Engineering metabolism and product formation in \textit{Corynebacterium glutamicum} by coordinated gene overexpression

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Abstract

Single gene overexpression in product pathways such as lysine synthesis has often been employed in metabolic engineering efforts aiming at pathway flux amplification and metabolite overproduction. This approach is limited due to metabolic flux imbalances that often lead to unpredictable physiological responses and suboptimal metabolite productivity. This deficiency can be overcome by the coordinated overexpression of more than one flux controlling genes in a production pathway selected by considering their individual contributions on the cell physiology. This concept is demonstrated by the simultaneous overexpression of pyruvate carboxylase and aspartate kinase, two key enzymes in central carbon metabolism and the lysine production pathway in \textit{Corynebacterium glutamicum}. Contrary to expectations based on the importance of each of these two genes in lysine production, the monocistronic overexpression of either gene results in marginal changes in the overall lysine productivity due to either reduced cell growth or reduced lysine specific productivity. In contrast, the simultaneous amplification of the activities of the two enzymes yielded more than 250% increase of the lysine specific productivity in lactate minimal medium without affecting the growth rate or final cell density of the culture. These results demonstrate that significant flux amplification in complex pathways involving central carbon metabolism is possible through coordinated overexpression of more than one gene in the pathway. This can be achieved either by external, gene expression inducing, controls or controls responding to the physiological cellular state.

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1. Introduction

Metabolic engineering was founded on the theme of industrial strain improvement using modern genetic tools (Bailey, 1999). Initial attempts focused on pathway flux increase through the overexpression of single genes that were believed to be important in pathway flux control. Despite some positive results (Colon et al., 1995; Ikeda and Katsumata, 1992), such approaches often introduce unpalatable metabolic imbalances leading to unpredictable physiological responses (Stephanopoulos and Simpson, 1997). These findings are also supported by theoretical results from Metabolic Control Analysis (Kacser and Burns, 1973, 1995) pointing to the absence of a single rate limiting step in most metabolic pathways and the need for coordinated amplification of more than one enzymes in a pathway in order to maintain, at an approximately steady level, the pools of intracellular metabolites (Kacser and Acerenza, 1993; Simpson et al., 1995). In response to this greater challenge, Metabolic Engineering emerged as the field concerned with the function of integrated metabolic pathways (Bailey, 1991; Stephanopoulos and Vallino, 1991), methods for the determination of metabolic fluxes as essential components of the metabolic phenotype (Stephanopoulos, 1999), and applications to the rational modification and design of metabolic networks to improve cellular properties. Some examples include: alleviation of the undesirable consequences of oxygen fluctuation by the cloning of the hemoglobin gene (\textit{vgh}) from the bacterium \textit{Vitreoscilla} (Khosla and Bailey, 1988a, b); the increase of recombinant protein production by redirecting carbon flow from acetate to acetalactate by overexpressing acetalactate synthase in \textit{E. coli} (Aristidou et al., 1995); and the successful alteration of the nitrogen assimilation pathway of \textit{Methylophilus methylotrophus} by cloning glutamate

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dehydrogenase in this organism that led to an enhancement of the yield of single cell protein (Windass et al., 1980).

Although generally accepted as necessary for effective pathway manipulation in strain improvement, overexpression of more than one gene by methods other than random mutagenesis is not a very common experimental approach. It places in doubt the popular “silver bullet” concept of single gene modification as means of improving industrial strain productivity or curing disease. It also raises the question of how to select multiple genes for overexpression that is, generally, a non-trivial issue. Despite these difficulties, theoretical analysis of pathway flux control and accumulating experimental evidence suggest that multiple gene overexpression is the key for industrial strain improvement and effective drug discovery (Bailey, 1999).

