysis approach including isotope labelling, metabolite balancing, enzymatic analysis and pool size determination techniques.

Detailed Analysis of the Anaplerotic Node in *C. glutamicum*

The anaplerotic reactions are of key importance for the synthesis of L-lysine and other amino acids of the aspartate family, as they supply oxaloacetate, a direct precursor of aspartate. *C. glutamicum* is a special organism with regard to its anaplerotic enzyme equipment. This organism possesses two C3-carboxylating enzymes (PEPcarboxylase and pyruvate carboxylase) in addition to the three C4-decarboxylating enzymes oxaloacetate decarboxylase, PEPcarboxykinase, and malic enzyme (Figure 4) (Jetten *et al.*, 1994; Lindley *et al.*, 1996). Therefore, the question of the *in vivo* usage of these enzymes has been addressed in several studies. PEPcarboxylase has long been considered the principal anaplerotic enzyme (Vallino and Stephanopoulos, 1993). However, overexpression of this enzyme in *C. glutamicum* had no effect on growth, nor on lysine synthesis (Peters-Wendisch *et al.*, 1993; Gubler *et al.*, 1994). [^{13}C]-labelling experiments on a PEPcarboxylase-deficient mutant proved that an alternative, C3-carboxylating reaction exists in this organism (Peters-Wendisch *et al.*, 1996). Subsequent studies finally succeeded in the identification, characterisation and cloning of pyruvate carboxylase as the second, anaplerotic enzyme (Peters-Wendisch *et al.*, 1997, 1998). Overexpression of pyruvate carboxylase resulted in a significantly increased lysine production. A number of studies using the integrated metabolite balancing/[^{13}C]-labelling approach demonstrated that in *C. glutamicum* there is in general a strong C4-decarboxylating activity *in vivo*, which varies according to fermentation conditions (Sonntag *et al.*, 1995; Marx *et al.*, 1996, 1997, 1999). Interestingly, these studies also showed that increased C4-decarboxylating activity correlates with decreased lysine production (Table 3).

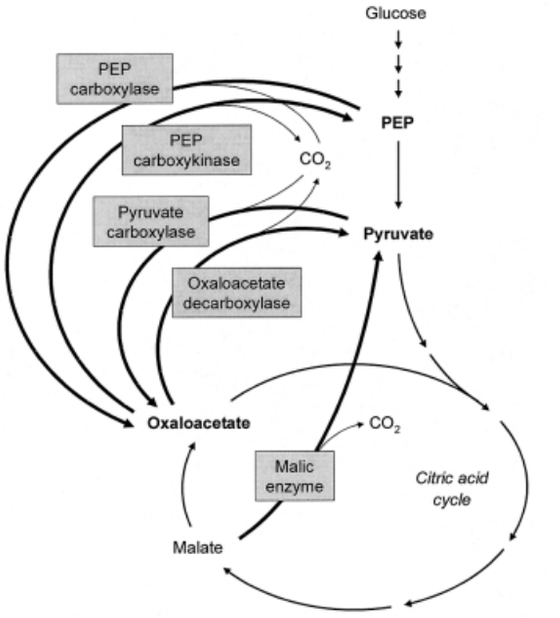

Fig. 4 Enzymes Involved in the Complex Anaplerotic Node of *C. glutamicum.*

These data strongly suggest that elimination of the reverse anaplerotic flux could result in increased lysine production. Therefore, it was of key importance to identify which enzyme(s) is/are responsible for the C4-decarboxylating flux *in vivo*. For this purpose, a special procedure combining [^{13}C]-isotopomer analysis and metabolite balancing was designed in order to determine the fluxes over all five C3-carboxylating and C4-decarboxylating enzymes *in vivo* (Petersen *et al.*, 2000). The rationale was to use a mixture of labelled substrates that, firstly, would produce a differential labelling of PEP and pyruvate in order to discriminate between PEPcarboxylase and pyruvate carboxylase activities, and, secondly, to introduce a specific labelling pattern of PEP and pyruvate resulting from decarboxylation of oxaloacetate/malate. The first goal was met by applying co-feeding of [3-^{13}C]lactate so as to gen-

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Table 3 Net C3-Carboxylating and C4-Decarboxylating Fluxes (in % of the Molar Glucose Uptake Rate) Determined by ^{13}C-NMR Analyses in *C. glutamicum* Strains LE4 and MH20-22B (Marx *et al.*, 1996, 1997, 1999).

