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

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ABSTRACT

Adaptive laboratory evolution has proven a valuable strategy for metabolic engineering. Here, we established an experimental evolution approach for improving microbial metabolite production by imposing an artificial selective pressure on the fluorescent output of a biosensor using fluorescence-activated cell sorting. Cells showing the highest fluorescent output were iteratively isolated and (re-) cultivated. The L-valine producer *Corynebacterium glutamicum* Δ*aceE* was equipped with an L-valine-responsive sensor based on the transcriptional regulator Lrp of *C. glutamicum*. Evolved strains featured a significantly higher growth rate, increased L-valine titers (~25%) and a 3-4-fold reduction of by-product formation. Genome sequencing resulted in the identification of a loss-of-function mutation (UreD-E188*) in the gene *ureD* (urease accessory protein), which was shown to increase L-valine production by up to 100%. Furthermore, decreased L-alanine formation was attributed to a mutation in the global regulator GlxR. These results emphasize biosensor-driven evolution as a straightforward approach to improve growth and productivity of microbial production strains.

1. Introduction

Mutation and selection are key components of evolution driving adaption and the development of novel traits. Short generation times and a natural mutation frequency of 10^{-10} to 10^{-9} mutations per base pair per replication cycle enable the selection of beneficial phenotypical traits from high genetic diversity (Barrick and Lenski, 2013). During the last few years, laboratory evolution strategies went more and more into the focus to adapt industrial producer strains to detrimental growth conditions such as oxidative and thermal stress (Lee et al., 2013; Oide et al., 2015; Sandberg et al., 2014; Tenaillon et al., 2012), to improve product formation (Raman et al., 2014; Reyes et al., 2014; Xie et al., 2015) or solvent tolerance (Atsumi et al., 2010; Lee et al., 2011; Oide et al., 2015) (for reviews discussing the use of adaptive evolution approaches in metabolic engineering, see Abatemarco et al., 2013; Portnoy et al., 2011).

Due to the high complexity of carbon and energy fluxes in living cells, classical strain engineering based on rational design approaches is often limited by the current knowledge of bacterial physiology. Alternatively, high-throughput engineering approaches based on random mutagenesis followed by an efficient screening strategy are applied to overcome the limits of rational strain development. In this context, the use of biosensors has proven to be a highly valuable tool by translating intracellular product formation into a screenable optical output, such as fluorescence (Dietrich et al., 2010; Eggeling et al., 2015; Schallmey et al., 2014). However, after random mutagenesis strains typically reveal several hundreds of genomic alterations representing a major challenge in identifying those mutations linked to the particular phenotype of interest (Binder et al., 2012; Chou and Keasling, 2013). Here, industrial strain development strongly benefits from adaptive evolution approaches, in which strains typically feature only a few mutations and which enable the enrichment of non-intuitive beneficial mutations by improving growth at the same time (Abatemarco et al., 2013; Portnoy et al., 2011). Up to now, laboratory evolution experiments of mainly fitness-linked phenotypes have been performed by exposing microorganisms to sequentially increasing levels of environmental stress (Eckdahl et al., 2015; Lee et al., 2013; Marietou et al., 2014; Oide et al., 2015; Reyes et al., 2014). Especially in the case of the yeast *Saccharomyces cerevisiae*, adaptation to an improved ethanol tolerance has been proven useful for increasing product formation (Alper et al., 2006; Jiménez and Benítez, 1987; Liu, 2006).

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The bottom line of almost all reported adaptive evolution approaches is selection for improved growth and survival, which usually coincides with increased product formation – especially in the case of growth-coupled processes (Feist et al., 2010). To expand the toolbox for metabolic engineering, we here report on a novel strategy capable of evolving the production of inconspicuous metabolites, which are not linked to fitness. This approach involves the implementation of an artificial selective pressure on the fluorescent output of transcription factor-based biosensors by fluorescence-activated cell sorting (FACS). In previous studies, several laboratories have successfully demonstrated the value of synthetic sensor constructs for small molecule detection as well as their application in high-throughput screening approaches and single-cell analysis (Binder et al., 2012; Dietrich et al., 2010, 2013; Mustafi et al., 2012, 2014; Siedler et al., 2014).

Recently, we developed an amino acid biosensor, based on the transcriptional regulator Lrp of *Corynebacterium glutamicum* (Lange et al., 2012), which enables the intracellular detection of L-methionine as well as branched-chain amino acids, and translates this information into a measureable fluorescent output (Mustafi et al., 2012). This biosensor system has already been successfully applied for online monitoring and live cell imaging studies of *C. glutamicum* L-valine production strains at the single-cell level to analyze phenotypic production heterogeneity (Mustafi et al., 2014).

C. glutamicum is an important industrial platform organism used for the large-scale industrial production of amino acids (e.g. L-glutamate, L-lysine and L-valine) (Eggeling and Bott, 2005; Wendisch, 2007). L-valine is an essential amino acid for vertebrates and is required for infusion solutions, cosmetics or as precursor for herbicides (Eggeling et al., 2001; Leuchtenberger, 1996). In order to engineer strains for L-valine production, mutants deficient in the E1p subunit (*aceE*) of the pyruvate dehydrogenase complex (PDHC) have been constructed and characterized in several studies (Blombach et al., 2008, 2007; Chen et al., 2015; Eikmanns and Blombach, 2014). Due to the inactivation of the PDHC, pyruvate accumulates in the cell and is channeled as a precursor towards L-valine production (Fig. 1). For cell growth, acetate is supplied to the medium to maintain the acetyl-CoA pool for the tricarboxylic acid (TCA) cycle.