We provide one such example in this work. We chose the lysine pathway as model to illustrate the above concept because lysine is a large volume commodity chemical where yield improvements are materially important and because lysine is synthesized in a branched pathway lacking isoenzymes, which makes it very suitable for metabolic engineering. Additionally, lysine productivity depends on carbon flux distributions in central carbon metabolism, an area that has exhibited remarkable rigidity to various modification attempts. Improvement of the specific lysine productivity of *C. glutamicum* cells by genetically engineering the lysine biosynthetic pathway has been the subject of intense research activity in academic and industrial laboratories during the past 15 years (Eggeling and Sahm, 1999). A major breakthrough in this research was the discovery that pyruvate carboxylase is the main anaplerotic pathway for lysine synthesis, supplying the carbon flux for lysine synthesis from central carbon metabolism (Park, 1996; Park et al., 1997a, b; Peters-Wendisch et al., 1998; Petersen et al., 2000). This finding was confirmed by the subsequent cloning and sequencing of the pyruvate carboxylase gene (*pyc*) in *C. glutamicum* (Koffas et al., 1998; Peters-Wendisch et al., 1998). As certain estimates placed the flux through pyruvate carboxylase to as much as 90% of the total lysine flux (the balance supplied by the route catalyzed by phosphoenolpyruvate carboxylase), it became of interest to examine the effect of *pyc* overexpression on the growth and specific lysine productivity in *C. glutamicum* fermentations.

Another enzyme of interest in the lysine biosynthesis pathway that has been the subject of extensive research in the past is aspartate kinase. Literature evidence suggests that this enzyme can be a potential bottleneck in the lysine biosynthesis and that elevated levels of aspartate kinase are detrimental for growth in production medium. A lower specific productivity has also been reported for the recombinant strain that overexpresses the aspartate kinase gene (*ask*) (Jetten et al., 1995; Hua et al., 2000).

In the present work, we are first reporting our results with the overexpression of the pyruvate carboxylase gene (*pyc*). Motivated by the data obtained from this work, the simultaneous overexpression of the pyruvate carboxylase gene together with the gene of aspartate kinase (*ask*) was performed and the data obtained are presented in this paper.

2. Materials and methods

2.1. Strains, media and growth conditions

*Corynebacterium glutamicum* ATCC strain 21253 was used as the main source of chromosomal DNA for PCR purposes, and also as host strain for plasmids constructed in this work and tested in shake flask experiments. Strains were grown on either brain–heart infusion (BHI) rich medium or on standard minimal medium (Park, 1996; Vallino and Stephanopoulos, 1993). Whenever necessary, the medium was supplemented with 30 μg ml⁻¹ kanamycin.

For lysine production, cells were cultivated in 300-ml flasks containing 50 ml of defined medium supplemented with 20 g L⁻¹ glucose or lactate (Park, 1996) in the shaking incubator (New Brunswick Scientific, Inc., Edison, NJ) at 250 rpm, 30°C. A proper amount of samples at specific time points were taken for further analysis.

*Escherichia coli* DH5α (*hsdR*, *recA*) (Hanahan, 1983) was used for transformations. The strain was routinely grown on LB medium at 37°C in shake flasks. Whenever recombinant *E. coli* was grown, the medium was supplemented with 100 μg ml⁻¹ of ampicillin.

A comprehensive summary of strains used in the present work is given in Table 1.

2.2. DNA manipulations

Transformations, cloning procedures and DNA isolation were performed using standard protocols (Sambrook et al., 1989). Polymerase chain reaction (PCR) amplifications were performed in 100 μl volumes containing primers (0.5 μM each) custom-made by Gibco-BRL, deoxynucleotide triphosphates (dNTPs) in different concentrations, chromosomal DNA (<0.5 μg), and either *Taq* or *Pfu* polymerase (0.5 U) in the buffer recommended by the manufacturer (Boehringer Mannheim, Germany; Stratagene, La Jolla, CA). Temperature cycling was performed by a programmable thermocycler (PTC-150 minicycler, MJ Research Inc., Watertown, MA) following standard protocols with minor modifications based on specific primers and amplification results.
Preparation of *C. glutamicum* electro-competent cells as well as electroporation was performed as previously described (Dunican and Shivnan, 1989).

