Protein Engineering

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Design and Application of a Mevalonate-Responsive Regulatory Protein**

*Shuang-Yan Tang and Patrick C. Cirino**

The discovery and engineering of genetic components involved in the synthesis of a desired metabolite are often limited by a lack of sufficiently sensitive and/or rapid screening methods (or genetic selections) for the identification of gene candidates from large natural or synthetic gene libraries.[1] Many bacterial regulatory proteins mediate direct coupling between the specific recognition of small molecules ("effectors") and changes in gene transcription at targeted promoters.[2] However the lack of available regulatory proteins that respond to effector molecules of interest has hindered their use in the endogenous reporting of effector synthesis through reporter-gene expression. Herein we demonstrate a new approach to metabolic-pathway engineering in which a regulatory protein is engineered to respond to an effector compound of interest and then used as a reporter in library screening for improved effector production (Figure 1).

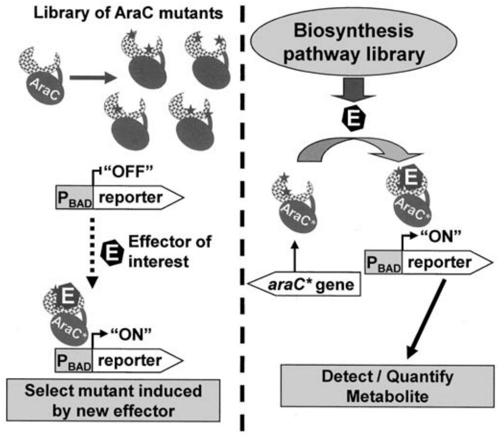

Figure 1. In this metabolic-engineering strategy, the *E. coli* regulatory protein AraC is first engineered to be induced by the product of interest (effector). The customized regulator (AraC*) is then used in high-throughput screening to report effector synthesis through reporter-gene expression. In the current study, the host organism is *E. coli*, the effector is mevalonate, and the gene library consists of randomized bases in a mevalonate-synthesis-pathway operon.

A variety of bacterial regulatory proteins that respond to small molecules have been characterized, and many have been engineered to show altered or relaxed effector specificity.[2,3] The *Escherichia coli* homodimeric AraC protein acts as a repressor of transcription at the promoter P_{BAD} in the absence of its effector molecule L-arabinose. Upon binding L-arabinose, the AraC dimer switches conformation and contacts alternative operator half-sites, which leads to the activation of transcription at P_{BAD}.[4] We recently described the use of multiple-site saturation mutagenesis and fluorescence-activated cell sorting (FACS) to isolate AraC variants with effector specificity switched from L-arabinose to D-arabinose.[3a] We next sought an AraC variant that could be used as a reporter for the screening of improved production of a desired metabolite.

Isoprenoids constitute a large class of industrially valuable secondary metabolites.[5] The mevalonate-dependent (MEV) isoprenoid pathway converts acetyl coenzyme A (acetyl-CoA) into the five-carbon-atom isoprenoid building block isopentenyl pyrophosphate (IPP). The reduction of hydroxymethylglutaryl-CoA (HMG-CoA) to mevalonate by HMG-CoA reductase is a committed step in this pathway (see Figure S1 in the Supporting Information).[6] The MEV pathway is found in eukaryotes and prokaryotes, but is not native to *E. coli*. The functional heterologous expression of an engineered MEV pathway in *E. coli* has been reported.[6,7] The "MevT" operon (contained in plasmid pMevT) is composed of *atoB* encoding *E. coli* acetoacetyl-CoA thiolase, *ERG13* encoding *Saccharomyces cerevisiae* 3-hydroxy-3-methylglutaryl-CoA synthase, and a truncated *HMG1* gene from *S. cerevisiae* (named herein *tHMGR*) encoding a soluble version of HMG-CoA reductase.[8]

The production of isoprenoids in *E. coli* through the heterologous MEV pathway is limited by mevalonate supply;[7] the improvement of mevalonate production is therefore an important step in the metabolic engineering of microbial isoprenoid biosynthesis.[9] Keasling and co-workers have described several approaches to the enhancement of mevalonate production in engineered *E. coli*.[7,9] When the expression of the MevT operon was simply increased, growth and productivity decreased as a result of a variety of potentially unfavorable factors, including decreased intracellular acetyl-CoA levels, inhibitory HMG-CoA levels,[9] the biosynthetic burden, and enzyme toxicity.[10] Therefore, for mevalonate production to be increased, fine-tuning of individual MevT gene-expression levels is required to balance intermediate metabolite levels and prevent unfavorable growth conditions. High-throughput screening is ideal for

[*] Dr. S.-Y. Tang, Prof. Dr. P. C. Cirino
Department of Chemical Engineering, Pennsylvania State University
226 Fenske Laboratory, University Park, PA16802 (USA)
Fax: (+1) 814-865-7846
E-mail: cirino@engr.psu.edu
Homepage: http://fenske.che.psu.edu/Faculty/Cirino/

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such nonintuitive metabolic optimization. Pfleger et al. developed an *E. coli* mevalonate auxotroph,[11] which was used as a reporter of mevalonate production in the screening of combinatorial libraries of tunable intergenic MevT regions containing genetic control elements that included mRNA secondary structures, RNase cleavage sites, and ribosome-binding-site-sequestering sequences.[7] This approach resulted in enhanced mevalonate production and a final titer of approximately 6 mM mevalonate in batch cultures (ca. 0.6 mM/cell OD_{600}) after 24 h. Reduced levels of *ERG13* and *tHMGR* mRNA generally correlated with increased mevalonate production.

