

Cheating the Cheater: Suppressing False-Positive Enrichment during Biosensor-Guided Biocatalyst Engineering

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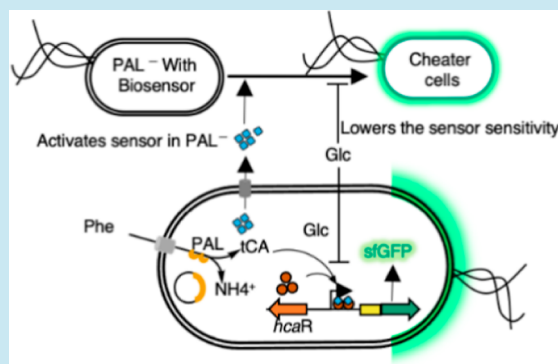
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ABSTRACT: Transcription factor (TF)-based biosensors are very desirable reagents for high-throughput enzyme and strain engineering campaigns. Despite their potential, they are often difficult to deploy effectively as the small molecules being detected can leak out of high-producer cells, into low-producer cells, and activate the biosensor therein. This crosstalk leads to the overrepresentation of false-positive/cheater cells in the enriched population. While the host cell can be engineered to minimize crosstalk (e.g., by deleting responsible transporters), this is not easily applicable to all molecules of interest, particularly those that can diffuse passively. One such biosensor recently reported for *trans*-cinnamic acid (tCA) suffers from crosstalk when used for phenylalanine ammonia-lyase (PAL) enzyme engineering by directed evolution. We report that desensitizing the biosensor (i.e., increasing the limit of detection) suppresses cheater population enrichment. Furthermore, we show that, if we couple the biosensor-based screen with an orthogonal prescreen that eliminates a large fraction of true negatives, we can successfully reduce the cheater population during the fluorescence-activated cell sorting. Using the approach developed here, we were successfully able to isolate PAL variants with $\sim 70\%$ higher k_{cat} after a single sort. These mutants have tremendous potential in phenylketonuria (PKU) treatment and flavonoid production.

KEYWORDS: gene circuits, protein engineering, directed evolution, phenylalanine ammonia-lyase, PAL, phenylketonuria, PKU



INTRODUCTION

While experimental and computational pathway along with biocatalyst designs have made major strides in past decades, screening for high target metabolite producing cells and enzymes remains a major bottleneck.¹ As with protein directed evolution, one of the bottlenecks in combinatorial metabolic engineering is the screening step.² Techniques such as high-performance liquid chromatography that are traditionally used to monitor concentrations of products or metabolic intermediates during strain development are too low throughput to screen large combinatorial libraries.³ Resultantly, significant effort has been expended to develop higher throughput screening methods to monitor metabolite levels.^{4–6} Most popular among these are genetically encoded biosensors,^{7–9} which link a phenotype to a readily detectable quantitative output signal. Biosensors are most frequently transcription factor- (TF) or riboswitch-based^{10–12}—although other modalities such as enzyme-based^{13–15} or protein–protein interaction-based¹⁶ are also possible. Among these, TF-based biosensors are most widely used due to their ease of construction and tunability. These TFs specifically bind a target metabolite (e.g., pathway intermediate, substrate, or final product) and activate or repress expression of a reporter gene—usually a fluorescent protein, which can then be screened using fluorescence-activated cell sorting (FACS).

Many of the biosensors described in the literature are based on natural TFs. However, genetic circuits controlling them often need to be engineered to reduce high basal expression, improve dynamic range, increase sensitivity, and ensure orthogonality.¹⁷ Examples for the application of these TF-based sensor-selector systems include screening campaigns for identifying improved producers of malonyl-CoA,^{18,19} naringenin,^{20,21} *cis,cis*-muconic acid,^{22,23} glucaric acid,²⁴ and fatty acyl-CoA,²⁵ vanillin,²⁶ protocatechuate,²⁷ butanol,²⁸ lactam,⁵ and so forth.

