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Biology of L-lysine overproduction by *Corynebacterium glutamicum*

Review Article

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Summary. The Gram positive bacterium *Corynebacterium glutamicum* is used for the production of L-lysine. This review focuses on the progress achieved in the past five years for a deeper understanding of lysine overproduction. This period also coincides with decisive progress in the use of genetic engineering techniques for analysing and increasing the metabolite flux in *C. glutamicum*. It was thus demonstrated that the *in vivo* activity of the allosterically controlled aspartate kinase is important for flux control, but in addition also the amount of the dihydrodipicolinate synthase. An outstanding feature of *C. glutamicum* is the split lysine biosynthesis pathway. NMR investigations have clearly shown that both pathways are simultaneously used *in vivo* and that the flux ratio depends on nitrogen availability. The cellular synthesized lysine is eventually exported into the external medium through a specific carrier. Interestingly lysine producers have other export characteristics so that the carrier properties also seem to be important for increased metabolite flux.

Keywords: Amino acids – Flux control – Flux quantification – Lysine production – Gene exchange – Metabolic design – Amino acid export

Introduction

The era of the bacterial production of amino acids started in 1957 by the discovery by Kinoshita et al. (1957) that "coryneform glutamic-acid bacteria" can be easily manipulated to secrete L-glutamate. Since then a variety of processes have been developed for the production of L-glutamate, L-lysine, L-threonine, L-tryptophane, L-isoleucine and other amino acids by various bacteria. The quantitatively largest amounts produced are L-glutamate and L-lysine, of which 350,000 tons and 180,000 tons, respectively are currently manufactured annually. This is done with the "glutamic-acid bacterium" *Corynebacterium glutamicum* or its subspecies *flavum* and *lactofermentum*, which are Gram-

positive aerob rods. Of course, mutants of *C. glutamicum* are used for the technical process to produce L-glutamate and L-lysine which resulted from extensive screening programmes. This classical, essentially iterative procedure resulted in a rapid improvement of strains and in remarkable productivities.

With the advent of the applicability of genetic engineering techniques to *C. glutamicum* (Katsumata et al., 1984; Martin, 1989; Schwarzer and Pühler, 1990; Eikmanns et al., 1991), it became possible to achieve a better understanding of the physiology on the molecular level. In the same way, new exciting characteristics of *C. glutamicum* were discovered and quantified by biochemical work (Ebbighausen et al., 1989a; Bröer and Krämer, 1991). It is the purpose of this review to summarize recent findings on the peculiarities of L-lysine synthesis with *C. glutamicum* and to discuss their use for further strain improvement.

A split pathway of lysine synthesis

The bacterial synthesis of L-lysine starts from the central metabolite L-aspartate, which is derived from the tricarboxylic acid cycle intermediate oxaloacetate by transamination. Aspartate is activated via phosphorylation by aspartate kinase and reduced to give aspartate semialdehyde (Fig. 1). This intermediate is at an important branch point to enter either L-threonine, L-methionine, and L-isoleucine synthesis or the further reactions of L-lysine synthesis. These are the condensation of aspartate semialdehyde with pyruvate by dihydrodipicolinate synthase and its reduction to piperideine-2,6-dicarboxylate. At this step the bacterial diaminopimelate pathway of L-lysine synthesis is at variance. *Escherichia coli* and other bacteria (Weinberger and Gilvarg, 1970) use the succinylase variant with its four specific reactions (see Figure) to make the ultimate L-lysine precursor D,L-diaminopimelate. The second variant, the acetylase variant (not included in Fig. 1), is a minor modification, where instead of the succinyl an acetyl group is used as blocking group. This is used e.g. in *Bacillus subtilis*. The third variant is the dehydrogenase variant, where D,L-diaminopimelate is synthesized in one single step by the action of D-diaminopimelate dehydrogenase (Misono and Soda, 1980), as is the case in *Bacillus sphaericus*. Metabolites of the three generally possible variants are rejoining at the step of D,L-diaminopimelate. This is at another important branch point since it is either used for cell wall synthesis or decarboxylated to L-lysine.

