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Screening for Enhanced Triacetic Acid Lactone Production by Recombinant *Escherichia coli* Expressing a Designed Triacetic Acid Lactone Reporter

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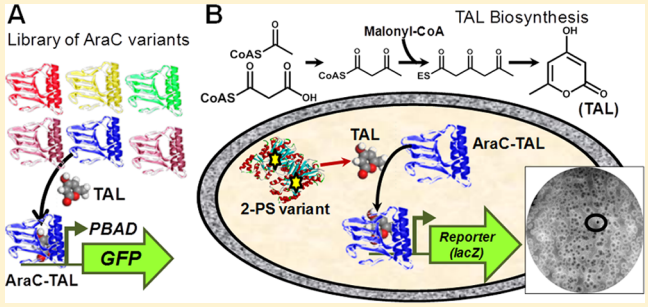
Supporting Information

ABSTRACT: Triacetic acid lactone (TAL) is a signature byproduct of polyketide synthases (PKSs) and a valuable synthetic precursor. We have developed an endogenous TAL reporter by engineering the *Escherichia coli* regulatory protein AraC to activate gene expression in response to TAL. The reporter enabled in vivo directed evolution of *Gerbera hybrida* 2-pyrone synthase activity in *E. coli*. Two rounds of mutagenesis and high-throughput screening yielded a variant conferring ∼20-fold increased TAL production. The catalytic efficiency (k_{cat}/K_m) of the variant toward the substrate malonyl-CoA was improved 19-fold. This study broadens the utility of engineered AraC variants as customized molecular reporters. In addition, the TAL reporter can find applications in other basic PKS activity screens.



INTRODUCTION

4-Hydroxy-6-methyl-2-pyrone, also referred to as triacetic acid lactone (TAL), is a natural compound of polyketide origin,[1,2] commonly identified as a triketide derailment product during polyketide biosynthesis (e.g., lovastatin[3] and 6-methylsalicylic acid[4]). Natural products bearing the 2-pyrone functionality exhibit biological activities and are valuable pharmaceutical precursors.[5−7] TAL is also a precursor in the chemical synthesis of phloroglucinol, used in the synthesis of the thermostable energetic material 1,3,5-triamino-2,4,6-trinitrobenzene (TATB), and resorcinol, used in resin and adhesive formulations.[8,9] Further catalytic upgrading of TAL has demonstrated its potential as a biorenewable platform chemical.[10] In *Gerbera hybrida*, TAL is a precursor in the biosynthesis of the pyrone glucosides gerberin and parasorboside.[11] The *g2ps1*-encoded type III PKS 2-pyrone synthase (2-PS) from *G. hybrida* (having 73% identity to the chalcone synthase (CHS) in this organism) uses acetyl-CoA as a starter substrate and catalyzes two condensation reactions with malonyl-CoA, resulting in TAL formation.[11]

Few studies have focused on improving microbial TAL production.[12,13] Two different mutants of *Penicillium patulum* 6-methylsalycilic acid synthase (6-MSAS) exhibit TAL-synthesizing activities, in contrast to their wild-type counterparts.[13] TAL yields from different TAL-synthesizing enzymes were compared by Zhao and co-workers. The highest TAL titers previously reported in controlled fermentations were 0.47 g/L (3.7 mM) from *E. coli* expressing wild-type 2-PS and 1.8 g/L (14.3 mM) from *Saccharomyces cerevisiae* expressing a variant of 6-MSAS.[13]

Engineering of genetic components involved in the synthesis of a target metabolite is often limited by the lack of sensitive and rapid screening/selection methods for identifying desirable candidates from large gene libraries.[14] Several studies, including those from our group, have sought to overcome this limitation by engineering a regulatory protein with altered effector specificity to enable direct coupling between recognition of the metabolite of interest (effector) and changes in reporter gene expression.[15−18] Owing to the lack of a well-developed high-throughput screen for TAL synthesis, the *Escherichia coli* regulatory protein AraC was engineered to recognize TAL as an effector. An endogenous TAL reporter system based on expression of *β*-galactosidase (LacZ) from AraC's cognate promoter P_{BAD} was subsequently used to screen for 2-PS variants conferring elevated TAL production by *E. coli*.