2.3. Cloning of the aspartate kinase gene

Polymerase chain reaction was used for the isolation of the aspartate kinase (*ask*) gene as a 1.3 kb DNA fragment. Flanking primers were designed based on the sequence published previously (Kalinowski et al., 1990; Thierbach et al., 1990). The upstream primer sequence was 5'-TTTATACCCGGGGAGTTG-3' and the downstream 5'-C-CTGGTCGACCTGGCCGGTGACCAACAC-3'. An artificial *Sma* I site was introduced at the 5'-terminal end of the beginning primer and a *Sal* I site at the 5'-terminal end of the end primer in order to facilitate subsequent cloning steps. The PCR amplification was performed in a way that minimizes the possibility of error during the amplification process. For that purpose, *Pfu Turbo* (Stratagene, La Jolla, CA) was used as the DNA polymerase enzyme while the nucleotide concentration was kept at a low level (1 mM) and 25 cycles were performed in total. PCR reaction was carried out using genomic DNA isolated from *C. glutamicum* strain 21253 as template.

After PCR, the *ask* gene was inserted into the *Srf* I site of the *E. coli* vector pCR-Script. The resulting 4.2 kb plasmid, named pCR253ask, contained the *ask* gene from strain 21253. The PCR product was next sequenced to confirm that the corresponding aspartate kinase gene of strain 21253 was identical with the one reported in the literature.

The aspartate kinase gene in the above constructs was removed from the *E. coli* vector pCR-Script by double-digestion with *Sma* I-*Sal* I and inserted into the *C. glutamicum–E. coli* shuttle vector pMG108 (Colon et al., 1995) that was also digested with the same enzymes. This step introduced the aspartate kinase gene immediately downstream of the *E. coli–C. glutamicum* tac promoter carried by plasmid pMG108 (Colon et al., 1995). The plasmid so constructed was designated as pMG253ask, which was further electroporated into strain 21253, yielding recombinant strain 21253(pMG253ask).

2.4. Construction of pyruvate carboxylase–aspartate kinase containing plasmid

For the overexpression of the pyruvate carboxylase and aspartate kinase genes, plasmid pKD7 harboring the gene of pyruvate carboxylase (Koffas et al., 2002) was digested with *Sac* I. The product of this restriction digest was further polished with *Pfu* according to manufacturer’s manual (Stratagene, La Jolla CA) and dephosphorylated according to standard procedures (Sambrook et al., 1989). Plasmid pMG253ask was digested with *Kpn* I-*Sal* I. The approximately 2 kb DNA fragment that contains the aspartate kinase gene was polished by *Pfu* and inserted into the previously digested, polished and dephosphorylated plasmid pKD7. This cloning strategy yielded the new plasmid construct, pKD7ask253, that carries both the *ask* and *pyc* genes of *C. glutamicum*. The introduction of the aspartate kinase gene into plasmid pKD7 was further verified by PCR analysis and restriction digests (Koffas, 2000). This construct was finally introduced into strain 21253 yielding recombinant strain 21253(pKD7ask253).

2.5. RNA isolation from *C. glutamicum*

RNA isolation from *C. glutamicum* was performed using the RNAqueous (Ambion, Austin, TX) isolation kit with a modification of the protocol provided by the company at the initial lysis step.

Cells were grown overnight in 3 ml cultures using glucose or lactate minimal medium ($OD_{600} ≈ 1$) and were harvested by centrifugation and resuspended in solution E (40 mM Tris; 2 mM EDTA, pH adjusted to 7.9 with acetic acid) and 250 μL 1M sorbitol that contains lysozyme at concentration 3 mg mL$^{-1}$. After the addition of 10 μL mutanolysin the suspension was left to incubate at 37°C for 1 h.

