Metabolic Engineering of the Tricarboxylic Acid Cycle for Improved Lysine Production by *Corynebacterium glutamicum*†

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In the present work, lysine production by *Corynebacterium glutamicum* was improved by metabolic engineering of the tricarboxylic acid (TCA) cycle. The 70% decreased activity of isocitrate dehydrogenase, achieved by start codon exchange, resulted in a >40% improved lysine production. By flux analysis, this could be correlated to a flux shift from the TCA cycle toward anaplerotic carboxylation.

With an annual market volume of more than 1,000,000 tons, lysine is one of the dominating products in biotechnology. In recent years, rational metabolic engineering has emerged as a powerful tool for lysine production (16, 18, 22). Hereby, different target enzymes and pathways in the central metabolism could be identified and successfully modified to create superior production strains (1, 2, 5, 8, 10, 17–20). The tricarboxylic acid (TCA) cycle has not been rationally engineered so far, despite its major role in *Corynebacterium glutamicum* (6). From metabolic flux studies, however, it seems that the TCA cycle might offer a great potential for optimization (Fig. 1), which is also predicted from in silico pathway analysis (13, 22). Experimental evidence comes from studies with *Brevibacterium flavum* exhibiting increased lysine production due to an induced bottleneck toward the TCA cycle (21). In the present work, we performed TCA cycle engineering by downregulation of isocitrate dehydrogenase (ICD). ICD is the highest expressed TCA cycle enzyme in *C. glutamicum* (7). Downregulation was achieved by start codon exchange, controlling ICD expression on the level of translation.

Microorganisms and strain construction. In the present work, two lysine-producing strains of *C. glutamicum* derived from the wild-type strain, ATCC 13032, were investigated. A detailed description is given in Table 1. All modifications implemented to yield the strains *C. glutamicum* BS87 and *C. glutamicum* BS205 were stably integrated into the genome by replacement of the wild-type allele by the mutant allele (1). Transformation was performed with an integrative vector, using kanamycin resistance and sucrose tolerance as positive selection markers (1, 9). Vector construction was carried out with standard cloning strategies involving PCR, enzymatic digestion, and DNA ligation. Depending on the implemented modification, strain validation was performed by PCR, determination of enzyme activity, Southern blotting, or sequencing.

Cultivation and growth conditions. Cultivation was performed as described previously in two precultures and a main culture (2). First, preculture was carried out in complex medium containing 10 g liter−1 peptone, 5 g liter−1 beef extract, 5 g liter−1 yeast extract, 2.5 g liter−1 NaCl, 10 g liter−1 glucose, and 2 g liter−1 urea. Second, preculture and main culture were performed in minimal medium as described previously (3). Medium composition was modified by reducing the glucose concentration to 10 g liter−1 and increasing the ammonium sulfate concentration to 15 g liter−1. Main cultivation was performed in triplicate using 2-liter baffled shake flasks with 200 ml minimal medium. During the cultivation, the pH remained constant within a range of 7.1 ± 0.1, and sufficient oxygen supply was ensured.

Substrate and product analysis. Glucose was quantified with a biochemical analyzer (YSI 2700 Select; Kreienbaum, Langeinfeld, Germany). Concentrations of organic acids and trehalose were determined by high-performance liquid chromatography on an Aminex HPX-87H column (300 by 7.8; Bio-Rad, Hercules, CA) at 45°C, with 10 mM H2SO4 as mobile phase and a flow rate of 0.5 ml min−1 and detection via refractive index (trehalose) or UV absorbance (organic acids) at 210 nm. Amino acids were quantified as described previously (14). Here, an optimized gradient profile was applied, increasing eluent B by 4% min−1. Cell concentration was determined as optical density at 660 nm (OD660) (Libra S11; Biochrome, Cambridge, United Kingdom) or gravimetrically as cell dry mass (CDM) (11). The correlation factor between OD660 and CDM was determined to be CDM (g liter−1) = 0.255 × OD660.