The peculiarity of *C. glutamicum* is that this bacterium uses the succinylase plus the dehydrogenase variant (Schrumpf et al., 1991). This is currently the only example of such an apparently luxurious equipment of parallel reactions leading to the final product of an anabolic pathway. Such a network of reactions is usually reserved for catabolic reactions like the generation of glycerol aldehyde-3-phosphate by glycolysis and the pentose phosphate pathway adapted for the varying demands of building blocks and energy. Whether the two variants in *C. glutamicum* have a specific function other than to supply D,L-diaminopimelate for cell wall synthesis and L-lysine synthesis is unknown. However, gene-directed inactivation of the dehydrogenase by integration of vector sequences (Schrumpf et al., 1991) verified the fact that the dehydrogenase variant is dispensable. Whether this is also the case for the opposite situation where the succinylase

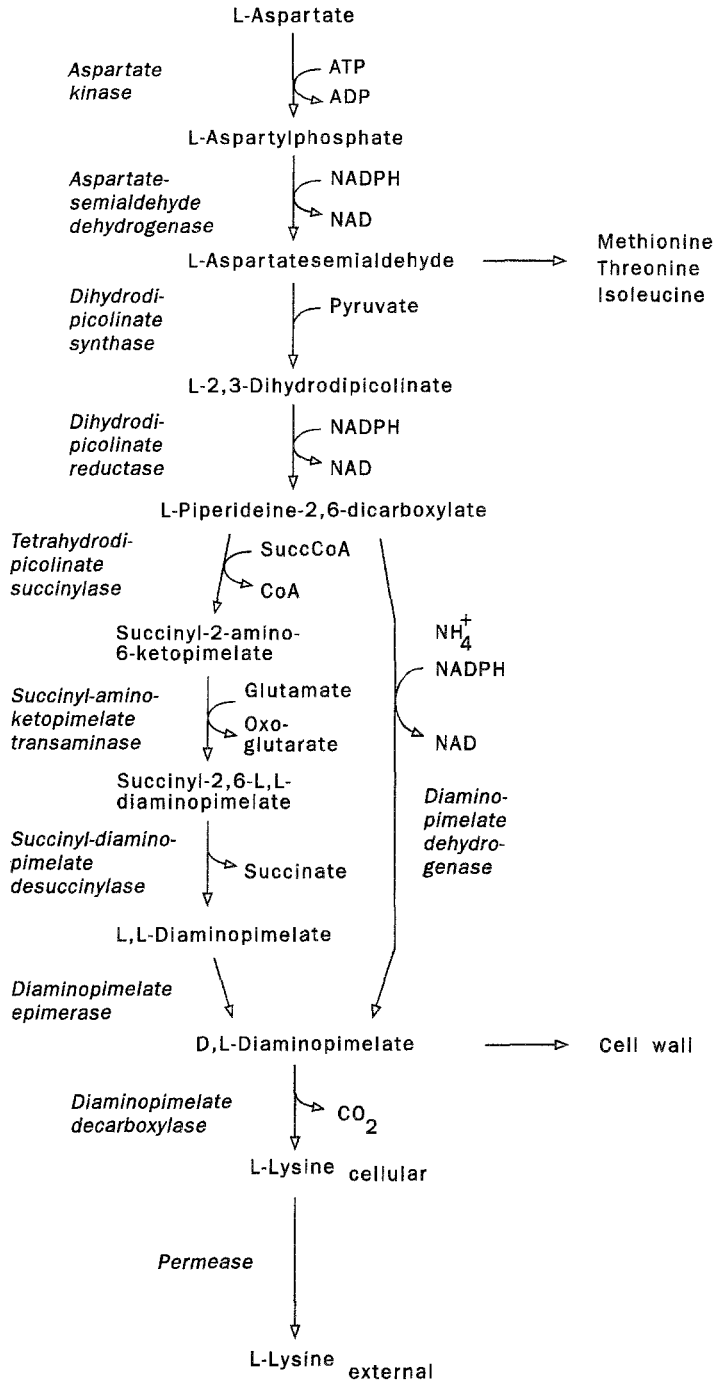

Fig. 1

variant is inactive cannot be answered at present since the corresponding genes have not yet been cloned to enable gene-directed mutagenesis. Apparently, the cloning of these genes is difficult, since in different attempts to clone genes of the split pathway by the use of an appropriate *E. coli* mutant the gene encoding the D-diaminopimelate dehydrogenase was always isolated (Ishino et al., 1988; Yeh

et al., 1988). This is probably due to specific promoter structures of the genes of *C. glutamicum* opposed to those used in *E. coli* (Eikmanns, 1992). Quantification of the contribution of each pathway in *C. glutamicum* for total flux towards lysine will be discussed further below.