A major shortcoming of many biosensor-based systems is the propensity of “cheater” cells or false positives to enrich.^{1,29–33} This is particularly concerning when the metabolite being sensed can be actively or passively transported in and out of cells. Recent examples include the use of biosensor-based engineering to identify overproducers of *trans*-cinnamic acid (tCA),³⁴ benzoic acid and derivatives,³⁵ 1-butanol,³⁶ and naringenin² or to improve synthetic methylo-

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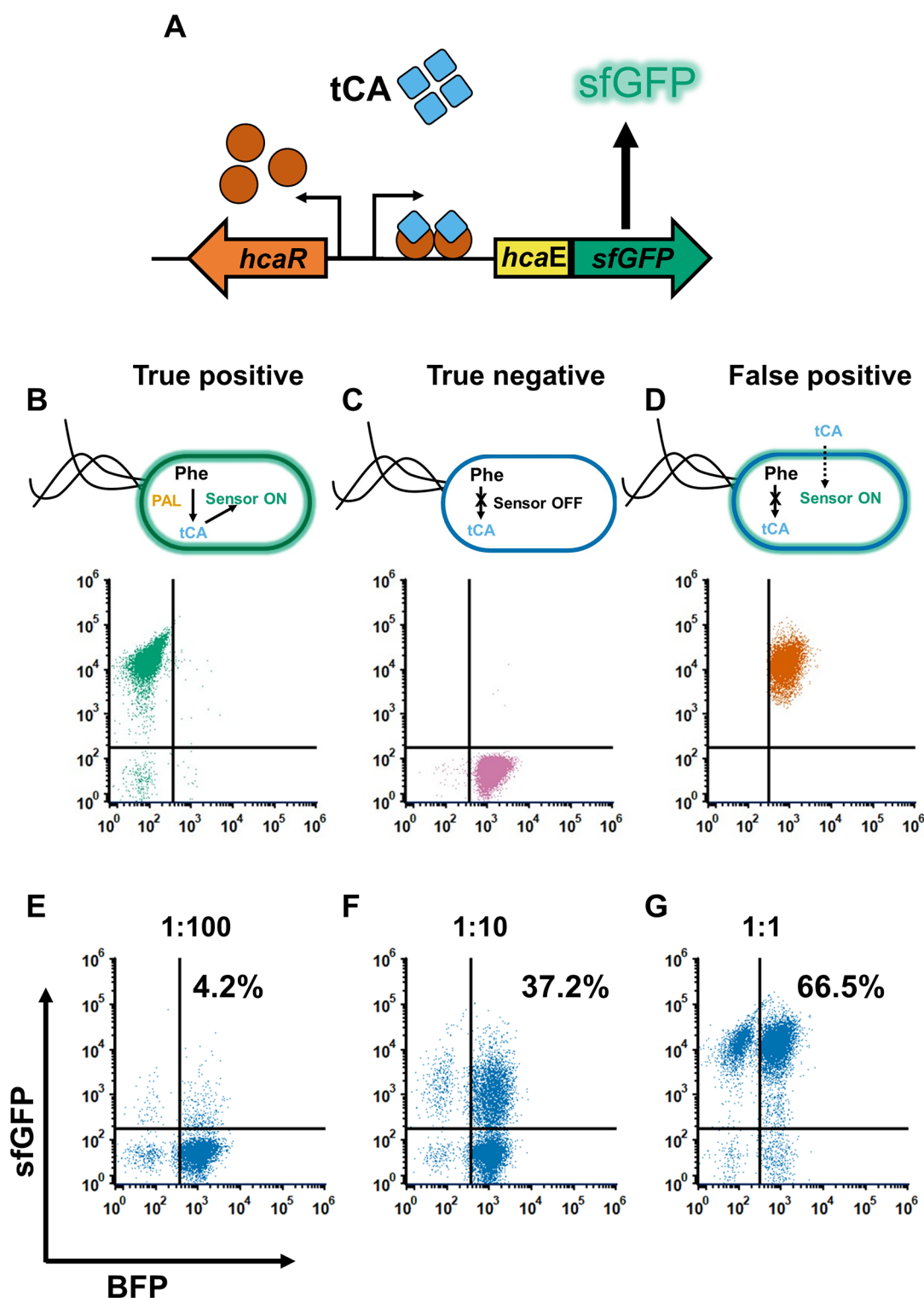


Figure 1. Quantification of cheater cell evolution using flow cytometry. (A) Schematic representation of the tCA biosensor. In the presence of tCA, HcaR serves as the transcriptional activator of P_{hcaE} -sfGFP. (B–D) Scatter plots of the control population, PAL⁺ (green, upper left quadrant) and PAL[−] (pink, lower right quadrant) and cheater cells (orange, upper right quadrant). (E–G) Mock libraries containing PAL⁺ and PAL[−] in ratios of 1:100, 1:10, and 1:1, respectively. The red-green gated population of in (D) was used to generate (G). The percentage of cheater cells (upper right quadrant) increased from 4.2 to 66.5% with the increase in relative abundance of true positives.

