Biosensor-driven adaptive laboratory evolution of L-valine production in Corynebacterium glutamicum

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I. Introduction

The selection of mutations are the key components of evolution driving adaption and the development of novel traits. The classical strain engineering based on rational design approaches is often limited by the current knowledge of bacterial physiology. Alternatively, is the random mutagenesis based on a high-throughput approach followed by an efficient screening strategy applied to overcome the limits of rational strain development. Up to now, laboratory evolution experiments of mainly fitness-linked phenotypes have been performed by exposing microorganisms to sequentially increasing levels of environmental stress. Especially in the case of the yeast Saccharomyces cerevisiae, adaptation to an improved ethanol tolerance has been proven useful for increasing product formation.

In this study, involves the implementation of an artificial selective pressure on the fluorescent output of transcription factor-based biosensors by fluorescence-activated cell sorting (FACS). Recently, it was developed an amino acid biosensor, based on the transcriptional regulator Lrp of Corynebacterium glutamicum, which enables the intracellular detection of L-methionine as well as branched-chain amino acids and translates this information into a measureable fluorescent output. The knockout strain (Figure 1) for the L-valine production, mutants deficient in the E1p subunit (ΔaceE) of the pyruvate dehydrogenase complex (PDHC). Due to the inactivation of the PDHC, pyruvate accumulates in the cell and is channeled as a precursor towards L-valine production.
It was shown a successfully established a biosensor-driven adaptive evolution approach to improve L-valine production of *C. glutamicum* ΔaceE.

II. Results

The biosensor driven evolution of *C. glutamicum* ΔaceE

The producer strain *C. glutamicum* ΔaceE containing the sensor plasmid pJC1-Lrp-sensor was used for several metabolic engineering studies and displays a characteristic growth-decoupled production phenotype. The cells were analyzed and sorted by FACS after in the stationary phase 28 h of cultivation and shown an increased intracellular L-valine pool than resulting in a measurable output of the Lrp-biosensor. After one evolution step, one million cells showing the top 10% fluorescent output signal, followed an re-cultivation in minimal medium. Within five evolution steps, the L-valine production of the culture increased by about 25% (from 13 mM to about 16 mM in average) and the level are remaining constant, while the production of L-alanine, as by-product, decreased by a factor of 3–4 to 3.5 mM after the second sorting step.

Analysis of isolated clones

The respective growth and production phenotype of isolated strains was analyzed and cultivated in the BioLector microtiter plate system. Compared to the non-evolved, parental strain *C. glutamicum* ΔaceE (μmax=0.36±0.007 h⁻¹), the evolved cells featured a shorten lag phase and an increased growth rate of μmax=0.41±0.019 h⁻¹. To verify growth and production of isolated clones, two strains with high (M1 showed 57 mM) and medium (M2 showed 42 mM) L-valine accumulation in the supernatant were analyzed in comparison to the parental ΔaceE strain.
Characterization of accumulated mutations

In order to identify the accumulated mutations in this experiment was the genomic DNA isolated form cultures after every evolution step and from two clones (M1 and M2) and analyzed by next-generation sequencing. Seven mutations were identified in the population after the 5th evolution step (Table 1).

<table>
<thead>
<tr>
<th>Variant data</th>
<th>C. glutamicum ATCC13032 ΔaceE (parental strain)</th>
<th>SNP frequency in the last population</th>
<th>Isolated clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>C96079T</td>
<td>aceD E188*</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>T307688A</td>
<td>glxR T93S</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C673216T</td>
<td>ppcD2 T201I</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>C2135276A</td>
<td>rmp D300D (e900)</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>Del C228302</td>
<td>glxR G801G</td>
<td>96</td>
<td>95</td>
</tr>
<tr>
<td>C265520287T</td>
<td>lrp T248T (g1046)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C2803292T</td>
<td>g26467</td>
<td>36</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 1 Accumulated mutations in the biosensor-driven evolution of C. glutamicum ATCC13032 ΔaceE.

For example, single-nucleotide polymorphisms (SNPs) leading to amino acid exchanges or a stop codon were identified in glxR (T93S), encoding cAMP-dependent global transcriptional regulator, in prpD2 (T201I), coding for 2-methylcitrate dehydratase, and in ureD (E188*), encoding the urease accessory protein UreD. After 33 h of cultivation from parental strain C. glutamicum ATCC13032 ΔaceE showed an YP/X of 1.54 mmol L-valine per g CDW, during after the introduction of ΔaceE ureD-E188* into the parental strain exhibits significantly increased L-valine production by about 100% with a YP/X of 3.03 mmol per g CDW.

Loss-of-function in urease activity significantly increases L-valine production

The mutation ureD-E188* results in a truncated version of the urease accessory protein UreD and this disruption was shown to disable the function of urease to degrade urea to carbon dioxide and ammonium. To test whether the loss-of-function in urease activity was C. glutamicum ΔaceE and ΔaceE ureD-E188* cultivated in minimal media with and without 5 g/l urea. Without urea exhibits the ΔaceE strain a significantly increased L-valine level of 36 mM and ΔaceE ureD-E188* a L-valine level of 50.5 mM. Without urea and/or in the presence of the ureD-E188*mutation, L-alanine production of the ΔaceE strain increased about four-to five-fold.

III. Discussion

In this present work could been successfully established a biosensor-driven laboratory evolution approach for the improvement of growth and metabolite production of industrially relevant strains by imposing an artificial selective pressure on the fluorescent output of the biosensor using FACS. The strain C. glutamicum ATCC 13032 ΔaceE containing the plasmid-encoded Lrp-biosensor was evolved toward an increased L-valine production, decreased by-product formation (L-alanine) and show a significantly improved growth behavior. After five sorting steps was the L-valine level remained constant, probably due to the fact that the Lrp-biosensor reached it is intracellular detection limit. In the further toward reduced the sensitivity or increased the dynamic range of biosensor engineering for a further
improvement of this approach. Through genome sequencing and insertion of selected SNPs into the parental strain resulted the identification of novel mutations, which directly affect product (ureD-E188*) or by-product (gtxR-T93S) formation. During the evolution of the ΔaceE strain, four mutations led to amino acid changes or to a stop codon in annotated genes. When mutations were inserted into the non-evolved ΔaceE parental strain, each mutation alone was found to increase the L-valine production. This finding strongly argues that biosensor-based evolution directly selects for strains that accumulate mutations according to the selective pressure. A further interesting mutation beneficial for L-valine production was in the gene encoding the urease accessory protein UreD. When replaced the codon for glutamate residue 188 in the accessory UreD by a stop codon leading to the formation of a truncated protein. The strong downregulation of the urease activity form 7.8 U (mg protein)$^{-1}$ to 0.1 U (mg protein)$^{-1}$ into the parental strain increased significantly the L-valine production by about 100%. After a metabolic analysis using GC-ToF exhibits an increase of the L-valine biosynthesis pathway intermediates 2-aceto-lactate and dihydroxyisovalerate suggesting an increased flux towards L-valine. It was observed a shift from lactate to pyruvate overflow in the absence of urea or in the ureD-E188 background. In conclusion, biosensor-driven evolution proved as an efficient strategy to balance metabolic fluxes according to the engineer’s purposes without any deep knowledge of the complex bacterial physiology. This approach might also be of great benefit for the establishment of heterologous pathways in order to adapt the organism to changed energy and metabolite fluxes.

IV. References