Strain	LE4	MH20-22B	MH20-22B with heterologous glutamate dehydrogenase	MH20-22B with homologous glutamate dehydrogenase
Net anaplerotic flux (%)	24.1	37.6	35.6	44.3
C4-decarboxylating flux (%)	72.1	30.8	29.2	10.3

erate an increased [3-^{13}C]isotopomer content in pyruvate as compared to PEP. The second goal was met by feeding [U-^{13}C]glucose against a background of unlabelled glucose, as explained in the legend of Figure 2. The [^{13}C] NMR spectra of PEP-derived phenylalanine and pyruvate-derived alanine isolated from *C. glutamicum* clearly showed an increased content of [3-^{13}C]fragments in pyruvate as compared to PEP. The presence of a similarly high abundance of [1,3-^{13}C2] and [3-^{13}C] fragments in oxaloacetate-derived aspartate demonstrated that pyruvate carboxylase is the most important C3-carboxylating anaplerotic enzyme *in vivo*. Significant amounts of [1,2-^{13}C2] fragments in PEP and pyruvate resulting from oxaloacetate decarboxylation were found. Since the relative abundancies of them in PEP and pyruvate were approximately equal, it could be concluded that C4-decarboxylation was towards PEP and not pyruvate. The flux distribution resulting from an analysis of the data by the recently developed isotopomer modelling software is shown in Figure 5. It was found that pyruvate carboxylase and PEPcarboxykinase are the most active enzymes catalysing more than 90% of the forward and reverse anaplerotic flux, respectively. These two enzymes thereby constitute a futile cycle in which pyruvate is carboxylated to oxaloacetate, oxaloacetate is decarboxylated to PEP, and PEP is metabolized to pyruvate by pyruvate kinase, with a concomitant net loss of one ATP per turn. The physiological role and possible growth advantage of such a cycle is the subject of further studies.

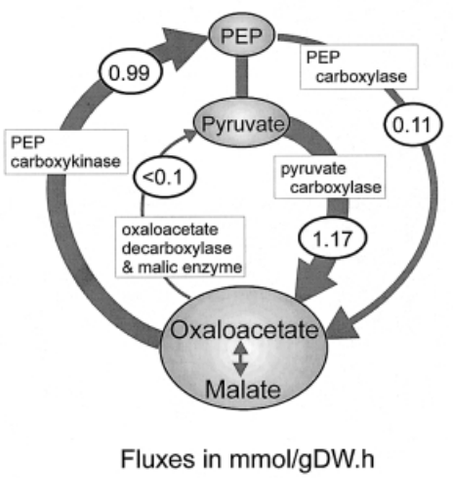

Fig. 5 Anaplerotic Fluxes in *C. glutamicum* (Petersen *et al.*, 2000).

Biosynthesis of L-Lysine *via* a Highly Branched Pathway

L-Lysine is part of the aspartate family of amino acids, which consists of L-lysine, L-threonine, L-methionine, L-isoleucine, and D, L- diaminopimelate (Figure 6). This latter non-proteinogenic amino acid is the precursor for the synthesis of L-lysine and at the same time it is also required for cell wall synthesis. The regulation of this pathway in *C. glutamicum* is rather simple as compared to that in *Escherichia coli* or *Bacillus subtilis*. This is evident, for instance, by the absence of isoenzymes in *C. glutamicum*. The major steps where flux regulation occurs are at the branch points in the highly structured network. Thus the

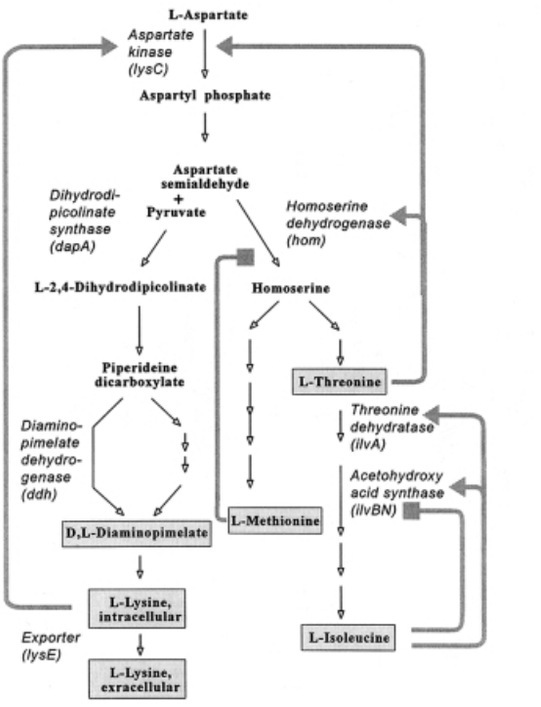

Fig. 6 The Biosynthesis of the Aspartate Family of Amino Acids and Its Regulation in *C. glutamicum*.
Feedback inhibition: shaded lines with arrowhead ends; repression: shaded lines with square ends.