Determination of ICD activity. Cell preparation was performed as previously described (1). Cell suspension was then aliquoted in 750-μl volumes in 2-ml Eppendorf tubes containing glass beads. Disruption was performed in a ribolizer (MM301; Retsch, Haan, Germany) at 30 Hz (5 min two times; 5-min break in between). Crude cell extracts were obtained by centrifugation for 10 min at 13,000 × g. Protein content was quantified by the method of Bradford (4).Activity was determined in a volume of 1 ml at pH 7.8 and 30°C. The assay mixture contained 100 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 1 mM isocitrate, 0.5 mM NADP, and 25 μl of crude cell extract. The change in absorbance at 340 nm due to NADPH formation was monitored online (Specord 40; Analytik Jena, Jena, Germany). Negative control and evaluation were performed as previously described (2).
corresponding citrate synthase fluxes (11, 12, 15, 23).

The data displayed represent values from 18 independent experiments with different Corynebacterium glutamicum strains. The flux mean values and confidence intervals were calculated from 100 statistically varied values for each strain. For BS205, the specific ICD activity was 141.3 ± 3.5 mmol/mol and Y_{X Sly} was 72.6 ± 2.0 g/mol; and for BS205, they were 200.4 ± 4.4 mmol/mol and 68.4 ± 2.5 g/mol, respectively (data represent mean values from three parallel cultivation experiments and corresponding deviations). The yields were determined as the slope of the linear best fit when plotting product formation against substrate consumption (see Fig. 2). As a response to the downregulation of ICD, the lysine yield on glucose was increased by 42%. Specific growth rate was slightly reduced in the mutant strain (0.28 h⁻¹) compared to that of the parent strain, BS87 (0.32 h⁻¹). Both strains produced small amounts of trehalose (<10 mmol mol⁻¹) but no other by-products. The yields of lysine and biomass, as well as the specific growth rates, were constant throughout the whole cultivation, showing that the strains were in metabolic steady state (Fig. 2). The observed differences between the two strains are therefore clearly attributed to the changed start codon sequence and the resulting reduction of the specific activity of ICD.

Estimation of in vivo fluxes. The in vivo flux through the TCA cycle was estimated by compiling a correlation between biomass yield, lysine yield, and TCA cycle flux, respectively. To this end, experimentally determined data for biomass yield (Y_{X Sly}) and lysine yield (Y_{X Sly}) from 18 independent experiments with Corynebacterium glutamicum were correlated to the corresponding citrate synthase fluxes (v_{CIS}) determined by 13C metabolic flux analysis (Fig. 1). A stoichiometric correlation was achieved by a paraboloid fitting of the data set with the following equation:

\[ v_{CIS} = Y_0 + aY_{LysS} + bY_{X Sly} + cY_{LysS}^2 + dY_{X Sly}^2 \]

Net flux through anaplerosis (PYC) was calculated as described previously (23). Flux mean values and confidence intervals were calculated from 100 statistically varied values for biomass yield, lysine yield, and anabolic fluxes, using a Monte Carlo approach.

Table 1. Description of Corynebacterium glutamicum BS87 and C. glutamicum BS205 constructed in the present work by stable genetic modification of the wild-type C. glutamicum ATCC 13032

<table>
<thead>
<tr>
<th>C. glutamicum strain</th>
<th>Genotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS87</td>
<td>sodp lyc(T311I)</td>
<td>T311I mutation in the lyc gene, encoding aspartokinase, and overexpression by replacement of the natural promoter by the promoter of sod, encoding superoxide dismutase</td>
</tr>
<tr>
<td></td>
<td>ddh ddh</td>
<td>Deletion of pck, encoding PEP carboxykinase</td>
</tr>
<tr>
<td></td>
<td>Δpck</td>
<td>Overexpression of pck, encoding dihydrodipicolinate reductase, by the sod promoter</td>
</tr>
<tr>
<td></td>
<td>sodp dapB</td>
<td>Implementation of a second gene copy of ddh, encoding diaminopimelate dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>lycA lycA</td>
<td>Implementation of a second gene copy of lycA, including the gene for diaminopimelate decarboxylase</td>
</tr>
<tr>
<td></td>
<td>hom(V59A)</td>
<td>V59A mutation in the hom gene, encoding homoserine dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>sodp pyc(ΔP458S)</td>
<td>Implementation of the pyc gene, encoding pyruvate carboxylase, and overexpression by sod promoter</td>
</tr>
<tr>
<td>BS205</td>
<td>BS87 icd(A17)</td>
<td>Replacement of the start codon ATG by GTG in the icd gene, encoding ICD</td>
</tr>
</tbody>
</table>

Strain construction and validation. Mutants from the second recombination were screened with regard to their specific ICD activity. Several clones exhibited a reduced specific activity of ICD compared to that exhibited by the parent strain, C. glutamicum BS87. In all mutants with reduced ICD activity, sequence analysis revealed substitution of the start codon ATG by GTG. Clone 1 was selected for further investigations in the present study and designated C. glutamicum BS205.

