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Comparison of the metabolic response to over-production of *p*-coumaric acid in two yeast strains

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ABSTRACT

The development of robust and efficient cell factories requires understanding of the metabolic changes triggered by the production of the targeted compound. Here we aimed to study how production of *p*-coumaric acid, a precursor of multiple secondary aromatic metabolites, influences the cellular metabolism of *Saccharomyces cerevisiae*. We evaluated the growth and *p*-coumaric acid production in batch and chemostat cultivations and analyzed the transcriptome and intracellular metabolome during steady state in low- and high-producers of *p*-coumaric acid in two strain backgrounds, S288c or CEN.PK.

We found that the same genetic modifications resulted in higher production of *p*-coumaric acid in the CEN.PK background than in the S288c background. Moreover, the CEN.PK strain was less affected by the genetic engineering as was evident from fewer changes in the transcription profile and intracellular metabolites concentrations. Surprisingly, for both strains we found the largest transcriptional changes in genes involved in transport of amino acids and sugars, which were downregulated. Additionally, in S288c amino acid and protein biosynthesis processes were also affected.

We systematically overexpressed or deleted genes with significant transcriptional changes in CEN.PK low and high-producing strains. The knockout of some of the downregulated transporters triggered a 20–50% improvement in the synthesis of *p*-CA in the CEN.PK high-producing strain. This study demonstrates the importance of transporters in the engineering of cell factories for production of small molecules.

1. Introduction

Plants produce a wide range of secondary metabolites as a protective mechanism to stresses caused by bacterial or viral infections, ultraviolet radiation, wounds, and other biotic and abiotic factors. Nearly 15% of these metabolites are phenolic compounds derived from the aromatic amino acids L-tyrosine, L-phenylalanine or L-tryptophan (Wink, 2010). Numerous aromatic secondary metabolites are available on the market as therapeutic agents, dyes, fragrances, and flavors. The majority of these compounds are currently synthesized chemically or isolated from plants (Bourgaud et al., 2001), however recently there have been significant advances in engineering industrial microbes, e.g., *Escherichia coli* and *S. cerevisiae*, for production of aromatic secondary metabolites by fermentation. A few biotech-derived aromatics are already on the market, such as phenylalanine, resveratrol, vanillin, steviol glucoside, and others. Additionally, many aromatic metabolites have been produced in microbial cell factories at proof-of-concept levels, but the strains, fermentation and downstream processes need further development before the production becomes economically feasible. These compounds include naringenin, genistein, kaempferol, fisetin, melatonin, and many others (Koopman et al., 2012; Trantas et al., 2009; Santos et al., 2011; Leonard et al., 2009; Stahlhut et al., 2015; Krivoruchko and Nielsen, 2015; Li et al., 2015; Germann et al., 2016).

An important step towards improved microbial cell factories is a better understanding of how the engineered cells respond to production of target compounds (Nielsen and Keasling, 2016). For this purpose, omic-level characterization of the strains is useful since the organism can be studied at different levels and the information can be assessed in

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the context of cellular metabolism (Kim et al., 2012; Curran and Alper, 2012). There are a few successful examples of applying systems biology for guiding metabolic engineering strategies. Otero et al. (2013) obtained a 30-fold improvement in succinic acid production in *S. cerevisiae* based on the integrative analysis of physiology and transcriptome data. Park et al. (2007) engineered an efficient L-valine-producing *E. coli* by using transcriptomic analysis together with *in silico* models. A multi-omic analysis of two different *E. coli* strains allowed Yoon et al. (2012) to identify an optimal strain for production of recombinant proteins. This study is one of the few that considered the differences between strains of the same species, when selecting the suitable host organism. In *S. cerevisiae*, a considerable number of differences have been found in the genomes of two widely used strains, CEN.PK and S288c. These differences are mainly related to the presence of 13,787 single nucleotide polymorphisms, 939 of them related to 158 genes involved in metabolic functions with enrichment in the galactose uptake and ergosterol biosynthetic pathways. Moreover, 83 genes, mainly located in sub-telomeric regions of S288c, are absent in the CEN.PK strain (Otero et al., 2010; Nijkamp et al., 2012).

The strain CEN.PK is widely used for industrial biotechnology research and applications, whereas the strain S288c is widely used in genetic studies. Recently the strain S288c has also been used for the production of some metabolites, such as vanillin-β-glucoside, 2-phenylethanol and methionol (Strucko et al., 2015; Yin et al., 2015a, 2015b). In the particular case of vanillin-β-glucoside, the engineered S288c strain produced 10-fold more product than the CEN.PK strain engineered in the same way and this effect was associated with several single nucleotide polymorphisms in the shikimate pathway genes.

p-Coumaric acid (*p*-CA) is a precursor for biosynthesis of a number of secondary metabolites, such as polyphenols, flavonoids, and some polyketides. We recently reported the engineering of *S. cerevisiae* as a cell factory for high-level production of *p*-CA with a titer of ~ 2 g/L (Rodriguez et al., 2015). In this study, we aimed to investigate firstly how the production of *p*-CA influences the host and secondly whether these effects depend on the strain background. To answer these two questions, we performed transcriptome and intracellular metabolome analysis on S288c and CEN.PK strains, which either only expressed an enzyme for making the product (low-producers) or were additionally optimized towards production of aromatic products (high-producers).