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aspartate kinase is controlled in its catalytic activity by feedback inhibition when L-lysine is present together with L-threonine (Shiio and Miyajima, 1969; Kalinowski *et al.*, 1991). Furthermore, the activity of the homoserine dehydrogenase is controlled by L-threonine feedback inhibition (Miyajima *et al.*, 1968; Follettie *et al.*, 1988), the threonine dehydratase by L-isoleucine (Miyajima *et al.*, 1972; Möckel *et al.*, 1992, 1994), and the acetohydroxy acid synthase by L-isoleucine, L-valine, and L-leucine (Eggeling *et al.*, 1987). The homoserine dehydrogenase synthesis is regulated by repression through L-methionine (Follettie *et al.*, 1988), and the acetohydroxy acid synthase by the branched-chain amino acids (Keilhauer *et al.*, 1993). A further control concerns the export of L-lysine from the cell. This is catalysed by a recently discovered transport carrier (Vrljic *et al.*, 1996). This carrier has a so far unknown topology, and it is the first member of a large new superfamily of transport proteins present in eubacteria and archaea (Aleshin *et al.*, 1999; Vrljic *et al.*, 1999). When either L-lysine or L-arginine are present intracellularly in high concentrations, it catalyses the export of these amino acids to the surrounding medium. The synthesis of this export carrier is controlled by the regulator LysG together with one of the basic amino acids (Bellman *et al.*, 2000).

In *E. coli* L-lysine is synthesised by a reaction sequence involving the conversion of piperideine dicarboxylate to D,L-diaminopimelate by 4 steps (Figure 6). It was a big surprise to discover a diaminopimelate dehydrogenase activity in *C. glutamicum* and other bacteria as well which is able to catalyse this conversion in only one step by direct ammonium incorporation (Bartlett and White, 1985). Enzyme analyses and gene isolations (Schrumpf *et al.*, 1991; Wehrmann *et al.*, 1994, 1995, 1998) verified the fact that *C. glutamicum* has both variants of D,L-diaminopimelate synthesis together. These are called the succinylase variant and dehydrogenase variant, respectively. The question of *in vivo* use was solved in a labelling study, since the fate of the carbon atoms *via* each of the variants is different (Yamaguchi *et al.*, 1986). Thus using [^{13}C]-enriched glucose as substrate and application of ^{13}C- and ^{1}H-NMR spectroscopy it was quantified that in the finally accumulated L-lysine 33% was synthesised in *C. glutamicum via* the dehydrogenase variant and 66% *via* the succinylase variant (Sonntag *et al.*, 1993). However, the flux distribution varied during L-lysine fermentation. At the beginning the major part of L-lysine was made *via* the dehydrogenase variant, but finally the newly synthesised L-lysine was almost exclusively synthesised *via* the succinylase variant (Figure 7). This correlated with the decreasing ammonium content during fermentation and the properties of the dehydrogenase which has a high K_m of 36 mM for ammonium (Misono and Soda, 1980). Thus the benefit of the NMR study was twofold. First of all, it enabled the *in vivo* use of the flux partitioning in the split pathway of L-lysine synthesis to be quantified, and most importantly, it permitted the discovery of a dynamic variation of pathway use with cultivation time.

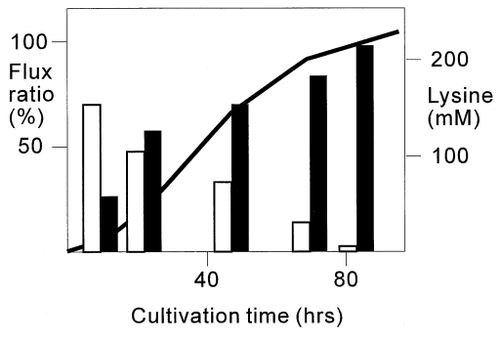

Fig. 7 The Varying Contribution of the Dehydrogenase Variant (Open Bars) and Succinylase Variant (Filled Bars) of the Diaminopimelate Pathway for L-Lysine Synthesis in *C. glutamicum*.