trophy with formaldehyde-responsive TFs.^{37,38} All these studies had to contend with a false positive when using the biosensor for strain or protein engineering. Flachbart et al.³⁴ recognized the high false rate and attempted to mitigate it by maintaining the cells at low cell densities to reduce

extracellular tCA concentrations and transport. However, their directed evolution campaign to identify improved variants of enzyme phenylalanine ammonia-lyase (PAL) was still severely hindered by the presence of false-positive cells (“cheaters”) in the FACS-enriched population, resulting in

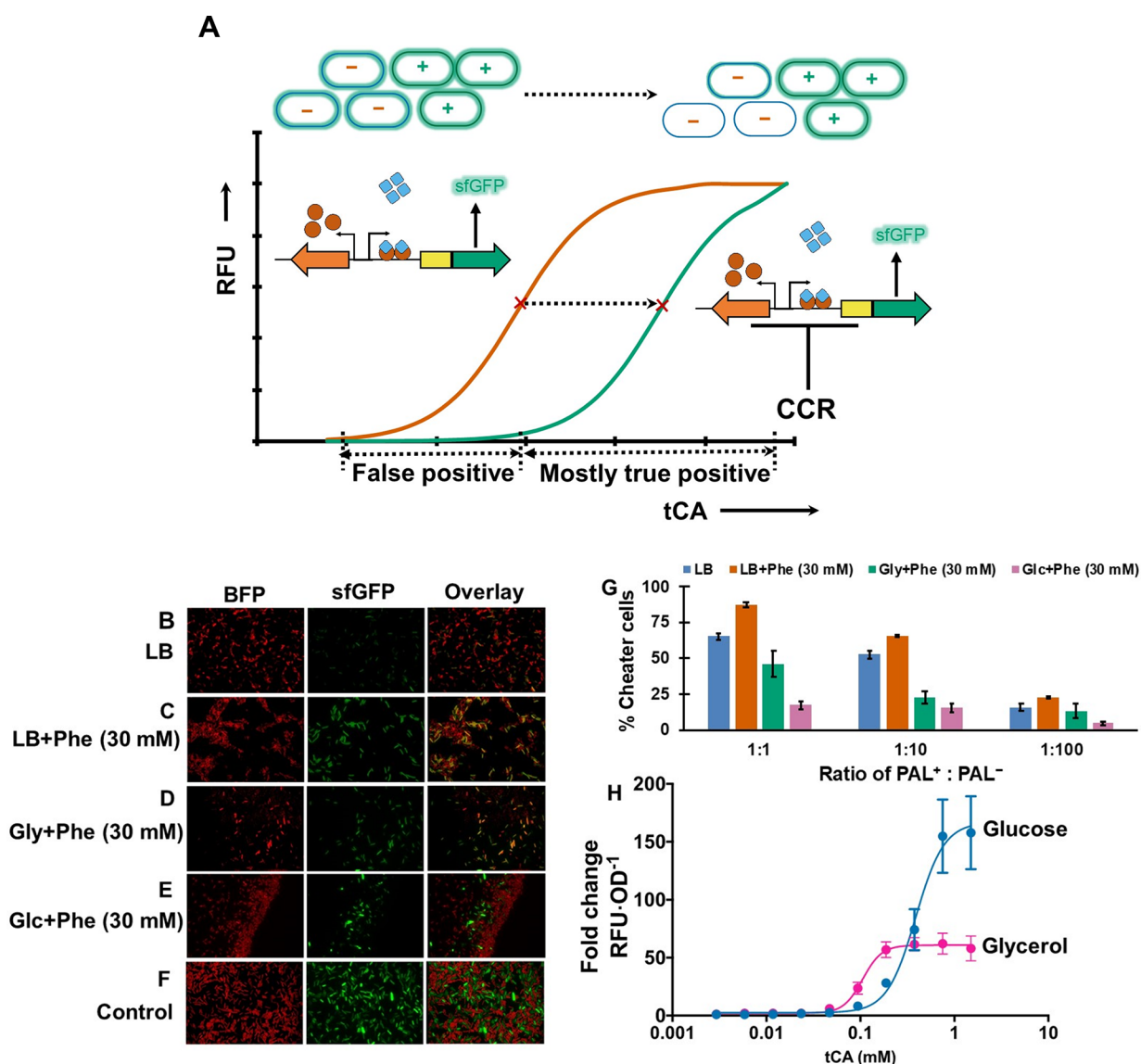


Figure 2. Evaluation of cheater cell evolution under CCR. (A) Schematic of how CCR can be exploited to suppress cheater cells by right-shifting the EC_{50} of the synthetic circuit. PAL⁻ cells (blue outline), cheater cells (green glow with blue outline), A co-culture of PAL⁺, and BFP-tagged PAL⁻ biosensor strain cells were mixed in a 1:1 ratio and grown for 8 h before imaging. Mixture grown in (B) LB, (C) LB + Phe (30 mM), (D) Gly + Phe (30 mM), and (E) Glc + Phe (30 mM). True positives are green (sfGFP) and true negatives are red (false-colored BFP). Cheater cells are yellow (red + green). (F) Control—cells not co-cultured together but mixed immediately before imaging. (G) Percent of cheater cells as determined from flow cytometry for the different growth conditions. The flow cytometry profiles are given in the Supporting Information (Figure S1 and Table S1). (H) Dynamic range of the fluorescent output in glucose or glycerol when supplemented with tCA (3 μ M–3 mM) after 8 h of growth in complete minimal media. The fluorescence output normalized to uninduced control of the respective carbon sources.