Specific enzyme activity. After validation of the nucleotide exchange by sequencing, specific ICD activity was determined in minimal medium by using glucose as the sole carbon source. Usage of the rare start codon GTG instead of the common ATG resulted in a drastically reduced specific activity of ICD. While the parent strain, C. glutamicum BS87, exhibited a specific ICD activity of 1.3 ± 0.06 U mg⁻¹, C. glutamicum BS205 exhibited a specific ICD activity of only 0.3 ± 0.04 U mg⁻¹.

Growth and production characteristics. Growth rates, lysine productions, and biomass formations of the different mutants were compared to investigate the impact of the reduced enzyme activity on production characteristics. For BS87, Y_{X Sly} was 141.3 ± 3.5 mmol/mol and Y_{X Sly} was 72.6 ± 2.0 g/mol; and for BS205, they were 200.4 ± 4.4 mmol/mol and 68.4 ± 2.5 g/mol, respectively (data represent mean values from three parallel cultivation experiments and corresponding deviations). The yields were determined as the slope of the linear best fit when plotting product formation against substrate consumption (see Fig. 2). As a response to the downregulation of ICD, the lysine yield on glucose was increased by 42%. Specific growth rate was slightly reduced in the mutant strain (0.28 h⁻¹) compared to that of the parent strain, BS87 (0.32 h⁻¹). Both strains produced small amounts of trehalose (<10 mmol mol⁻¹) but no other by-products. The yields of lysine and biomass, as well as the specific growth rates, were constant throughout the whole cultivation, showing that the strains were in metabolic steady state (Fig. 2). The observed differences between the two strains are therefore clearly attributed to the changed start codon sequence and the resulting reduction of the specific activity of ICD.

In vivo fluxes. To study the impact of the modified enzyme activity on the metabolic pathways in more detail, we estimated the flux through the TCA cycle and anaplerotic carboxylation in the two strains. Based on the experimental yield for biomass and lysine, the resulting TCA cycle and anaplerotic net fluxes for C. glutamicum BS87 and BS205 were determined from the

FIG. 1. Stoichiometric correlation of lysine yield (%), biomass yield (g/mol) and TCA cycle flux (%; entry flux through citrate synthase) determined by 13C metabolic flux analysis achieved by paraboloid fitting of the data set (parameters were determined with Y_0 = 105.1, a = -1.27, b = 0.35, c = -9.35 × 10⁻³, d = -11.16 × 10⁻³). The data displayed represent values from 18 independent experiments with different C. glutamicum strains taken from previous studies (1, 3, 11, 12, 15, 23).
established correlation (Fig. 1). The TCA cycle flux was reduced in the icd mutant, while the anaplerotic flux was increased (Fig. 3). The performed t test clearly revealed a significant difference between the strains considering the TCA cycle flux (t = -10.1) and the anaplerotic flux (t = 43.6). In sum, these flux changes resulted in a flux redirection from the TCA cycle toward anaplerosis.

In the present work, rational strain optimization was achieved by TCA cycle engineering. By replacing the common start codon ATG by the rare GTG in the icd gene, the activity of ICD could be reduced by 70%. This resulted in flux redirection from the TCA cycle to anaplerosis, which enhanced lysine production by more than 40%. Previous metabolic flux studies revealed that strains with increased lysine production exhibit a reduced TCA cycle flux, suggesting this key metabolic pathway as a promising target for engineering (Fig. 1). A complete shut-off of the TCA cycle, suggested from metabolic simulations, can, however, hardly be realized since this does not support biomass formation. In this regard the present study displays an important contribution, since it reduced the TCA cycle flux to a well-balanced extent. TCA cycle engineering nicely complements other successful targets in C. glutamicum, focusing on the biosynthetic pathway of lysine (5, 10), the complex network around the pyruvate node (19, 20), or the pentose phosphate pathway, supplying reducing power in the form of NADPH (1, 2, 17).

Beyond the present study, targeted downregulation of the TCA cycle seems also promising for various other products, since the high carbon loss through CO2 formation by the TCA cycle is generally undesired with respect to carbon yield for the desired product.

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REFERENCES