To the slurry, 50 μL of 0.5 M EDTA and 100 μL of 10% SDS were added. The mixture was then boiled for 2 min and immediately returned to ice where it was left to incubate for 10 min. The cell debris was removed and to the supernatant an equal volume of 64% ethanol was added and thorough mixing was performed. After centrifugation at 15,000 rpm for 15 min the supernatant was discarded and the precipitated RNA was subsequently treated according to manufacturer’s instructions (Ambion, Austin, TX). RNA was quantified by measuring absorbance at 260 nm.
The procedures for RNA electrophoresis and Northern Blot hybridization were based on the protocols provided for Southern Blot hybridization from Roche Molecular Biochemicals (DIG-based procedure, Roche Molecular Biochemicals Inc., Germany), Schleicher & Schuell (Transblotter, S&S Inc., Germany) as well as the methods by Sambrook et al. (1989) with necessary adjustments (Koffas, 2000).

2.6. Pyruvate carboxylase and aspartate kinase enzymatic assays

The enzymatic assay of pyruvate carboxylase was performed as previously described in the literature (Koffas et al., 2002). The activity of aspartate kinase was measured by the modified method based on the procedures proposed by Thierbach et al. (1990) and Folletie et al. (1993). Cells grown on the defined medium described above were harvested at mid-exponential phase and washed twice with washing buffer containing 100 mM Tris-Cl (pH 7.5), 1 mM dithiothreitol and 800 mM ammonium sulfate. Washed cells were homogenized using glass bead mill (5100 Mixer Mill, SpexCentriprep Inc., Metuchen, NJ). Crude extract obtained was mixed with 5 vol of saturated ammonium sulfate and collected precipitate by centrifugation (8000g, 10 min). Precipitate was resuspended in 1 ml assay buffer containing 100 mM Tris-Cl (pH 7.5), 1 mM dithiothreitol, 400 mM ammonium sulfate, 400 mM hydroxylamine, 300 mM L-aspartate, 40 mM ATP and 20 mM magnesium chloride and incubated for 60 min at 37°C. After the incubation, the amount of formed aspartyl hydroxamate was measured by spectrophotometry at 540 nm and one unit of aspartate kinase activity was defined as the amount of enzyme to produce one nmole of aspartyl hydroxamate per min under the reaction condition described above.

3. Results

3.1. Overexpression of the pyc gene enhances growth but reduces specific lysine productivity

The pyc gene was overexpressed on a plasmid vector that was constructed using plasmid pEP2 as the backbone (Koffas et al., 2002; Zhang et al., 1994). The pyruvate carboxylase gene was cloned from one of the four positive cosmids of our C. glutamicum cosmid library that contain this gene, designating pKD7, as previously described (Koffas et al., 2002) and its overexpression in C. glutamicum was confirmed by Western blot analysis as shown in Fig. 2a.

Contrary to initial expectations based on the importance of the pyruvate carboxylase catalyzed pathway to lysine synthesis (Park et al., 1997a, b), pyc overexpression resulted in a reduction of specific lysine productivity while the growth rate of the cells was enhanced. Specifically, as summarized in Table 2, the final cell concentration of the recombinant strain was 75% higher than that of the control strain while the specific lysine productivity was reduced by 63% in cells with 2.5 fold greater pyruvate carboxylase activity relative to the control. (Koffas, 2000; Koffas et al., 2002) We hypothesize that this result is due to an imbalance between the higher pyruvate carboxylase activity resulting from the single pyc overexpression and the native activity of other downstream enzymes in the lysine pathway. As illustrated in Fig. 1b, this imbalance would lead to accumulation of tricarboxylic acid (TCA) cycle metabolite intermediates that, as biosynthetic precursors, fuel further cellular growth instead of product formation. Since aspartate phosphorylation has been identified in the past as one of the three steps in lysine synthesis where control is exerted (Cremer et al., 1991), aspartate kinase became the next target for improving lysine flux.