Further studies on the split pathway of L-lysine synthesis in *C. glutamicum* showed that the succinylase variant is essential to enable growth on organic nitrogen sources (Wehrmann *et al.*, 1998). Thus when a mutant devoid of the succinylase variant was supplied with a low ammonium concentration the cells were enlarged up to 6 μm and often club-shaped at their ends (Figure 8). They were also less resistant to mechanical stress in comparison to the wild type. This is consistent with a limited availability of D,L-diaminopimelate for peptidoglycan synthesis. D,L-diaminopimelate serves to link the glycan backbones in the murein sacculus of many bacteria, giving them their shape and rigid structure. The vital role of this compound is probably the reason that *C. glutamicum* and several other bacteria, like *Bacillus macerans*, possess the split pathway to respond flexibly to alterations in the nitrogen supply in their natural habitat (Bartlett and White 1985; Misono *et al.*, 1979). As already mentioned, the dehydrogenase has a low affinity. The enzymes of the succinylase variant have a high affinity and this variant use is energetically more costly than use of the dehydrogenase variant. In serveral aspects D,L-diaminopimelate synthesis thus resembles other bacterial systems where two pathways exist, like glutamate dehydrogenase (low affinity) and glutamine synthetase (high affinity) for ammonium assimilation.

Flux Control at the Aspartate Semialdehyde Branch Point

An important flux control step within L-lysine synthesis is the aspartate semialdehyde branch point. This aldehyde is either used as a substrate for the homoserine dehydrogenase, or together with pyruvate as a substrate for the dihydrodipicolinate synthase (Figure 6). Whereas the homoserine dehydrogenase is allosterically controlled in its catalytic activity by the L-threonine concentration and repressed by L-methionine (Follettie *et al.*, 1988), no such control is known for the dihydrodipicolinate synthase (Cremer *et al.*, 1988). Overexpression of the dihydrodipicolinate synthase gene *dapA* resulted in increased L-lysine accumulation (Cremer *et al.*, 1991). At first sight this could be interpreted as the 'opening of a bottleneck'. However, as will be outlined subsequently, *dapA* overex-

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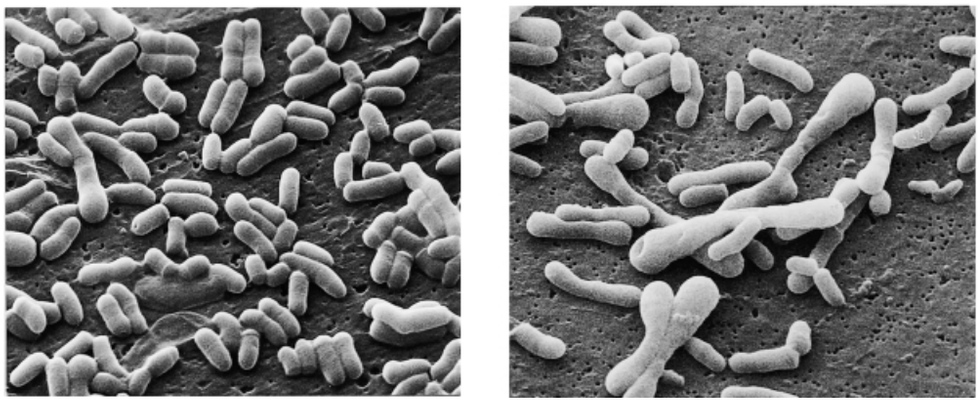

Fig. 8 Electron Micrographs of Wild-Type (Left) and *dapD* Mutant Cells of *C. glutamicum* (Right).

Table 4 Effects of *dapA* Overexpression on the Growth Rate and the Synthesis of L-Threonine, L-Valine, and L-Lysine.