MATERIALS AND METHODS

Fluorescence-Based Positive and Negative Screening for AraC Mutants. Construction and screening of the AraC mutant

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library were carried out as previously described.[18] Strain HF19 harboring P_{BAD}-*gfpuv* reporter plasmid pPCC442 was transformed with the *araC* gene library contained in plasmid pPCC423. In the first round of screening, cells were grown in the absence of TAL and the least fluorescent 1.5×10^8 cells were collected (representing 82% of all cells sorted). This was done to eliminate AraC variants having high levels of leaky expression. In the second round, the most fluorescent 1.5×10^6 cells were sorted from a total of 1.5×10^8 cells after growth in the presence of 5 mM TAL. This dual screening procedure was repeated two more times and then two additional times with the concentration of TAL at 2.5 mM during positive screening, followed by a final round of negative screening. Twenty clones were then selected for rescreening. These were cultured separately and characterized in green fluorescent protein (GFP) expression fluorescence assays (described below). Three clones showing increased fluorescence in the presence of 2.5 mM TAL were selected for sequencing. The sequencing results revealed that all the clones contained the same mutations.

GFP Expression Fluorescence Assays. The fluorescence assays were performed as described previously.[17] Briefly, HF19 cells harboring plasmids pPCC442 and either pFG1-AraC or pFG1-TAL were grown overnight in lysogeny broth (LB) containing chloramphenicol, apramycin, and 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), then diluted to OD_{600} = 0.01 (Table 1) or OD_{600} = 0.2 (Figure 1) in the same medium containing the appropriate concentration of inducer, and allowed to grow under inducing conditions for 14 h. A total of 500 μL of the resulting culture was centrifuged, and the cells were washed with phosphate-buffered saline (PBS) (pH 7.4) and resuspended in 1000 μL of the same buffer. The OD_{600} of the suspension was measured using a NOVOstar microplate reader (BMG LABTECH), and the fluorescence emission was measured using a SpectraMax Gemini EM microplate reader (Molecular Devices Corp.) (400 nm excitation filter, 510 nm emission filter). The fluorescence emission was normalized with respect to OD_{600}, and the background fluorescence due to buffer was subtracted from all measurements. Reported data represent the average of three independent data points, and the standard deviations are shown in Figure 1. The coefficient of variation (CV) for all measurements was less than 15% and less than 10% for most data.

β-Galactosidase Activity-Based Solid-Phase Screening of 2-PS Random and Saturation Mutagenesis Libraries for Increased TAL Production. Strain HF22[18] was transformed with plasmid pPCC704, which contains the 2-PS random or saturation mutagenesis library. Cells were plated onto LB containing 400 μM IPTG, 274 mM glycerol (as the carbon source for TAL production), 40 μg/mL X-Gal, and apramycin. The plates were incubated at 37 °C for ~20 h, and the colonies which were obviously darker blue (by the eye) than those expressing wild-type 2-PS (for random mutagenesis library screening) or the 2-PS E3 mutant (for saturation mutagenesis library screening) were picked for quantifying TAL production in liquid culture.

HPLC Quantification of TAL. A single colony of strain HF22 harboring wild-type or mutant plasmid pPCC704 (carrying wild-type or mutant *g2ps1* genes) was grown in 3 mL of LB containing apramycin for 10 h. The cultures were then diluted to OD_{600} = 0.2 in 20 mL of LB containing 220 mM glycerol, 400 μM IPTG, and apramycin. At different time points, 700 μL of culture was centrifuged and the supernatant was passed through a 0.45 μm filter and acidified with 1% acetic acid. A Shimadzu LC-20AD HPLC system with an SPD-20A dual-wavelength UV–vis detector and a Phenomonex Luna C18 column (25 cm × 4.6 mm, 5 μm) was used for TAL quantification, following the protocol of Xie et al.[13] The TAL elution time was ~15 min, and concentrations were determined from a TAL standard curve. Reported data represent the average of three independent data points, and standard deviations are shown in Figure 2. The compound eluted at 15 min was collected and verified as TAL by comparison to an authentic standard using a Bruker MicroToF mass spectrometer operated in the positive ESI mode. HPLC chromatograms and mass spectra are available in the Supporting Information.