3.2. Aspartate kinase overexpression results in reduced growth and marginal increase of specific lysine productivity

Aspartate kinase overexpression was verified both by Northern blot analysis as well as in vitro enzymatic analysis (Fig. 2) as described earlier. As shown in Fig. 2b, the amounts of ask transcripts in two recombinant strains, 21253(pMG253ask) (harboring the

<table>
<thead>
<tr>
<th>Strain</th>
<th>q0 (mg lysine g cell⁻¹h⁻¹)a</th>
<th>Xf (g L⁻¹)b</th>
<th>Q0 (mg lysine L⁻¹h⁻¹)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>21253d</td>
<td>63</td>
<td>2.5</td>
<td>157.5</td>
</tr>
<tr>
<td>21253(pKD7)d (pyc overexpressing strain)</td>
<td>23</td>
<td>4.0</td>
<td>92</td>
</tr>
<tr>
<td>21253'e</td>
<td>66</td>
<td>15</td>
<td>900–1000g</td>
</tr>
<tr>
<td>21253-33 (ask overexpressing strain)</td>
<td>90</td>
<td>10</td>
<td>900–1000g</td>
</tr>
</tbody>
</table>

a Maximum specific lysine production rate.
b Final cell concentration.
c Maximum volumetric lysine production rate.
d Cells were grown on lactate defined media (Koffas et al., 2002).
e Data adopted and recalculated from the 2-l batch fermentation operated under control of pH and DO by Hua et al. (2000).
f Results are for growth on complex media. No growth has been reported for ask overexpressing strains on defined media.
g Range is due to small changes in cell density during lysine accumulation.
regulated *ask* gene) and 21253(pKD7ask253) (harboring both *ask* and *pyc* genes), were significantly higher than the control strain 21253. The larger transcript levels of *ask* apparently translate into greater enzymatic activities: As shown in Fig. 2c, aspartate kinase activities in the recombinant strains were 3.5–4.5 times greater than the control strain consistent with the Northern blot results.

The purpose of overexpressing the *ask* gene was to enhance lysine productivity in *C. glutamicum* cultures grown on defined media. However, none of the *ask*-overexpressing constructs described above was able to grow on defined media supplemented with glucose or lactate. This is consistent with prior researches (Jetten et al. 1995; Hua et al., 2000) reporting similar unsuccessful results, the growth on defined media of *C. glutamicum* transformants overexpressing aspartate kinase gene. Table 2 shows that overexpression of aspartate kinase was detrimental for cell growth in complex media while yielding a marginal increase in specific lysine productivity (Hua et al., 2000). These results are corroborated by similar finding in Jetten et al. (1995) In accordance with our previous hypothesis this result is probably due to an imbalance between the higher aspartate kinase activity and the native activity of the anaplerotic enzymes (pyruvate carboxylase and PEP carboxylase) that replenish the TCA cycle intermediates. This imbalance would drain biosynthesis precursors and impair growth as depicted in Fig. 1c.

### 3.3. Simultaneous overexpression of the *pyc* and *ask* genes yields significant increase of specific lysine productivity without impacting growth in *C. glutamicum*

As it can be seen from the above results, the effects of pyruvate carboxylase overexpression are exactly the opposite of those of aspartate kinase overexpression. In the first case, pyruvate carboxylase overexpression apparently increases the carbon flux from glycolysis to the anaplerotic pathway (Fig. 1). However, the higher carbon flux cannot eventually translate into more lysine production because of the presence of rate limitations in the lysine pathway downstream of oxaloacetate. On the other hand, the increased anaplerotic flux enhances the availability of biosynthesis precursors. The overall result of this imbalance is less lysine production but better growth. In the case of aspartate kinase overexpression, the product pathway is drawing an increased flux from the biosynthesis precursor pool impacting negatively growth, especially in defined media (Fig. 1c). Increases in specific lysine productivity are not reflected in similar increases of volumetric productivities due to the loss in biomass resulting from the limitation in biosynthesis precursors. The question that naturally arises is whether it is possible to counterbalance the negative effect of aspartate kinase overexpression on growth by increasing the anaplerotic activity through *pyc* overexpression. In other words, could we expect an improved cell physiology in terms of *both* robust growth and greater...
specific lysine production if *both* pyruvate carboxylase and aspartate kinase are simultaneously overexpressed? The expression of both pyruvate carboxylase and aspartate kinase genes in the double recombinant strain 21253(pKD7ask253) was confirmed by Western blot analysis, Northern blot analysis and in vitro enzymatic analysis, as shown in Fig. 2.