Strain	*dapA* copies	Dihydrodipicolinate synthase activity ($U \times mg^{-1}$)	Homoserine dehydrogenase activity ($U \times mg^{-1}$)	Growth rate (h^{-1})	Intracellular L-threonine (mM)	Intracellular L-valine (mM)	L-lysine excretion rate ($nmol \times min^{-1} mg^{-1}$ dw)
13032	1	0.05	0.62	0.43	9	3	0.0
13032::*dapA*	2	0.08	0.85	0.37	3	6	0.2
13032 pKW3::*dapA*	6	0.25	0.62	0.36	< 1	8	2.7
13032 pJC24	20	0.63	0.67	0.22	< 1	10	3.8

The four strains analysed are the wild-type (13032) and recombinant strains carrying several copies of *dapA* encoding for the dihydrodipicolinate synthase.

pression affects the flux at the entire aspartate semialdehyde branch point.

As can be seen in Table 4, the wild type with one *dapA*-copy did not excrete L-lysine, whereas introduction of a second copy already resulted in increased L-lysine synthesis and its excretion. A further increase in the copy number increased the dihydrodipicolinate synthase activity and L-lysine excretion as well (Eggeling *et al.*, 1998). This is due to two effects. The first is the kinetic properties of the competing enzymes at the branch point. Thus the dihydrodipicolinate synthase has a high affinity for the aspartate semialdehyde (K_m = 0.37 mM) and a low maximal specific activity (V_{max} = 0.09 μmol min^{-1} mg $protein^{-1}$), whereas the corresponding values for the homoserine dehydrogenase are nearly one order of magnitude higher (K_m = 2.08 mM; V_{max} = 0.75 μmol min^{-1} mg $protein^{-1}$). These data, as well as the concentration of aspartate semialdehyde in the cell of about 0.05 mM, show that the flux towards L-lysine is determined by the high affinity of the dihydrodipicolinate synthase. Since this flux control could not be operative if the homoserine dehydrogenase had high affinity and low activity, in fact both the homoserine dehydrogenase and the dihydrodipicolinate synthase together are elements of flux control for aspartate semialdehyde distribution.

The second effect resulting in increased flux towards L-lysine as a consequence of *dapA* overexpression is more subtle. As can be seen in Table 4, gradual *dapA* overexpression also resulted in a gradual reduction of the growth rate. As the quantification of the intracellular amino acid concentrations revealed (Table 4) the L-threonine concentration was reduced upon *dapA* overexpression. This unexpected finding was confirmed by the fact that addition of L-homoserine, for instance, restored growth of a *dapA*-overexpressing strain (Eggeling *et al.*, 1998). This growth limitation resulted in an increased availability of intracellular precursors, like pyruvate for instance. This is evident from the increased L-valine concentration which is synthesised from two pyruvate molecules (Table 4). An additional advantage of increased L-lysine synthesis due to *dapA* overexpression is the reduced extracellular accumulation of some minor concentrations of the byproducts formed (Kircher, 1998). For instance, plasmid-encoded *dapA* overexpression resulted in an increased L-lysine accumulation from about 230 mM to 280 mM, accompanied by a reduction of L-isoleucine and L-alanine from concentrations of 6 mM to concentrations below 1 mM.

Conclusion

In recent years the biochemistry, physiology, and molecular biology of amino acid biosynthesis in *C. glutamicum* have been studied intensively, and a substantial amount

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of information has been obtained, especially by the development and use of recombinant DNA techniques. The current knowledge on the biosynthesis of amino acids and their regulatory features shows that the regulation of these pathways is much simpler than the corresponding pathways, for example, in *Escherichia coli*. In *C. glutamicum* only very few of the involved enzymes are controlled, and up to now no isoenzymes could be detected. This may be the reason why this bacterium can be manipulated quite well for the production of various amino acids.

The determination of *in vivo* metabolic fluxes is a demanding task for metabolic engineering. For the quantitative assessment of metabolic fluxes various methods have been developed. Isotopic tracer methods such as [^{13}C]-labelling and NMR spectroscopy are very well established to determine split ratios of flux distributions at important metabolic branch points. Furthermore, molecular study of the genes has allowed the analysis of gene expression and its regulation. Disruption or overexpression of certain genes in *C. glutamicum* enabled the analysis of carbon flux control in response to removal or elevation of the respective enzyme activity. Based on these analyses, new strategies for the manipulation of this industrially important amino acid producer become possible. Also the DNA microarray technology developed recently can speed up the process for monitoring the transcription levels of all the genes in the bacterium and their global control, thus making it possible to relate changes in gene expression to changes in cellular metabolism e.g. in amino acid biosynthesis. To properly interpret the complete molecular repertoire involved in these cellular programs during overproduction of amino acids will be a tremendous but also very exciting challenge.

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