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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY - SHORT COMMUNICATION

Engineering *Escherichia coli* to increase triacetic acid lactone (TAL) production using an optimized TAL sensor-reporter system

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Abstract

Triacetic acid lactone (TAL) (4-hydroxy-6-methyl-2-pyrone) can be upgraded into a variety of higher-value products, and has potential to be developed into a renewable platform chemical through metabolic engineering. We previously developed an endogenous TAL sensor based on the regulatory protein AraC, and applied it to screen 2-pyrone synthase (2-PS) variant libraries in *E. coli*, resulting in the identification of variants conferring up to 20-fold improved TAL production in liquid culture. In this study, the sensor-reporter system was further optimized and used to further improve TAL production from recombinant *E. coli*, this time by screening a genomic overexpression library. We identified new and unpredictable gene targets (*betT*, *ompN*, and *pykA*), whose plasmid-based expression improved TAL yield (mg/L/OD_{595}) up to 49% over the control strain. This work further demonstrates the utility of customized transcription factors as molecular reporters in high-throughput engineering of biocatalytic strains.

Keywords Triacetic acid lactone (TAL) · Biosensor · High-throughput screen · Gene library · Combinatorial metabolic engineering

Introduction

Metabolic engineering strategies can be broadly categorized as "rational" or "combinatorial" [26], though nowadays in practice experimental approaches are often a combination of these. One form of combinatorial metabolic engineering involves the search for and identification of novel gene targets whose altered expression confers enhanced production of a desired metabolite. A common bottleneck to the identification of rare mutants showing enhanced biochemical production, particularly when the biosynthesis of interest is not coupled to cell growth, is the availability of a suitable, high-throughput screen or selection system [5]. Customized, endogenous molecular reporting schemes in the form of transcription factors and other gene regulation systems have thus become the focus of much research [11, 19, 25].

TAL is a natural product and a potential biorenewable platform chemical, capable of being converted to a variety of commercially valuable intermediates and end products, including phloroglucinol (an important intermediate) [9], acetylacetone (a fuel additive), and sorbic acid (a food preservative) [3]. TAL can be chemically synthesized from dehydroacetic acid [22] or ethyl acetoacetate [8]. Sustainable microbial biosynthesis of TAL has promise to replace chemical synthesis, and has been pursued by several groups [2, 10, 14, 21, 23, 27, 29]. These efforts have involved the use of *Escherichia coli*, *Saccharomyces cerevisiae* or *Yarrowia lipolytica* as host, and expression of either 2-pyrone synthase (2-PS) or 6-methylsalicylic acid synthase (6-MSAS) for TAL production and accumulation in the culture medium.

Our interests in improving TAL production by recombinant *E. coli* expressing 2-PS led to the design of a TAL sensor-reporter system based on the *E. coli* AraC regulatory protein. AraC variant "AraC-TAL" contains five amino acid substitutions and activates reporter gene expression at promoter P_{BAD}, in response to low-millimolar concentrations of TAL [23]. We used this reporter in a high-throughput screen to identify variants of 2-PS that enhance TAL production in

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E. coli. Recently, this same AraC-TAL reporter was used to screen a library of transposons randomly inserted throughout the *E. coli* genome, to identify knockouts conferring enhanced TAL specific titer [10]. As an alternate strategy to identify non-obvious gene targets, here we employ the AraC-TAL reporter system to screen an *E. coli* genomic overexpression library for enhanced TAL yield. Through this strategy we found that plasmid-based expression of *betT* (encoding choline: H^+ symporter), *ompN* (encoding outer membrane porin N), and *pykA* (encoding pyruvate kinase II) result in up to 49% increased TAL yield, relative to the control strains. These results add to a growing list of genes whose controlled expression has potential to improve biosynthesis of polyketide natural products.

Results and discussion

Acetyl-CoA and malonyl-CoA are precursors in TAL biosynthesis, and many studies have described "rational" *E. coli* metabolic engineering efforts to enhance flux towards and/or availability of these metabolites to improve production of malonyl-CoA-dependent compounds [4, 28, 30]. We tested the effects of several such genetic modifications on TAL yield by *E. coli* expressing our previously reported 2-PS "S1" variant. Briefly, up-regulation of acetyl-CoA carboxylase or acetyl-CoA synthetase, and down-regulation of genes involved in fatty acids biosynthesis, were all met with little success. Details are provided in the supplementary material.