Flux increase by deregulated aspartate kinase

The aspartate kinase reaction has to handle the substantial amount of 25% of the carbon flux of total carbon incorporated into cellular material (Neidhardt, 1990). This is due to the fact that four amino acids arise from the activated aspartate molecule. Of these amino acids L-lysine, L-threonine, L-methionine and L-isoleucine are of biotechnological importance as feed additives and for pharmaceutical purposes (Kleeman et al., 1985). The large flux and the localization of the kinase at the entry of the C-4 unit into the highly branched pathway suggests a sensitive control of the overall activity of the enzyme. In fact, in *C. glutamicum* the enzyme activity is allosterically controlled by the combined presence of L-threonine plus L-lysine (Shiio and Miyajima, 1969), whose concentrations in the cell monitor the availability of these amino acids in the two major branches of the pathway. In the presence of 2.5 mM L-threonine the K_i for L-lysine is 2.4 mM. Isoenzymes as in *E. coli* or *B. subtilis* (Graves and Switzer, 1990) are not present in *C. glutamicum*, nor does the expression of the enzyme vary in response to the presence of any amino acid of the aspartate family (Cremer et al., 1988). The structure of the enzyme is unusual. It consists of four subunits, two of each being identical ($\alpha_2\beta_2$). As shown by genetic investigations (Kalinowski et al., 1990) there is one open reading frame, named *lysC*, which is identical to the structural gene *lysCα*, and whose 3′ end, within the same reading frame as *lysCα*, encodes the structural gene *lysCβ*. Therefore, the β subunit of the kinase is identical to the 172 amino acids of the carboxy end of the α subunit (consisting of 421 amino acids), suggesting an interesting architecture of the active protein, since this contains four regions corresponding to the β peptide sequence.

The tight control of the kinase activity can be overcome by using the general procedure of isolation of mutants which are resistant towards an analogue of a regulatory amino acid. Mutants of *C. glutamicum* resistant to the lysine analogue *S*-(2-aminoethyl)-L-cysteine can easily be obtained (Sano and Shiio, 1970). Among these are clones impaired in the uptake of *S*-(2-aminoethyl)-L-cysteine by the lysine import system (Seep-Feldhaus et al., 1991), and those with increased availability to degrade the analogue (Rossol and Pühler, 1992). Important are those with mutated aspartate kinase gene, rendering the protein insensitive to allosteric control by lysine plus threonine. One such mutation was sequenced (Kalinowski et al., 1991) and found to be a nucleotide exchange within *lysCβ* (and of course *lysCα*, too) resulting in an exchange of amino acid in position 63 of the β subunit from serine to tyrosine. Expressing the mutated β-subunit (which has been done by using a truncated *lysCα* gene) in the wild type of *C. glutamicum* renders the kinase partially resistant to allosteric control. It must therefore be concluded that the β subunit is of major importance for activity control, and that hybrid proteins occur in the recombinants.

The availability of the cloned mutated aspartate kinase gene (*lysC*FBR) enabled a study of the effect of this gene expression on total flux. Using homologous recombination, *lysC*FBR was integrated in one copy in the chromosome of the wild type, which resulted in excretion of 29 mM L-lysine without excretion of threonine (Bröer et al., 1993). This shows the sufficient, but simple control of L-lysine synthesis in the wild type of *C. glutamicum*, whereas more than one enzyme is tightly controlled on the flux of the C-4 unit aspartate towards L-threonine or L-isoleucine. An additional second copy of *lysC*FBR integrated in a lysine producer (unpublished) resulted in an increase from 40 to 46 mM L-lysine accumulated, and even with more then ten copies (*lysC*FBR on plasmid) only a minor increase to 48 mM lysine was found in this producer (Cremer et al., 1991). Since in all cases the total enzyme activity paralleled the gene dose, it must be concluded that even the one *lysC*FBR mutation in the chromosome already resulted in such high catalytic in vivo activity (compared to the regulated enzyme) that a massive flux alteration occurred with the shift of control to other reactions, or according to metabolite flux control theory, to sets of reactions (Niederberger et al., 1992). These have yet to be identified. Also another experiment performed with a hyperproducer confirmed the major flux control towards L-lysine by aspartate kinase. Again the *lysC* locus was modified, but in this case *lysC*FBR was replaced in the producer by wild type *lysC* (Schrumpf et al., 1992). The result was that with the constructed strain a dramatic reduction in accumulated L-lysine from 240 mM to 10 mM was obtained.