2-PS Activity Assays. TAL production was quantified by monitoring the corresponding increase in absorbance at 298 nm when incubating purified 2-PS variants with acetyl-CoA and/or malonyl-CoA using a molar extinction coefficient of 2540 M^{-1} cm^{-1} to quantify TAL.[13] The reaction mixture (200 μL) contained 100 mM HEPES buffer (pH 6.0) and the indicated concentration of acetyl-CoA and/or malonyl-CoA, and the reaction was initiated by the addition of 20 μL of purified enzyme. The increase in absorbance at 298 nm at 37 °C was measured using a NOVOstar microplate spectrophotometer. No background absorbance increase in the absence of the enzyme was detected, and the absorbance at 298 nm was verified to correspond to the TAL product measured by HPLC. The protein concentration was determined by the Quick Start Bradford Protein Assay (Bio-Rad Laboratories). All assays were performed in triplicate, and standard deviations are reported with the data. The mean values of the initial TAL production rate divided by the enzyme concentration under different malonyl-CoA concentrations are plotted in Figure S2 (Supporting Information). Kinetic parameters in Table 4 were obtained by nonlinear regression of the Michaelis–Menten equation.

RESULTS

Development of a TAL-Responsive AraC Variant. TAL does not induce expression of green fluorescent protein gene (*gfpuv*) from promoter P_{BAD} in *E. coli* expressing wild-type AraC (Figure 1). We previously described the use of multiple-site saturation mutagenesis, a P_{BAD}-*gfpuv* reporter plasmid, and iterative fluorescence activated cell sorting (FACS) to isolate AraC variants having altered effector specificity (e.g., specificity switched from its natural inducer L-arabinose to D-arabinose or mevalonate).[17,18] In a similar fashion, an *araC* library in which codons for five amino acid positions located in the effector binding pocket (P8, T24, H80, Y82, and H93)[18] were subjected to simultaneous saturation mutagenesis was screened for AraC variants responding to the presence of exogenous 5 or 2.5 mM TAL. Variants showing high levels of constitutive (uninduced) expression were eliminated by "negative" sorting, in which the least fluorescent cells are collected in the absence of TAL. After several rounds of screening, AraC variant "AraC-TAL" (P8V, T24I, H80G, Y82L, H93R) was isolated. The exogenous TAL dose response of GFPuv expression under control of AraC-TAL at promoter P_{BAD} is shown in Figure 1. TAL concentrations exceeding ~8 mM reduce growth and specific GFP expression, making it difficult to quantify the response of the AraC-TAL reporter system to a saturating exogenous TAL dose. As shown in Table 1, phloroglucinol (chemically similar

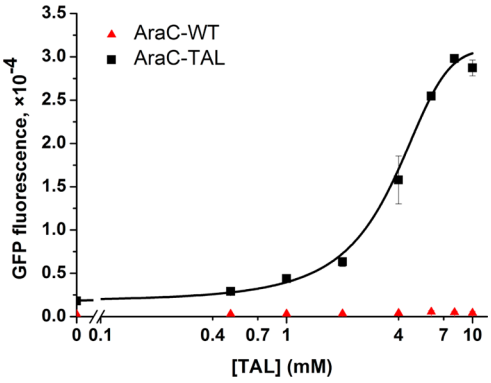

Figure 1. AraC-TAL TAL dose response. GFP expression from strain HF19 harboring P_{BAD}-GFP reporter plasmid and expressing either AraC-TAL (squares) or wild-type AraC (triangles) as a function of the TAL concentration (added to the culture).