Before using our AraC-TAL sensor to identify new gene targets for increasing TAL yield, we first sought to improve the ON/OFF ("contrast") ratio of our sensor-reporter system. Briefly, *lacZ* under control of the P_{BAD} promoter is our reporter gene, with blue colony formation owing to β-galactosidase activity on substrate X-Gal indicating effector-induced reporter gene expression. "Leaky" expression of *lacZ* increases background color formation and reduces the contrast ratio, while weak expression under inducing conditions similarly reduces contrast. To enhance the contrast ratio, we compared the use of plasmid-based vs. chromosomal reporter gene expression, and tested different ribosome binding site (RBS) sequences, corresponding to a variety of predicted translation initial rates (designed using the RBS calculator [6, 20]), placed upstream of *lacZ.* This led to our use of new strain SQ8 containing a single, chromosomal copy of the P_{BAD}-lacZ reporter, with the weakest RBS tested. Figure 1 summarizes the results from this optimization, using WT-AraC and its effector L-arabinose. Details of reporter strain construction, validation, and calibration by expression of different 2-PS variants (with AraC-TAL), are provided in the supplementary material.

To identify genes whose up-regulation improves TAL yield (defined as extracellular TAL concentration in liquid culture, per cell density), we constructed and screened three *E. coli* libraries (named GL-1, GL-2 and GL-3 kb), which differ by average size of overexpressed genome fragments. Details of library construction and characterization are given in supplementary material. The library sizes are roughly 1.5×10^5 (GL-1 kb), 3.2×10^5 (GL-2 kb) and 3.2×10^5 (GL-3 kb) (this estimate considers the use of two four-base cutters for partial genome digestion, and the ranges of DNA fragments recovered). The libraries were constructed such that each gene fragment was placed on a plasmid also expressing 2-PS variant S1, with no external promoter to control expression of cloned genes. For each library, sequencing eight naïve clones confirmed the randomness of gene fragments. Clones were spread onto LB-agar plates containing reagents for TAL production and screening (refer to supplementary material), and ~47,000 colonies were screened (by eye) for blue color development. After about 16 h of growth, the 14 darkest blue colonies from each library were isolated for re-screening in liquid culture and HPLC analysis. These 14 re-screened clones from GL-1 kb, GL-2 kb and G-3 kb had average TAL yields of 101 ± 24, 91 ± 28 and 86 ± 34 mg/L/OD_{595}, respectively. This trend of decreasing average yield may be attributed to the impact of increased plasmid size, which can reduce 2-PS expression levels and generally enhance the burden of plasmid maintenance. However, the 14 clones from GL-3 kb

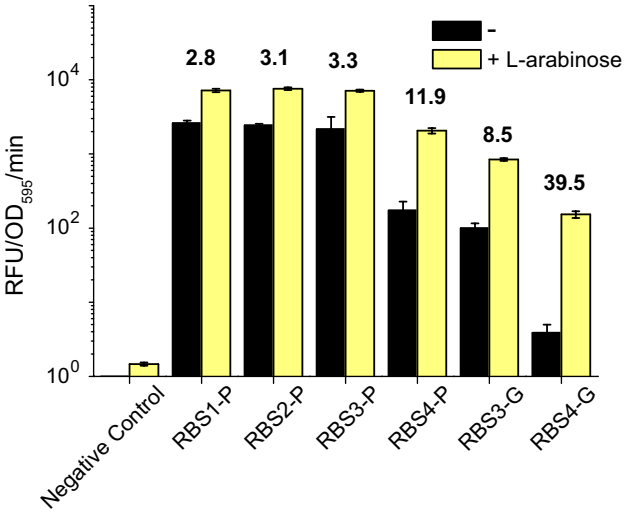

Fig. 1 Selection of an optimal P_{BAD}_*lacZ* reporter construct. Four RBS sequences representing relatively high (RBS1) to low (RBS4) translation initiation rates were placed upstream of *lacZ*. Each reporter construct was tested as multi-copy (on a plasmid, "P") or as a single copy (integrated into the genome, "G"). LacZ expression in the presence versus absence of L-arabinose was measured as the rate of fluorescence increase using β-D-galactopyranoside as substrate. Negative control is strain HF19 harboring pPCC423 (*lacZ* is not present in this strain). The ON/OFF ratio (values given above each data set) is highest for a single copy of *lacZ* with RBS4 (RBS4-G)