Cultures of the strain 21253(pKD7ask253) were carried out as described in Materials and Methods. As shown in Figs. 3 and 4, threonine (an auxotrophic requirement for strain 21253) is depleted right before the cells reach their stationary phase. At that time lysine starts accumulating at an almost constant rate. Specific lysine production rates were calculated using the data of lysine accumulation in the culture broth during the time period where lysine concentration increased linearly with time and the average cell concentrations did not change significantly. Volumetric production rates ($Q$) were estimated using least-squares method. The following equation was used to calculate the specific production rate $q$:

$$q \text{ (mg lysine g cell}^{-1} \text{h}^{-1}) = \frac{Q \text{ (mg lysine L}^{-1} \text{h}^{-1})}{\text{avg.cell concentration (g L}^{-1})}.$$  

Specific rates thus calculated for the control and the double recombinant strain, along with final cell concentrations, are summarized in Table 3.

As it can be seen, the recombinant strain 21253(pKD7ask253) has similar growth characteristics with the control strain carrying the empty vector. The specific growth rates and final cell concentrations are identical (0.35 h$^{-1}$ and 2.6 g L$^{-1}$, respectively) when lactate is used as a sole carbon source in the minimal medium and quite similar with glucose limiting media (Fig. 4). On the other hand, both final lysine concentrations as well as specific lysine productivity are significantly increased in the case of the recombinant strain. This is significantly higher when lactate is used as the sole carbon source in which case the highest pyruvate carboxylase expression occurs (Table 3, Koffas, 2000).

4. Discussion

Good amino acid producers have been isolated in the past by classical breeding and random mutagenesis. As a result it has become challenging to increase the lysine yield in such producers by applying recombinant DNA
techniques. A series of experiments (Cremer et al., 1991; Jetten et al., 1995; Malumbres and Martin, 1996) identified aspartate kinase as a potential lysine limiting step, together with dihydrodipicolinate synthase and the lysine exporter. Indeed, the flux control coefficient for aspartate kinase is higher than those for other enzymes especially in the early period of lysine production (Hua et al., 2000). This was confirmed experimentally whereby elevated aspartate kinase activities yielded higher specific lysine productivity (Jetten et al., 1995) albeit at the cost of reduced cell growth. Similar results were obtained with other prior attempts, such as with the overexpression of dihydrodipicolinate synthase that resulted in higher lysine yield, however the flux redistribution also introduced a growth limitation (Eggeling et al., 1998). It is thus seen that overexpression of enzymes in the product pathway gives rise to an inverse relation between flux increase towards product and an intracellularly introduced growth limitation. In order to explain this type of physiological behavior, the following metabolic model is proposed: increase in one of the limiting steps in lysine biosynthesis results in higher carbon flux towards lysine. However this also lowers the carbon flux that replenishes the TCA cycle intermediates reducing, as a consequence, the rate at which other amino acids (such as glutamate), porphyrins and ATP are generated (Fig. 1c). As a result, attenuation in growth is observed in ask and dapA overexpressing recombinant strains.