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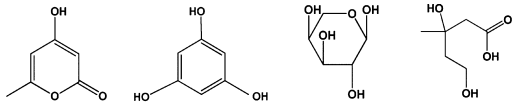
Table 1. Fluorescence of Strain HF19 Harboring P_{BAD}-*gfpuv* Reporter Plasmid and Expressing AraC-TAL in the Presence of the Indicated Effector Compound[a]



effector	fluorescence	induction fold	effector	fluorescence	induction fold
none	1420 ± 70		6 mM L-arabinose	1230 ± 60	0.9
6 mM TAL	19660 ± 680	13.9	60 mM mevalonate	1830 ± 50	1.3
6 mM phloroglucinol	2830 ± 130	2.0			

[a]The concentration indicated corresponds to the maximum fluorescence response.

Table 2. Amino Acid Substitutions in Improved 2-PS Variants

variant	amino acid substitutions	variant	amino acid substitutions
E1	L261F, K58K (silent mutation)	S1	L202G, M259L, L261N
E3	L261F, G221G (silent mutation)	S2	L202G, M259M, L261N
E8	L261F, D248G		

to TAL), L-arabinose (the native AraC inducer), and mevalonate (which AraC was previously engineered to recognize) are much poorer inducers of GFPuv expression, indicating that the expression response of the AraC-TAL reporter is relatively specific to TAL (presumably a result of negative sorting in which endogenous metabolites can act as decoy ligands). Complete dose responses for all ligands are shown in Figure S1 (Supporting Information).

Autodock[19] was used to estimate binding energies and identify potential AraC-TAL binding pocket interactions with TAL, L-arabinose, and mevalonate (Supporting Information). We were unable to rationalize inducer specificity from this analysis (binding energies for the ligands varied by <0.7 kcal/mol, while a typical error associated with such predictions is ±2 kcal/mol[20]), though it should be noted that ligand binding does not imply induced gene expression (a tight-binding ligand can inhibit activation) and the determinants of AraC molecular recognition resulting in activation remain unclear.[21]

2-PS Mutagenesis and Library Screening. Plasmid-based expression of wild-type 2-PS from strong promoters in *E. coli* resulted in production of ∼0.8 mM TAL in a shake-flask culture with excess glycerol (data presented here) and ∼3.7 mM TAL in a glucose-limited fermentation (previously reported[13]). Using AraC-TAL as an endogenous reporter of TAL, we next sought 2-PS variants conferring improved TAL production in *E. coli*. In our previous study involving an AraC-based mevalonate sensor, a visual colony screen on agar plates using strain HF22, which contains a chromosomal P_{BAD}-*lacZ* reporter, identified mutants that produce enhanced levels of mevalonate in liquid culture.[18] This screening approach reduces unwanted reporter expression due to the presence of effector (mevalonate or in this case TAL) that has been secreted by other high-producing clones, e.g., if the clones were pooled and screened directly from liquid culture via FACS.

The *g2ps1* gene was first randomly mutated using error-prone polymerase chain reaction (PCR), and strain HF22 was transformed with the library. Colonies expressing AraC-TAL and growing on LB agar supplemented with glycerol express LacZ when TAL is produced, and the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-GAL) results in blue color formation. After ∼20 h colonies expressing wild-type 2-PS were faintly blue, while the majority of library clones were white. Thirteen of the darkest blue colonies (identified by the eye) were selected (from a total of 4×10^4 colonies screened) and rescreened by quantifying TAL production from liquid cultures of these clones. Three clones, E1, E3, and E8, produced ∼2-fold more TAL than wild-type 2-PS in liquid culture (TAL produced by all 13 clones is provided in Table S3, Supporting Information). Thus, the qualitative visual screen rapidly identifies improved clones from a large library, as was the case for our mevalonate reporter system.

Plasmids from these clones were purified, sequenced, and confirmed to improve TAL production by HF22. All three variants contained amino acid substitution L261F, with an additional D248G substitution appearing in mutant E8 (Table 2). The time courses of TAL production and cell growth (OD_{600}) for clone E3 are presented in Figure 2 and Figure S3 (Supporting Information), respectively. At 12 h, E3 produced 2.3 mM TAL, compared to 0.8 mM by strain HF22 expressing wild-type 2-PS.