Often there is no clear strategy for the construction of a microbial strain that synthesizes and is auxotrophic for a compound of interest (particularly for nonnative compounds and secondary metabolites).[1] The creation of an endogenous reporter of product formation through engineering of the effector specificity of a regulatory protein is potentially a more general approach to the development of high-throughput screening or selection systems for combinatorial metabolic engineering in microorganisms. As a first demonstration of this approach, a mevalonate-responsive AraC variant was isolated and then used to screen for improved mevalonate production as a result of MevT-operon mutations.

Mevalonate does not act as an inducer for wild-type AraC (Figure 2a). On the basis of our previous studies,[12] five codons encoding amino acids in the AraC effector-binding pocket (P8, T24, H80, Y82, and H93) were selected for simultaneous saturation mutagenesis (corresponding to 3.2×10^6 AraC variants). Placement of the *gfpuv* gene encoding green fluorescent protein (GFPuv) downstream of promoter P_{BAD} (plasmid pPCC442) in *E. coli* clones expressing the AraC variants (from plasmid pPCC423) enabled the use of FACS to isolate clones in which GFP expression is significantly higher in the presence of 30 mM mevalonate than in its absence (all strains and plasmids are described in Table S1 of the Supporting Information, and plasmid maps are depicted in Figure S2). After several rounds of dual screening, the *araC* mutant "*araC-mev*" encoding variant AraC-mev (P8P, T24L, H80L, Y82L, H93R) was isolated. The exogenous-mevalonate-dose response of GFP expression under the control of AraC-mev at promoter P_{BAD} is shown in Figure 2a. Although the half-maximal response to exogenous-mevalonate concentrations is high (ca. 150 mM), Figure 2b shows that the GFP-expression response to mevalonate is almost linear in the range of 10–100 mM. GFP expression controlled by AraC-mev is also specific to mevalonate; this effect was not observed with the chemically similar compounds L-arabinose, triacetic acid lactone, and succinate (see Table S2 in the Supporting Information). Likewise, the expression of AraC-mev in strain HF22, which contains a chromosomal copy of the *lacZ* gene downstream of promoter P_{BAD}, resulted in a LacZ-expression (β-galactosidase activity) response to exogenous mevalonate similar to that for GFP (Figure 2). Finally, coexpression of the MevT operon (plasmid pMevT-PBR) and AraC-mev in strain HF22 showed that LacZ activity increases with endogenous mevalonate production beyond that observed for the control strain, which does not produce mevalonate (see Figure S3 in the Supporting Information).

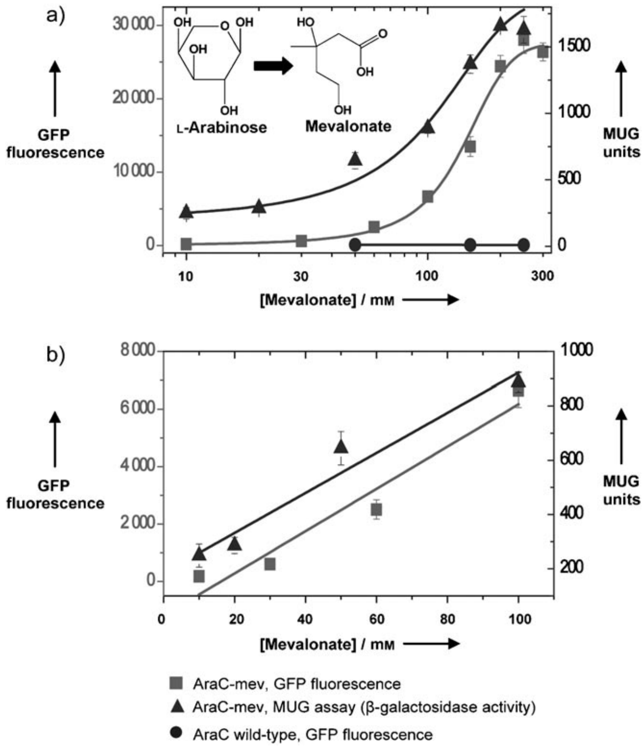

Figure 2. AraC-mev mevalonate-dose response. a) GFP expression from strain HF19 harboring the P_{BAD}–GFP reporter plasmid and expressing either AraC-mev (squares) or wild-type AraC (circles), and β-galactosidase activity (measured in MUG units[13]; MUG = 4-methylumbelliferyl β-D-galactoside) from strain HF22 expressing AraC-mev (triangles), reported as a function of mevalonate concentration (concentration of melavonate added to the culture). b) Response of GFP expression (squares) and β-galactosidase activity (triangles) in the range of 10–100 mM mevalonate. Data have been normalized to cell density and corrected by subtracting background values measured in the absence of an inducer.