As a direct consequence of high catalytic aspartate kinase activity, Schrumpf et al. (1991) monitored the intracellular aspartate concentration and the L-lysine excretion rates. As expected from the theory of flux control, the intracellular substrate pool is decreased at high catalytic in vivo activity of the kinase (compare wild type and DG52-5 in Table 1). The final result is lysine excretion at a rate of 0.13 mmol/g h. In further experiments the cell internal aspartate concentration was increased by overexpressing the phosphoenolpyruvate carboxylase or by the addition of precursors. Although a more than threefold increase in the cytosolic concentration was achieved, the flux response was weak

Table 1. Influence of aspartate kinase activity and cellular aspartate availability on lysine excretion

Strain	Aspartate kinase regulation	Additional characteristics	Aspartate conc. (mM)	Lysine excretion rate (mmol/g h)
Wild type	FBS[1]		19	0
DG52-5	FBR[1]		9	0.13
DG52-5	FBR	*ppc* overexpressed[2]	13	0.19
DG52-5	FBR	Asparagine added[3]	14	0.19
DG52-5	FBR	Fumarate added	29	0.21
MH20-22B	FBR		9	0.17
MH20-22B	FBR	Fumarate added	15	0.29

[1] FBS/FBR, Feedback-sensitive and feedback-resistant aspartate kinase, respectively.
[2] Plasmid-encoded phosphoenol pyruvate carboxylase overexpressed.
[3] Substances were added to growth medium.

and non-linear (Table 1). This is in accord with the conclusion that in strain DG52-5 the kinase is no longer of major importance for flux control, whereas in strain MH20-22B under the conditions assayed the kinase reaction still carries substantial control.

It should be mentioned that besides the presence of *lysC*FBR, which renders the enzyme resistant to combined inhibition by L-threonine plus L-lysine, strains unable to synthesize L-threonine can also be profitably used for lysine formation due to high aspartate kinase activity when the L-threonine concentration is low (Shiio and Sano, 1969). The problem is then to limit constantly for L-threonine in actively metabolizing cells, as is possible by chemical engineering techniques (Kiss and Stephanopoulos, 1991), since otherwise no excretion occurs. Therefore leaky phenotypes are advantageous. It also has to be considered that a general limitation might be advantageous for overproduction, as has long been known for *Klebsiella pneumoniae*, which can excrete pyruvate, oxoglutarate or acetate depending on the kind of growth limitations (Tempest and Neijssel, 1992). The results obtained with the recent gene directed inactivation of the isopropylmalate synthase gene *leuA* in lysine producing strains, which makes them leucine auxotrophic are in accord with the idea that a general limitation is advantageous for overproduction (Pátek et al., 1994). With the various strains investigated, higher lysine accumulations were obtained when the strains constructed were limited for leucine.

Flux increase by increasing the dihydrodipicolinate synthase activity

Within the pathway of lysine synthesis there are two drain offs to other amino acids. One is the branching at the level of D,L-diaminopimelate to cell wall synthesis. Virtually currently nothing is known on the regulation of this branching point in *C. glutamicum*, except that diaminopimelate decarboxylase and arginyl t-RNA synthetase form an operon (Marcel et al., 1990, Sharp and Mitchell, 1993). A biological role for this connection is unclear, but the gene arrangement is suggestive for an interconnection of amino acid synthesis at a different level than carbon flux. The second branching point is at the level of aspartate semialdehyde where it has long been known that homoserine dehydrogenase is inhibited in its activity by L-threonine and repressed in its synthesis by methionine (Miyajima et al., 1968; Follettie et al., 1988). Only recently has it been shown that the dihydrodipicolinate synthase is also involved in flux control. This, however, is more subtle and cannot simply be discerned from activity determinations. A hint came from oversynthesis of the wild type enzyme, which surprisingly resulted in lysine excretion in the wild type background (Cremer et al., 1991). It must therefore be concluded that the total amount of enzyme is critical. If a stationary picture of flow control can be drawn at all, then the synthase could function as a kind of barrier. The homoserine dehydrogenase has a high affinity towards the aldehyde (K_m = 0.7 mM), but the synthase a low one (K_m = 6.2 mM) (Tosaka et al., 1979). The dehydrogenase therefore preferentially converts the aldehyde to threonine. When the dehydrogenase activity decreases in response to threonine, an increased aspartate semialdehyde amount becomes available by the synthase to be converted to lysine. In accord with this critical