The three-dimensional structure of *G. hybrida* 2-PS shows L261 located in the enzyme's polyketide initiation/elongation cavity.[22] To further improve TAL production in *E. coli* and considering the results from the random mutagenesis study, the

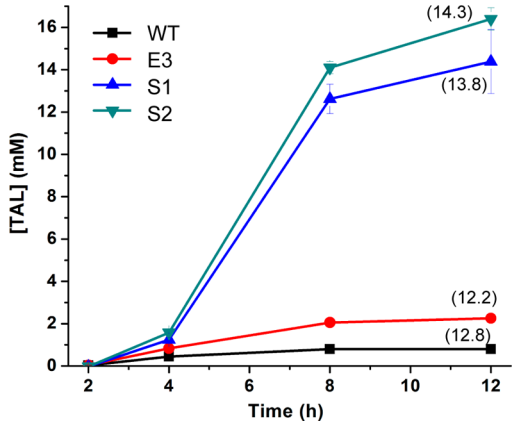


Figure 2. Time profiles of TAL concentration measured from cultures (LB + 220 mM glycerol) of strain HF22 harboring pPCC704 expressing wild-type 2-PS or its indicated variant (E3, S1, and S2). Final cell densities are in parentheses (OD_{600}).

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g2ps1 gene was next subjected to simultaneous saturation mutagenesis at three codons encoding amino acids in the 2-PS initiation/elongation cavity (L202, M259, and L261) (refer to Figure 6 in ref 22), corresponding to 8×10^3 2-PS variants. AraC-TAL and the P_{BAD}-*lacZ* reporter were again used to screen this 2-PS library. Eight dark blue colonies were selected (from a total of 4×10^4 colonies screened) and rescreened by quantifying TAL production from liquid cultures of these clones (Table S4, Supporting Information). Seven of the eight clones produced more TAL than 2-PS variant E3. Plasmids from the two top-producing clones, S1 and S2, were purified, sequenced, and confirmed to improve TAL production by HF22. The amino acid substitutions in variants S1 and S2 are shown in Table 2. The time courses of TAL production and cell growth (OD_{600}) of clones S1 and S2 are presented in Figure 2 and Figure S3 (Supporting Information), respectively. At 12 h, they produced significantly more TAL (14.4 ± 1.5 and 16.4 ± 0.5 mM) than wild-type 2-PS and variant E3. Improved TAL production did not appreciably affect cell growth, and cultures were near the saturating OD_{600} before TAL concentrations reached potentially inhibiting levels (~8 mM).

2-PS Variant Kinetic Parameters. His_6-tagged wild-type 2-PS and variants E3 and S1 were purified for activity measurements, which are summarized in Tables 3 and 4. The

Table 3. Initial Activity (mM TAL/min/mg of protein) of Purified Wild-Type 2-PS and Its E3 and S1 Variants in the Presence of Malonyl-CoA plus Acetyl-CoA

	wild type	E3	S1
1 mM malonyl-CoA + 0.5 mM acetyl-CoA	0.046 ± 0.004	0.16 ± 0.02	0.23 ± 0.01

Table 4. Kinetic Parameters of Wild-Type 2-PS and Its E3 and S1 Variants Using the Substrate Malonyl-CoA

enzyme	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
wild type	0.075 ± 0.012	0.32 ± 0.02	4.4 ± 0.5
E3	0.040 ± 0.006	0.28 ± 0.03	7.1 ± 0.4
S1	0.026 ± 0.001	2.18 ± 0.07	84.1 ± 6.4