2. Materials and methods

2.1. Plasmids and strains

E. coli DH5*α* was used for cloning procedures. The fragments used for overexpression of genes were amplified by PCR using primers and templates as described in the Tables S1 and S2. The fragments were amplified from the genomic DNA of *S. cerevisiae* CEN.PK102-5B *(*MATa *ura*3-52 *his*3Δ1 *leu*2-3/112 MAL2-8c SUC2*)* and *E. coli* NST 74. The gene encoding tyrosine ammonia-lyase from *Flavobacterium johnsoniae* (FjTAL) was as described in (Jendresen et al., 2015). The amplified gene-encoding fragments were cloned together with strong constitutive promoters into EasyClone integrative plasmids by USER cloning (Jensen et al., 2014; Jessop-Fabre et al., 2016). The clones were tested for correct insertion of gene/promoter fragments by colony PCR using the primers summarized in the Table S1 and the resulting plasmids were verified by sequencing. The list of the constructed vectors can be found in Table 1.

S. cerevisiae CEN.PK113-7D was obtained from Peter Kötter (Johann Wolfgang Goethe-University Frankfurt, Germany). The strain BY4741, a derivative of strain S288c, was obtained from EUROSCARF. Transformation of yeast cells was performed using the lithium acetate method (Gietz et al., 2002). The strains were selected on synthetic drop-out medium (Sigma-Aldrich) and the genetic modifications were confirmed by colony PCR. The yeast strains used in this study are listed in Table 2 and Table S3.

2.2. Media and cultivations.

We prepared a mineral medium for the batch fermentation according to Verduyn et al. (1992). Glucose concentration in batch medium was 40 g l^{-1}. The feed medium for chemostats was prepared in the same way, but the amount of glucose was reduced to 10 g l^{-1} and the medium was supplemented with 0.2 ml L^{-1} of 2 M KOH and one drop of antifoam 204 (Sigma A-8311) per 20 L of medium. The pre-culture was done by inoculating a yeast colony into 50 ml of mineral medium in a 250-ml baffled shake flask and incubating the culture with shaking at 200 rpm at 30 °C for around 12 h. When the pre-culture reached OD_{600} of ca. 2, it was used to inoculate a bioreactor to a starting optical density of 0.05.

The fermentations were performed in DasGip 1-L stirrer-pro vessels (Eppendorf, Jülich, Germany), using the working volume of 500 ml. The temperature was 30 °C, agitation was at 600 rpm and aeration at 1 vvm. pH was monitored with a pH sensor (Mettler Toledo, Switzerland) and pH was maintained at 5.0 ± 0.05 by automatic addition of 2 M KOH. Dissolved oxygen was above 30% throughout the fermentation as measured by the polarographic oxygen sensor (Mettler Toledo, Greifensee, Switzerland). The completion of the batch phase was determined by monitoring CO_2 in the exhaust gas, when the second CO_2 peak, corresponding to ethanol consumption phase, declined. We then initiated constant feed to obtain glucose-limited steady-state with dilution rate of 0.100 ± 0.005 h^{-1}. The volume was kept constant using an overflow pump. The samples for transcriptome and metabolome analysis were taken after 4 residence times of steady-state growth. Four technical replicates were taken from each reactor for transcriptome and metabolome analyses. Each strain was fermented twice to obtain 2 biological replicates.

2.3. Analytical methods

For analysis of extracellular metabolites and the biomass, we withdrew ca. 3-ml samples from the reactor. 1 ml of the sample was centrifuged at 11,000*g* for 5 min and stored at −20 °C until HPLC analysis for glucose and organic acids. For *p*-CA analysis in the optimized strains (ST4288 and ST4353) we mixed 1 vol of sample with 9 volumes of 50% ethanol, whereas for the non-optimized strains (ST4408 and ST4397) we mixed 1 vol of sample with 1 vol of 50% ethanol. This was done to dissolve the *p*-CA that may have precipitated from the broth due to poor solubility in water. These samples were also centrifuged at 11,000 × *g* for 5 min and stored at −20 °C until further analysis.

The analysis of glucose, glycerol, ethanol, and organic acids was performed on Dionex Ultimate 3000 high-performance liquid chromatography (HPLC) system (Dionex Softron GmbH, Germany), with an Aminex HPX-87H column (Bio-Rad) at 65 °C, using 5 mM H_2SO_4 as the mobile phase with a flow rate of 0.6 ml/min.

Quantification of *p*-CA was performed as described in Rodriguez et al. (2015) using a HPLC (Thermo Fisher Scientific), with a Discovery HS F5 150 mm × 2.1 mm column (particle size 3 μm). The samples were analyzed using a gradient method with two solvents: (A) 10 mM ammonium formate pH 3.0 and (B) acetonitrile at 1.5 ml min^1. The *p*-CA was detected by absorbance at 277 nm and the retention time was 4.7 min. The area under the curve was integrated with Chromeleon software 7. The quantification of *p*-CA was performed based on 5 points calibration curve in the range of 0.1–1 mM. For the dry cell weight measurement 5 ml of culture broth was filtered through a 0.45 μm filter membrane, after that the membrane was dried at 95 °C for 24 h and cooled down in a desiccator. The dry cell weight was calculated by measuring the weight increment of the dried filter.

2.4. Transcriptome analysis

Samples for RNA extraction were taken after four retention times of