variants with only modest improvement ($\leq 11\%$) in k_{cat} even after five rounds of stringent sorting. Very recently, Adolfsen et al.³⁹ reported the use of the tCA biosensor with a microdroplet-based screen to identify mutants of PAL with twofold improvement in the whole cell activity. In this work, we describe conditions that help mitigate enrichment of cheaters during high-throughput screening campaigns using the tCA biosensor system. We first demonstrate that decreasing sensitivity (i.e., increasing the limit of detection, LOD) of the biosensor circuit output significantly reduces false positives. Next, we show that the incorporation of a prescreen can further mitigate cheater enrichment. With the modified workflow, we undertook a directed evolution campaign to improve PAL (from *Anabaena variabilis*⁴⁰) activity on its native substrate, phenylalanine (Phe), and were able to identify

variants with $\sim 70\%$ higher activity (k_{cat}) after a single round of sorting—the highest reported for a PAL by biosensor-guided engineering.

RESULTS AND DISCUSSION

Abundance of Cheater Cells and True Positives are Highly Correlated. The *Escherichia coli* HcaR TF, which induces the expression of the hydroxycinnamic acid (*hca*) catabolic operon, forms the basis of our biosensor design and is similar to the previous design³⁴ (Figure 1A). We placed *sfGFP* under the control of an HcaR-responsive promoter (P_{hcaE}) in a plasmid (pVDT46) and transformed in *E. coli* MG1655 *rph*⁺ to create a strain hereon referred to as Ecvdt46. However, unlike in the published design, we did not knockout the native *hcaREFCABD* operon in *E. coli*, enabling catabolism of tCA.⁴¹