control of the synthase, the amplitude of its variation is at best threefold (Cremer et al., 1988). Also the structural elements in front of the gene (Bonnassie et al., 1990; Pisabarro et al., 1993) point to a tight control mechanism. For technical purposes the aldehyde branch is a very good candidate for manipulation. In fact, synthase overexpression also gives increased lysine yields in strains with feedback-resistant aspartate kinase (Cremer et al., 1991). This enzyme is also a good candidate to direct the flux of the aldehyde towards threonine. Thus, using classical procedures, strains with very low synthase activity were obtained, which excrete higher amounts of L-threonine than their ancestor strains (Shiio et al., 1989). Consequently, this branch is in fact a promising target not only with respect to simply switching the flux, but also to obtaining higher final yields of the respective amino acid with producers.

Flux ratio through the split pathway is nitrogen dependent

The enzymatic and genetic analyses have undoubtedly shown the presence of both variants of D,L-diaminopimelate and L-lysine synthesis in *C. glutamicum.* However, such methods are not suited to quantify in vivo use. First evidence of the simultaneous use of both variants came from the use of 13-C glucose and the determination of the resulting specific enrichments in excreted L-lysine (Ishino et al., 1984). The basis for such experiments is the fact that L,L-diaminopimelate in the succinylase variant has a C-2 rotational axis of symmetry. Therefore, an equimolar mixture of two physically different D,L-diaminopimelate species arises in the following epimerase reaction if specifically labelled glucose is used. In contrast, in the dehydrogenase variant only one of these species arises due to label retainment by D-diaminopimelate dehydrogenase activity (Misono et al., 1979). Such experiments were recently carried out in detail by Sonntag et al. (1993), who determined labelling in the intermediate pyruvate in addition to lysine, thus enabling the determination of unequivocal flux distribution coefficients by a simple linear equation. Surprisingly, it turned out that only 30% of the L-lysine finally accumulated is synthesized over the dehydogenase variant (though the *in vitro* specific activity of the dehydrogenase is high). Therefore the succinylase variant is the most used pathway of the two. This is not only the case in the wild type, but also in the investigated overproducers which accumulate up to 240 mM L-lysine. These data agree with those determined approximately for *C. glutamicum* ssp. *flavum* (Yamaguchi et al., 1986). Since strains with widely different lysine production were analysed, it might be suggested that the flux ratio is independent of the total flux.

The accumulation of L-lysine in fermentations occurs over several days. The instantaneous partition coefficient determined at various cultivation times revealed another surprising fact, namely that at the beginning of cultivation the flux partition coefficient over the dehydrogenase variant is 72% but decreases to 0% at the end of cultivation. Use of varying initial ammonium concentrations and replacement of free ammonium by an organic nitrogen source verified the fact that the dynamic changes during cultivation are due to a decrease in the ammonium concentration. Since Misono et al. (1979) have shown that the D-diaminopimelate dehydrogenase has weak affinity for ammonium (K_m = 38

mM), the in vivo activity of the dehydrogenase is apparently directly decisive for the flux distribution. Therefore, use of the dehydrogenase variant of L-lysine synthesis is kinetically controlled by the availability of ammonium. In contrast to the well known control of enzymes of nitrogen metabolism in *C. glutamicum* at the genetic level (Sung et al., 1985), this is thus a rare example of flux variation in direct response to changing environmental conditions, since the enzyme composition of both pathway variants remains constant under different growth conditions (Cremer et al., 1988; Schrumpf et al., 1991). It is unclear whether the different energy demand of both variants has an effect on total L-lysine formation and whether this can be profitably used.