In this study, we successfully established a biosensor-driven adaptive evolution approach to improve L-valine production of *C. glutamicum* Δ*aceE*. Isolated evolved clones exhibited significantly increased product formation and reduced formation of the by-product L-alanine. This approach demonstrates the power of biosensor-driven laboratory evolution approaches to select for beneficial and non-intuitive mutations leading to an improved production phenotype.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Strain *C. glutamicum* ATCC 13032 was used as the wild-type strain (Kalinowski et al., 2003).Unless otherwise specified, *C. glutamicum* Δ*aceE* cells were picked from a brain heart infusion (BHI) agar plate containing 85 mM acetate, inoculated in 4 ml BHI medium with 85 mM acetate and incubated for eight hours at 30 °C and 170 rpm. Subsequently, the cells from the first pre-culture were used to inoculate a second pre-culture in a shake flask containing 20 ml CGXII minimal medium (Keilhauer et al., 1993) with 222 mM glucose and 254 mM acetate. The cells were incubated overnight at 30 °C and 120 rpm. The following day, the cells were washed with 0.9% (w/v) saline, adjusted to an optical density (OD_{600}) of 1 in fresh 50 ml CGXII minimal medium

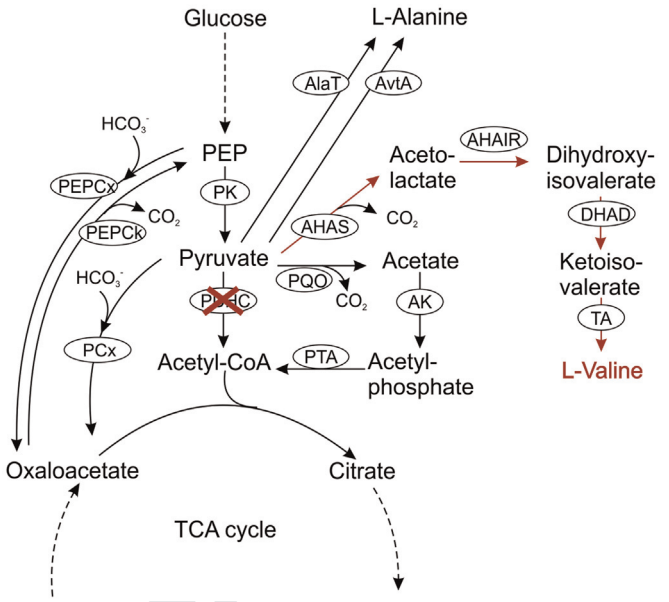

Fig. 1. Schematic of the central metabolism of *C. glutamicum* Δ*aceE* and the L-valine biosynthetic pathway. Due to the deletion of the Ep1 subunit (Δ*aceE*) of the PDHC, pyruvate is not converted to acetyl-CoA by the activity of the PDHC (red cross). Abbreviations: acetohydroxy acid isomeroreductase (AHAIR), acetohydroxy acid synthase (AHAS), acetate kinase (AK), alanine aminotransferase (AlaT), alanine aminotransferase (AvtA), dihydroxy acid dehydratase (DHAD), pyruvate decarboxylase (PCx), pyruvate dehydrogenase complex (PDHC), pyruvate kinase (PK), phosphoenolpyruvate (PEP), PEP carboxykinase (PEPCk), PEP carboxylase (PEPCx), pyruvate:quinone oxidoreductase (PQO), phosphotransacetylase (PTA), transaminase B (TA).

containing 222 mM glucose and 254 mM acetate, and incubated at 30 °C and 120 rpm. Unless otherwise specified, CGXII minimal medium was prepared without the addition of urea, which is part of the original CGXII recipe (Keilhauer et al., 1993). In this study, acetate was added to the medium as potassium acetate salt. Biomass formation was monitored by measuring OD_{600}, while the cell dry weight (CDW, g L^{-1}) was calculated by following equation: CDW = $OD_{600} \times 0.3$ g L^{-1} (Buchholz et al., 2013). *Escherichia coli* DH5α cells were incubated in lysogeny broth (LB) medium by agitation at 120 rpm in shake flasks or grown on LB agar plates at 37 °C (Sambrook et al., 2001). If appropriate, kanamycin was added to the media in a final concentration of 25 μg/ml for *C. glutamicum* and 50 μg/ml for *E. coli*.

2.2. Procedure of the biosensor-driven evolution experiment

For the evolution experiment, *C. glutamicum* Δ*aceE* containing the plasmid-encoded Lrp-biosensor was picked from an agar plate and cultivated overnight in 4 ml BHI medium with 85 mM acetate and 25 μg/ml kanamycin. The following day, 2 ml of the preculture was used to inoculate a 200 ml shake flask with 50 ml CGXII minimal medium, 222 mM glucose, 254 mM acetate and 25 μg/ml kanamycin. As the strain *C. glutamicum* Δ*aceE* displays a growth-decoupled production phenotype (Blombach et al., 2007), cells were analyzed and sorted by FACS after 28 h of cultivation. At this time, the cells typically entered the stationary phase and had initiated L-valine production some hours previously. One million cells showing the top 10% sensor output were sorted on MultiScreen HTS filter plates (Millipore, Billerica, USA) to separate cells from the FACSFlow™ buffer (Becton Dickinson, San Jose, USA). We isolated 10^6 cells with the top 10% sensor output to ensure a high genomic variability in the propagated culture. Furthermore, we found that inoculating the culture with a lower number of cells resulted in unstable growth. In parallel, the supernatant of the

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