On the other hand, overexpression of the key anaplerotic enzyme, pyruvate carboxylase, results in an increase of carbon flux into the TCA cycle. If the other enzymes involved in lysine biosynthesis are not similarly amplified, this can lead to accumulation of aspartate, a metabolite that usually functions as a pyruvate carboxylase inhibitor (Fig. 1b). The fact that an increase of anaplerotic carbon flux does not translate into more lysine can be due to various reasons related to the regulatory characteristics of the phosphoenolpyruvate-pyruvate-oxaloacetate triangle or to the presence of a kinetic bottleneck within the lysine biosynthesis pathway. As such, intracellular interactions are quite complex and poorly described and our metabolic engineering strategy is to minimize

Fig. 3. Effect of simultaneous overexpression of pyc and ask on the (a) growth and (b) lysine production and threonine consumption of C. glutamicum grown on glucose.

Fig. 4. Effect of simultaneous overexpression of pyc and ask on the (a) growth and (b) lysine production and threonine consumption of C. glutamicum grown on lactate.
intracellular metabolite perturbations through the coordinated amplification of key pathway enzymes. In this context, a natural question is whether it is possible to counterbalance the negative effect on growth from aspartate kinase overexpression by increasing the anaplerotic activity through the pyruvate carboxylase gene overexpression. From the data presented in Table 3 we can conclude the following: the simultaneous overexpression of *pyc* and *ask* yields a recombinant strain with growth characteristics similar to those of the control strain (parental strain carrying only the empty vector). Specific growth rates are almost identical while final cell concentrations are very close to each other, especially when lactate is used as a sole carbon source in the minimal medium. Additionally, both final lysine titer as well as lysine productivity increase to significant levels in the case of the recombinant strain. Productivity enhancement is significantly higher when lactate is used as the sole carbon source presumably because of pyruvate carboxylase activation by lactate (Peters-Wendisch et al., 1998). The importance of *pyc* and *ask* can be found in two studies reported recently. Peters-Wendisch et al. (2001) showed that, in a strain of C. glutamicum which has a different type of regulatory characteristics and relatively free from the kinetic bottleneck in lysine synthesis pathway with deregulated aspartate kinase, *pyc* overexpression itself enhanced the lysine titer in the culture broth to some extent without significant change of growth. This clearly indicates that *pyc* overexpression is highly responsible for lysine production combined with *ask*. Ohnishi et al. (2002) reported that enhanced lysine production could be obtained by “genome-based mutagenesis” creating several mutations in *hom*, *ask*, and *pyc*. Since ATCC 21253 used in this study is homoserine dehydrogenase deleted (*hom−*) as described in Table 1, the importance of both genes, *pyc* and *ask* in lysine production of *C. glutamicum* can be confirmed in this study. The level of lysine accumulation in this study, however, is relatively lower than industrial level which has been achieved under the extremely optimized cultivation condition. However, this approach can give a chance to improve the productivity in industrial level of lysine production.

Table 3
Summary of cultivation results of *C. glutamicum* strains 21253, and 21253(pKD7ask253) (*pyc* and *ask* overexpressing strain) grown on glucose and lactate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose</th>
<th></th>
<th></th>
<th>Lactate</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu_m) (h(^{-1}))(^{a})</td>
<td>(q_m) (mg lysine g cell(^{-1}) h(^{-1}))(^{b})</td>
<td>(X_f) (g L(^{-1}))(^{c})</td>
<td>(\mu_m) (h(^{-1}))</td>
<td>(q_m) (mg lysine g cell(^{-1}) h(^{-1}))</td>
<td>(X_f) (g L(^{-1}))</td>
</tr>
<tr>
<td>21253</td>
<td>0.54</td>
<td>5.5</td>
<td>5.8</td>
<td>0.35</td>
<td>42.3</td>
<td>2.6</td>
</tr>
<tr>
<td>21253(pKD7ask253)</td>
<td>0.5</td>
<td>7.9</td>
<td>6.7</td>
<td>0.35</td>
<td>106.6</td>
<td>2.6</td>
</tr>
</tbody>
</table>

\(^{a}\)Maximum specific growth rate.

\(^{b}\)Maximum specific lysine production rate.

\(^{c}\)Final cell concentration.