showed the highest variation and also included the top three TAL-producing mutants (TAL yields of 125, 130 and 146 mg/L/OD_{595}). Another 20 clones were therefore selected from library GL-3 kb for re-screening. The larger fragment library offers a higher probability that individual fragments are able to fully encode single genes and their promoters, which may explain the apparent advantage of this library, in spite of the larger plasmid size.

Among the 34 clones from GL-3 kb rescreened by liquid culture and HPLC analysis, the three most productive (TAL yield increased by 44.1, 20.5, and 49.2% over the strain carrying a control plasmid) were selected for further analysis. Results are summarized in Table 1. For all three clones, the corresponding gene fragment contained in the expression vector consisted of a single, full-length gene, including its upstream promoter. These genes are *betT* encoding choline: H^+ symporter, *ompN* encoding outer membrane porin N, and *pykA* encoding pyruvate kinase II (Table 1). To confirm that each gene was responsible for the observed increased in TAL yield, each gene was then cloned under the control of promoter P_{ltetO1}, inducible by addition of anhydrotetracycline (aTc). We additionally cloned *pykF* encoding pyruvate kinase I, since this enzyme catalyzes the same reaction as pyruvate kinase II (ADP + phosphoenolpyruvate + $H^+ \rightarrow$ pyruvate + ATP) [7, 17]. TAL production resulting from induced expression of these genes is shown in Fig. 2. Whereas induced expression of *ompN* and *pykA* increased TAL yield compared to the uninduced cultures, expression of *betT* or *pykF* did not improve TAL yield.

Pyruvate kinase I encoded by *pykF* and pyruvate kinase II encoded by *pykA* catalyze the same glycolytic reaction, but the enzymes have distinct physical, chemical [24] and kinetic properties [13]. The enzyme pyruvate kinase I is activated by fructose 1,6-bisphosphate, while pyruvate

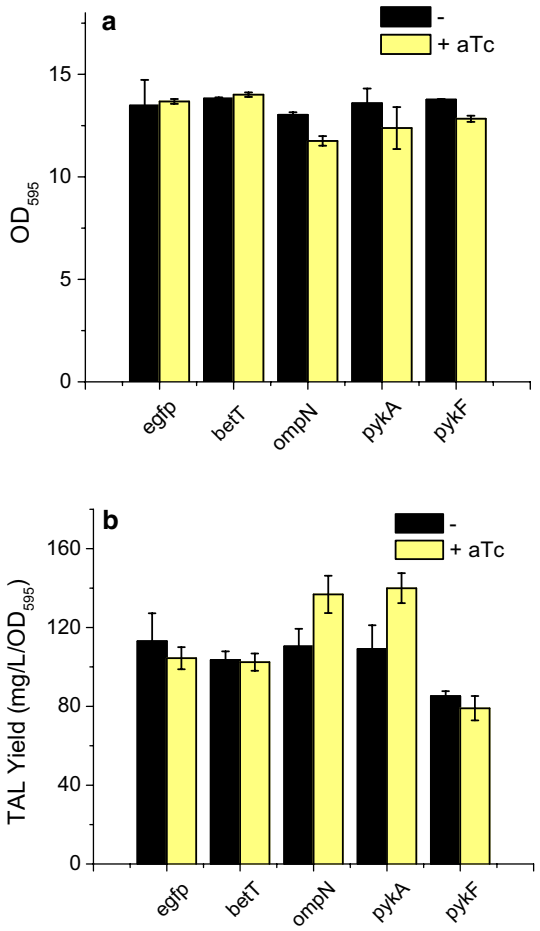

Fig. 2 Effects of induced gene overexpression (by aTc addition) on TAL production by *E. coli* strain SQ8 expressing 2-PS variant S1 at 16 h: **a** culture optical density (OD_{595}); **b** yield