acetyl-CoA starter molecule for TAL synthesis can be supplied by decarboxylation of malonyl-CoA by 2-PS, although in vitro activity using malonyl-CoA alone is 2−3-fold lower than in the presence of acetyl-CoA and malonyl-CoA.[11] In the presence of 1 mM malonyl-CoA plus 0.5 mM acetyl-CoA, variants E3 and S1 showed 3.5- and 5-fold higher initial activity than wild-type 2-PS, respectively. The kinetic parameters of wild-type 2-PS and variants E3 and S1 using malonyl-CoA as the substrate are reported in Table 4 (Michaelis−Menten curves are plotted in Figure S2, Supporting Information). Variant E3 has a nearly 2-fold lower K_m compared to wild-type 2-PS, while k_{cat} is not significantly different. Variant S1 has a further reduced K_m relative to the wild type and E3 and also a k_{cat} that is 6.7-fold higher than that of wild-type 2-PS, resulting in 19-fold increased catalytic efficiency (k_{cat}/K_m). Improved activities of the variants on malonyl-CoA therefore reflect the resulting improvements in TAL production.

Malonyl-CoA is a major building block for natural products such as fatty acids, polyketides, and flavonoids, and increasing intracellular malonyl-CoA levels using metabolic engineering strategies have improved malonyl-CoA-dependent production of a variety of these compounds in *E. coli*.[23−28] The intracellular malonyl-CoA concentration in an *E. coli* K-12 "wild-type" strain in exponential growth was reported to be 35 μM,[29] and previous measurements range from 4 to 90 μM[30] (in contrast, acetyl-CoA concentrations are 10-fold higher). Given the similarity of these values to the malonyl-CoA K_m of wild-type 2-PS (75 μM), the reduced K_m for the 2-PS variant S1 (26 μM) may therefore contribute significantly to more efficient utilization of this metabolite in *E. coli* (in addition to the elevated k_{cat}). L261N lies within the 2-PS initation/elongation cavity, opposite the catalytic C169.[22] This substitution may enhance binding of malonyl-CoA and help to polarize malonyl-CoA for decarboxylation (during elongation and/or initiation).

CONCLUSIONS

We have demonstrated the design and application of a novel endogenous TAL reporter, which facilitated screening for improved TAL production by engineered *E. coli*. Additional improvements in TAL production are foreseeable through further directed evolution of 2-PS or combinatorial host strain engineering using the TAL reporter, rational strain engineering (e.g., see ref 26), high-density fermentation, and optimized medium formulation. Moreover, the TAL reporter could serve as a useful tool for high-throughput identification of functional, minimal PKSs. Further directed evolution of AraC-TAL toward alternate 2-pyrone derivatives should also prove useful for screening and engineering polyketide biosynthetic pathways that incorporate alternate extender units.[31] Finally, this study further demonstrates the applicability of engineered AraC variants as customized endogenous molecular reporters in protein and metabolic engineering applications.

ASSOCIATED CONTENT

Supporting Information

Additional materials and methods, strain, plasmid, and primer descriptions, dose response profiles of wild-type AraC and AraC-TAL with phloroglucinol, L-arabinose, and mevalonate, AraC-TAL ligand docking analyses, enzyme Michaelis−Menten plots, HPLC traces, and TAL mass spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Bentley, R.; Zwitkowits, P. M. *J. Am. Chem. Soc.* **1967**, *89*, 681.