The MevT-pathway-engineering studies described above demonstrate that cell growth and mevalonate production are highly sensitive to the *tHMGR* expression level (overexpression is not necessarily beneficial), and that different genetic backgrounds or growth conditions alter the range of optimal *tHMGR* expression. Next, to screen for improved mevalonate synthesis by mutants with more optimal *tHMGR* expression, we randomized nucleotides in the ribosome-binding-site (RBS) region and the interval sequence between the RBS and the start codon of *tHMGR* in the MevT-operon plasmid pMevT-PBR. The library of 1.05×10^6 *tHMGR* RBS mutants was transformed into strain HF22. Solid-phase visual screening of individual clones (colonies) that expressed AraC-mev and grew in the presence of the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) enabled identification of the darkest-blue colonies corresponding to the highest levels of β-galactosidase activity due to mevalonate-induced *lacZ* expression.

Six dark-blue colonies (identified by the eye) were selected (from a total of approximately 1×10^5 colonies screened) and rescreened by quantifying mevalonate production from liquid cultures of these clones. Three plasmids (named "RBS2", "RBS4", and "RBS5") from three of the top-producing clones were purified, sequenced, and retransformed into strain HF22 for more-detailed analysis. The time courses of mevalonate production and cell growth (OD_{600}) are presented in Figure 3. All three mutants produced significantly more mevalonate (up to 17 mM) than the strain harboring pMevT-PBR (which produced 4.5 mM mevalonate), and two of the three mutants showed higher final cell densities. HMG-CoA reductase activity levels measured from lysates of the mutant strains (see Table S4 in the Supporting Information) show that significantly lower levels of active HMG-CoA reductase were expressed by two mutants (RBS2 and RBS5; see Table S3 for the sequences of the *tHMGR* RBS regions) than by the culture with the original pMevT-PBR plasmid (a decrease of 55 and 64%, respectively). However, the activity observed for the third mutant (RBS4) is essentially the same as that of pMevT-PBR. Although it is difficult to interpret how the different activities result in similar improvements in mevalonate production, it is possible that the RBS mutations also influence the relative expression levels of *atoB* and *ERG13* in the different constructs. To verify that this screening approach effectively identified the improved mutants, we also randomly selected and rescreened ten colonies from the RBS library. Nine mutants produced mevalonate at a concentration less than 1 mM in liquid culture and expressed less than 7% of the HMG-CoA reductase activity observed for the culture with pMevT-PBR, whereas the remaining mutant behaved similarly to the pMevT-PBR clone (see Tables S4, S5, and S6 in the Supporting Information).

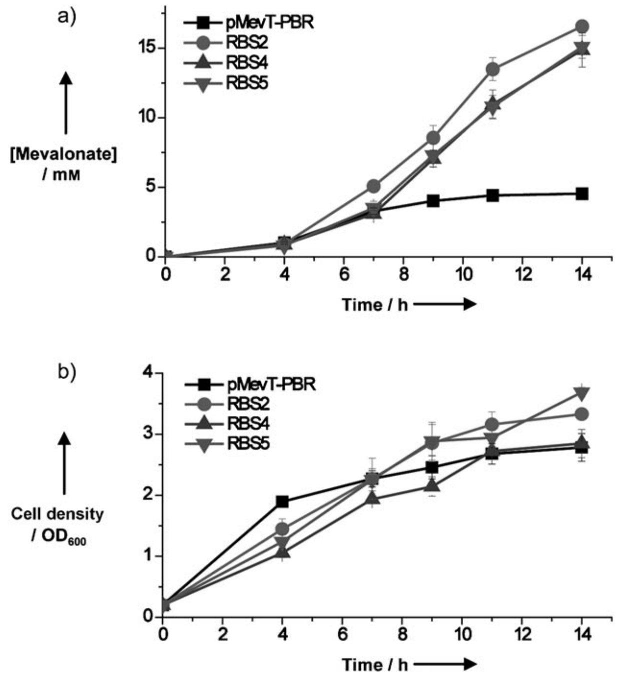

Figure 3. Time profiles of a) mevalonate concentration and b) cell density measured for cultures of strain HF22 harboring pMevT-PBR or the indicated *tHMGR* RBS mutant plasmid.

These results clearly demonstrate the successful application of a novel mevalonate reporter based on the AraC–P_{BAD} regulatory system as a screening tool for the improvement of mevalonate production by engineered *E. coli*. The mevalonate reporter system should also be useful in other applications related to the regulation, screening, or alteration of the activity of MEV-pathway enzymes. Examples include the application of combinatorial methods to the engineering of the microbial synthesis of HMG-CoA reductase inhibitors (cholesterol-lowering compounds) and the screening of pathway inhibitors as potential antimicrobial agents.[14] Finally, the general approach outlined herein (the use of AraC–P_{BAD} or other regulator–promoter pairs) should prove useful for the design of molecular reporters and high-throughput screening systems specific to a large variety of target compounds.

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