Table 1 Plasmid-cloned genomic fragments that lead to improved TAL yield

Clone	Improvement	Chromosomal fragment (size, genes included, and chromosome location)	Notes
GL01	44.1 ± 5.2%	3.3 kb 328,358 … 331,696 *betT*	*betT*: encodes choline: H^+ symporter
GL02	20.5 ± 2.6%	2.4 kb 1,433,253 … 1,435,673 *ompN*	*ompN*: encodes outer membrane pore protein N
GL03	49.2 ± 6.0%	3.1 kb 1,935,255 … 1,938,351 *pykA* *lpxM*	*pykA*: encodes pyruvate kinase II *lpxM*: encodes myristoyl-ACP transferase (no promoter was present)

kinase II is activated by AMP [13]. Under conditions of relatively low fructose 1,6-bisphosphate levels owing to growth on glycerol [12] (as in this study), pyruvate kinase II is up-regulated and the dominant form to divert carbon flux from PEP to pyruvate [16]. Since pyruvate is a precursor to acetyl-CoA and malonyl-CoA (required for TAL biosynthesis), this may explain the observation that overexpression of *pykA*, but not *pykF*, leads to improved TAL yield. Note that TAL production by strain SQ8 grown on LB medium supplemented with glycerol is more than tenfold greater than when instead supplemented with glucose (refer to Table S4). To the best of our knowledge, up-regulation of *pykA* as a metabolic engineering strategy to increase malonyl-CoA-dependent flux when using glycerol has not been previously reported.

betT encodes inner membrane protein choline: H^+ symporter, which is responsible for choline uptake by *E. coli* to synthesize glycine betaine [1]. *ompN* encodes outer membrane porin N, one of several porins responsible for transport of small polar molecules (< 600 Da) [18]. While induced expression of *betT* did not improve TAL yield, increasing the copy of this gene with its native promoter (carried on a plasmid) was beneficial (Table 1). Since both *betT* and *ompN* encode transport-related membrane proteins, it is likely their altered expression influences either TAL transport across the cell membrane, or perhaps TAL-related toxicity. We therefore tested whether expressing these proteins reduced TAL toxicity. Briefly, growth rate and cell density were monitored in the presence of various concentrations of TAL which impair growth of *E. coli*, with and without induced expression of *betT* or *ompN*. No significant effect of induced expression on growth was observed.

In a recent study by Li et al., the same AraC-TAL sensor was used to screen an *E. coli* transposon insertion library, resulting in the identification of several gene deletions (*ΔrcsA*, *ΔfhuA*, *ΔcsgA* and *ΔtonB*) that led to improved intracellular malonyl-CoA accumulation and hence TAL yield [10]. We similarly constructed, verified, and screened libraries of colonies with randomly inserted chromosomal transposons (methods are provided in supplementary material). However, upon re-screening using the same culturing methods as for the genome fragment libraries, we only identified one gene deletion, *ΔzapE*, that led to improved TAL yield by 35.8%. This discrepancy in results may be related to the significantly higher cell densities (> 10-fold) and TAL yields (> 4-fold) that we observed, as compared to this previous study. When *ompN* and/or *pykA* were overexpressed in the *ΔzapE* strain, no further TAL yield improvement was obtained. *zapE* encodes an ATPase involved in cell division [15], and the mechanism by which its deletion affects TAL yield is not clear.

Conclusion

In this study, the contrast ratio of our previously developed AraC-TAL sensor-reporter system was enhanced, and the improved reporter was applied in high-throughput screening to identify new and unpredictable gene targets (*betT*, *ompN*, and *pykA*), whose plasmid-based expression improved TAL yield. Screening random transposon insertions similarly identified a target for gene deletion (*zapE*). This work further demonstrates the utility of customized transcription factors as molecular reporters for library screening and engineering of biocatalytic strains.

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