Clearly, the two enzymes have counterbalancing effects that eventually lead to an improved strain with respect to both growth and productivity. In fact, overexpression of two genes has been applied previously in amino acid production in *Corynebacterium*, namely in optimizing strains for threonine production through the coordinated amplification of homoserine dehydrogenase and homoserine kinase (Colon et al., 1995). In that work, the activities of the two enzymes were optimized by fixing the overexpression of the first using a native promoter and modulating the second through a regulated promoter and external addition of the IPTG inducer. In that case, the overexpressed genes had minimal impact on central carbon metabolism and insignificant effect on cell growth. The overexpression of these genes affected only the redistribution of carbon flux between the lysine and threonine pathways. Subsequent studies should evaluate the effect of simultaneous overexpression of pyruvate carboxylase and other potential bottlenecks in the lysine biosynthesis, such as diaminopimelate synthase and the lysine export system.

Many attempts to increase productivity by genetic engineering are unsuccessful. There are many reasons contributing to this, including the fact that targeted enzymes have small flux control coefficients. Another possibility is disruptive perturbations of intracellular metabolites from their steady state. The present work demonstrates that this can be avoided by the simultaneous amplification of sequential enzymes, in accordance with the Universal Method of Kacser and Acerenza. This method suggests the overexpression of multiple enzymes in a metabolic pathway such as to minimize changes in the concentrations of metabolite intermediates (Kacser and Acerenza, 1993; Kacser and Burns, 1973, 1995).

It has been observed that high level induction of recombinant proteins leads to growth attenuation and reduced metabolic activity (Farmer and Liao, 2000; Kurland and Dong, 1996). The reason is that changes in metabolic fluxes imposed to satisfy production demands, disrupt metabolism and alter intracellular metabolite levels, both of which interfere with growth requirements. A different approach, termed metabolic control engineering, has been presented recently for
overcoming this problem and has been applied successfully in secondary metabolite production, such as lycopene. According to this, the burden imposed on the cell for overexpressing recombinant proteins can be alleviated by designing dynamic controllers that are able to sense the metabolic state of the cell and regulate the expression of the recombinant protein accordingly (Farmer and Liao, 2000). Due to their strong connection to growth, this metabolic control engineering approach cannot be applied in primary metabolite production. The present work demonstrates that by carefully selecting and overexpressing multiple genes, recombinant strains can be constructed that overproduce primary metabolites with minimal growth limitations. This was accomplished without altering the transcriptional regulation of the genes of interest, a process that can be quite complicated considering the tight relationship between primary metabolite production and cell growth.

The idea of multiple gene overexpression has been applied before. It has been used successfully in various cases of fermentative production of chemicals. In the case of Colon et al. (1995) fusion of the C. glutamicum homoserine kinase thrB gene to the tac promoter and regulated expression of this gene with that of homoserine dehydrogenase (hom+) led to an increase of the final threonine titer by 120%. In a similar mode, metabolic engineering of the early non-mevalonate terpenoid pathway of E. coli was carried out to increase the supply of phenyl pyrophosphates as precursor for carotenoid production. Transformation with the genes dxs for overexpression of 1-deoxy-d-xylulose 5-phosphate synthase, ddxr for 1-deoxy-d-xylulose 5-phosphate reductoisomerase and idi encoding an isopentenyl pyrophosphate stimulated carotenogenesis up to 3.5 fold. Cotransformation of idi with either ddxs or ddxr had an additive effect on β-carotene and zeaxanthin production which reached 1.6 mg g⁻¹ of dry weight. (Albrecht et al., 1999). In the case of lysine, production improvement has been attempted by overexpressing single as well as multiple genes in the lysine biosynthesis pathway (Cremer et al., 1991). These examples of multiple gene overexpression, have concerned enzymes within the product pathway. The ability to increase carbon flux towards product formation by overexpressing genes in the central carbon metabolism of the host cell is one of the contributions of this work. Clearly this approach can be extended with further overexpression of other downstream genes in the lysine pathway.

References


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