(2) Bentley, R.; Zwitkowits, P. M. *J. Am. Chem. Soc.* **1967**, *89*, 676.
(3) Ma, S. M.; Li, J. W.; Choi, J. W.; Zhou, H.; Lee, K. K.; Moorthie, V. A.; Xie, X.; Kealey, J. T.; Da Silva, N. A.; Vederas, J. C.; Tang, Y. *Science* **2009**, *326*, 589.
(4) Maia, T.; Richardson, N. L. P. *Metab. Eng.* **1999**, *1*, 180.
(5) Groutas WC, S. M.; Brubaker, M. J.; Huang, T. L.; Moi, M. K.; Carroll, R. T. *J. Med. Chem.* **1985**, *28*, 1106.
(6) Ridley, C. P.; Khosla, C. *ACS Chem. Biol.* **2007**, *2*, 104.
(7) Fairlamb, I. J.; Marrison, L. R.; Dickinson, J. M.; Lu, F. J.; Schmidt, J. P. *Bioorg. Med. Chem.* **2004**, *12*, 4285.
(8) Hansen, C. A.; Frost, J. W. *J. Am. Chem. Soc.* **2002**, *124*, 5926.
(9) Agrawal, J. P. *Prog. Energy Combust. Sci.* **1998**, *24*, 1.
(10) Chia, M.; Schwartz, T. J.; Shanks, B. H.; Dumesic, J. A. *Green Chem.* **2012**, *14*, 1850.
(11) Eckermann, S.; Schroder, G.; Schmidt, J.; Strack, D.; Edrada, R. A.; Helariutta, Y.; Elomaa, P.; Kotilainen, M.; Kilpelainen, I.; Proksch, P.; Teeri, T. H.; Schroder, J. *Nature* **1998**, *396*, 387.
(12) Zha, W. J.; Shao, Z. Y.; Frost, J. W.; Zhao, H. M. *J. Am. Chem. Soc.* **2004**, *126*, 4534.
(13) Xie, D.; Shao, Z.; Achkar, J.; Zha, W.; Frost, J. W.; Zhao, H. *Biotechnol. Bioeng.* **2006**, *93*, 727.
(14) Gredell, J. A.; Frei, C. S.; Cirino, P. C. *Biotechnol. J.* **2012**, *7*, 477.
(15) van der Meer, J. R.; Belkin, S. *Nat. Rev. Microbiol.* **2010**, *8*, 511.
(16) Galvao, T. C.; de Lorenzo, V. *Curr. Opin. Biotechnol.* **2006**, *17*, 34.
(17) Tang, S. Y.; Fazelinia, H.; Cirino, P. C. *J. Am. Chem. Soc.* **2008**, *130*, 5267.
(18) Tang, S. Y.; Cirino, P. C. *Angew. Chem., Int. Ed.* **2011**, *50*, 1084.
(19) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. *J. Comput. Chem.* **2009**, *30*, 2785.
(20) Cosconati, S.; Forli, S.; Perryman, A. L.; Harris, R.; Goodsell, D. S.; Olson, A. J. *Expert Opin. Drug Discovery* **2010**, *5*, 597.
(21) Schleif, R. *FEMS Microbiol. Rev.* **2010**, *34*, 779.
(22) Jez, J. M.; Austin, M. B.; Ferrer, J.; Bowman, M. E.; Schroder, J.; Noel, J. P. *Chem. Biol.* **2000**, *7*, 919.
(23) Lu, X.; Vora, H.; Khosla, C. *Metab. Eng.* **2008**, *10*, 333.
(24) Davis, M. S.; Solbiati, J.; Cronan, J. E., Jr. *J. Biol. Chem.* **2000**, *275*, 28593.
(25) Zha, W. J.; Rubin-Pitel, S. B.; Shao, Z. Y.; Zhao, H. M. *Metab. Eng.* **2009**, *11*, 192.
(26) Cao, Y.; Jiang, X.; Zhang, R.; Xian, M. *Appl. Microbiol. Biotechnol.* **2011**, *91*, 1545.
(27) Leonard, E.; Lim, K. H.; Saw, P. N.; Koffas, M. A. *Appl. Environ. Microbiol.* **2007**, *73*, 3877.
(28) Xu, P, R. S.; Fowler, Z. L.; Maranas, C. D.; Koffas, M. A. G. *Metab. Eng.* **2011**, *13*, 578.
(29) Bennett, B. D.; Kimball, E. H.; Gao, M.; Osterhout, R.; Van Dien, S. J.; Rabinowitz, J. D. *Nat. Chem. Biol.* **2009**, *5*, 593.
(30) Takamura, Y.; Nomura, G. *J. Gen. Microbiol.* **1988**, *134*, 2249.
(31) Koryakina, I.; McArthur, J. B.; Draelos, M. M.; Williams, G. J. *Org. Biomol. Chem.* **2013**, *11*, 4449.