Attempts to quantify total metabolite flux

Efforts have naturally also been made to analyse the entire flux in the cell from the glucose substrate to the amino acid product. Of course, this is much more complicated. Final products were analysed and quantified and models used for flux prediction in initial attempts to do this. The main problems are the many variables of the different biochemical reactions and the fact that no simple equations can be applied as in the favourable situation prevailing for the split pathway of lysine synthesis as described above. Nevertheless, Ishino et al. (1991) compared glutamate and lysine excreted by mutants of *C. glutamicum* again by the use of specifically labeled 13-C glucose. The authors calculated from the label in the finally accumulated lysine that 60–70% of the carbon flux is via the pentose phosphate pathway, whereas this is only 20% for glutamate formation. This seems reasonable due to the high NADPH demand for lysine synthesis. In an entirely different approach Vallino and Stephanopoulos (1993) used metabolite balances to calculate absolute flux rates through the main reactions within *C. glutamicum*. Uptake rates for substrates were determined (i.e. glucose, ammonium, oxygen) and product formation rates (i.e. for lysine, cell mass, carbon dioxide). Assuming a simplified metabolism (no difference can be made between the succinylase and dehydrogenase variant) several fluxes were calculated for one strain in the course of lysine fermentation. The data are in accord with the general view that the pentosephosphate pathway (Yamaguchi et al., 1986) and phosphoenolpyruvate carboxylase (O'Reagan et al., 1989) might support significant fluxes for lysine oversynthesis. However, the recent surprising discovery that phosphoenolpyruvate carboxylase inactivation does not effect lysine or glutamate secretion in *C. glutamicum* (Peters et al., 1993) shows that either assumptions used for modelling were invalid, or the cell has an enormous flexibility by means which still have to be discovered. The metabolite balance technique is experimentally simple and yields absolute rates. A combination of this technique with that essentially utilized by Sonntag et al. (1993) would result in tremendously increased detailed and reliable information on intracellular fluxes.

Flux increase by increased export activity?

When the regulatory mechanisms of lysine biosynthesis are overcome lysine is excreted. What is the mechanism? In contrast to earlier hypotheses on passive

lysine efflux, Bröer and Krämer (1991) found that *C. glutamicum* possesses a specific lysine export carrier. This carrier functions according to a secondary transport mechanism, the driving forces for this process being the membrane potential, the lysine gradient, and the proton gradient. At low external lysine concentration total export activity is mainly dependent on the membrane potential, whereas the other two forces come into play at high external lysine concentrations. This is due to the mechanistically different steps of binding, release, and reorientation of the carrier, which are influenced differently by the three forces. This carrier has a low K_M of approximately 20 mM for (internal) lysine which ensures that in the wild type, where such a concentration is hardly reached, no substantial amounts of lysine are excreted. Also a specific lysine import carrier has been characterized in *C. glutamicum*, which however does not play a role when lysine is excreted (Bröer and Krämer, 1990). The simultaneous presence of both an import and export system is also known for isoleucine transport in *C. glutamicum* (Ebbighausen et al., 1989a,b). For non-producing strains the lysine export carrier is required when cells grow on peptide-containing media (Erdmann et al., 1993). These peptides can be taken up and degraded. However, due to the lack of a lysine degrading enzyme, lysine would pile up if the export carrier were not active at the higher cytosolic lysine concentration. This export carrier is of course a prerequisite so that after overcoming the biosynthetic limitations a producer strain excretes lysine. Interestingly, in one hyperproducer analysed a very active lysine export activity was found (Schrumpf et al., 1992). In growing cells the hyperproducer MH20-22B excretes lysine at a rate of 0.57 mmol/g dry weight and hour, whereas for its ancestor strain a rate of 0.19 mmol/g h was determined. In a biochemical comparison of three producing strains notable differences in the total activity of the export system and its dependence on the three forces given above were determined (Bröer et al., 1993). This suggests that during undirected strain development the export carrier might also be mutated to obtain high yields.

Outlook

The molecular investigation of *C. glutamicum* revealed a wide range of new insights into the biology of this organism. The basic pathway of the synthesis of the aspartate family of amino acids has now been almost completely characterized biochemically. Also most of the corresponding genes have been cloned. In one type of study this equipment is currently used to perform directed mutations to study their consequences and profitable use. A different direction of studies is the analysis of hyperproducers, by biochemical or preferably genetical analyses. From this type of studies totally new characteristics of *C. glutamicum* might be discovered, which do not directly concern the flow schemes of amino acid synthesis, as is evident from the discovery of the altered export carriers. A third type of study, where the whole cell is considered, is the quantification of the major fluxes within the cell. This requires elaborate physical and mathematical techniques. A combination of these types of studies will further improve the understanding of lysine formation (and other amino acids) with *C. glutamicum*,

and finally not only be profitable for scientific purposes but also for the use of this interesting bacterium in industry.

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