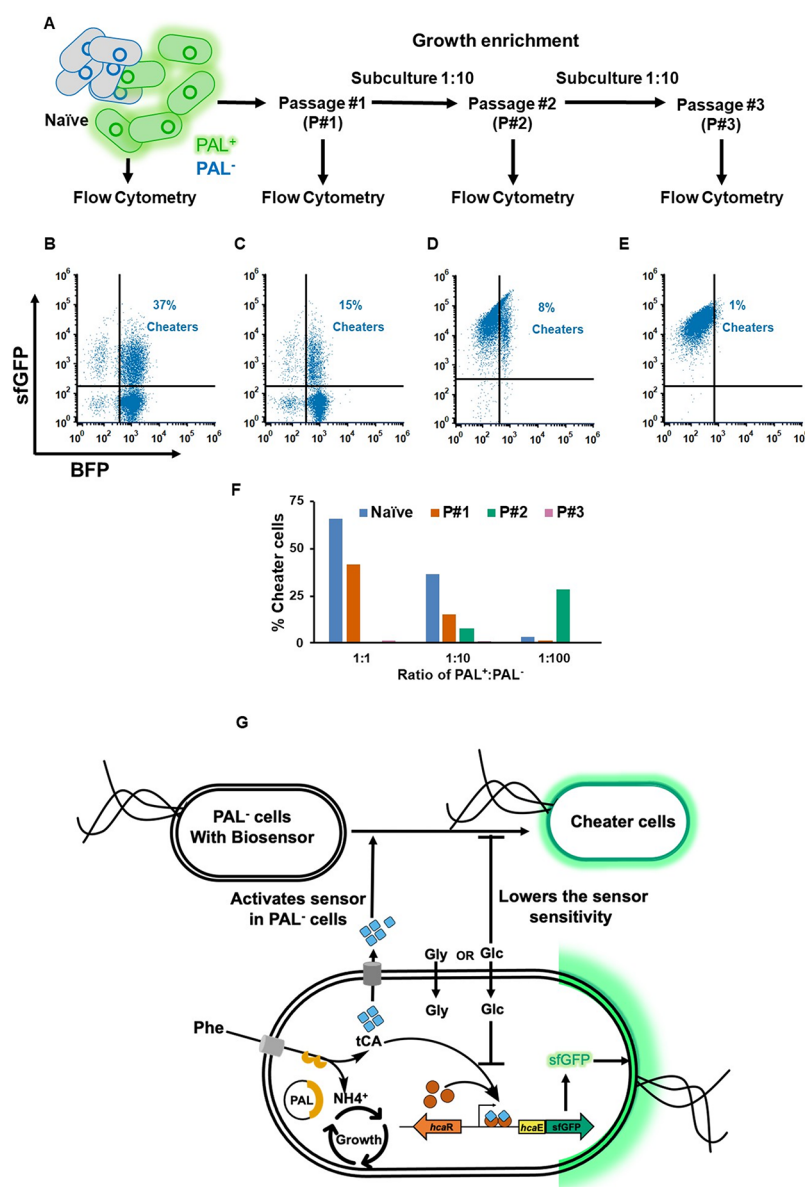


Figure 3. Growth-coupled enrichment mitigates cheater evolution. (A) Overview of the method used to demonstrate that growth-coupled prescreen eliminates cheater cells. PAL⁺ and BFP-tagged PAL⁻ cells were mixed in 1:1 or 1:10 ratio and subjected to selection for three passages in selective media. Presence of cheater cells were shown for 1:10 mock library using flow cytometry for (B) naive, (C) passage #1 (P#1), (D) passage #2 (P#2), and (E) passage #3 (P#3, gating was adjusted for P#2 and #3 to account for increased sfGFP fluorescence which could not be corrected by compensation). See Figure S3 for scatter plots of the 1:1 mock library. (F) Percent of cheater cells after different passages relative to naive for 1:1 and 1:10 mixtures. (G) Proposed design to suppress activation of the biosensor in cheater cells through CCR and growth-coupled prescreen. In the presence of active PAL, intracellular phenylalanine (Phe) is deaminated to ammonium (NH₄⁺) and *trans*-cinnamic acid (tCA). tCA binds to HcaR to activate the transcription of *P_{hcaE}-sfGFP*. Because tCA can be transported in and out of cells, it can enter PAL⁻ cells (that inherently do not produce any tCA) and activate the expression of sfGFP. These PAL⁻ cells show up as “cheater” cells during FACS and flow cytometry. While glycerol (Gly) and glucose (Glc) can serve as substrates for growth, only Glc engages CCR of the *hca* operon, decreasing basal activation. CCR also lowers the sensitivity of the biosensor, which is beneficial in preventing activation of the sensor by low intracellular tCA concentrations that may be present in PAL⁻ cells. Utilization of NH₄⁺ for growth produced by PAL can further enrich true positives over false positives in mixed populations.

To assess the evolution of cheater cells by tCA cross-feeding, we investigated how they evolve as a function of true positive population. We call true positives biosensor-encoding cells that generate tCA through PAL-mediated deamination of phenylalanine (Phe) (i.e., PAL⁺) (Figure 1B) and true negatives as those without functional PAL (i.e., PAL⁻). We integrated a blue fluorescent protein (BFP) reporter in PAL⁻ cells to aid in tracking their population (Figure 1C). Thus, PAL⁻ cells are unable to generate intracellular tCA (true negatives, Figure

1C) and would only activate their biosensor if they import exogenous tCA (false positives, Figure 1D). Next, we generated a mock library by mixing PAL⁻ and PAL⁺ cells in different ratios and monitored the evolution of cheater cells during co-culture. We observed that the abundance of cheater cells was dependent positively and monotonically on PAL⁺ cell concentration (Figure 1E–G). These cells show up in the GFP⁺ and BFP⁺ channels (top right), whereas true positives are GFP⁺ only and are simply upshifted (top left). This

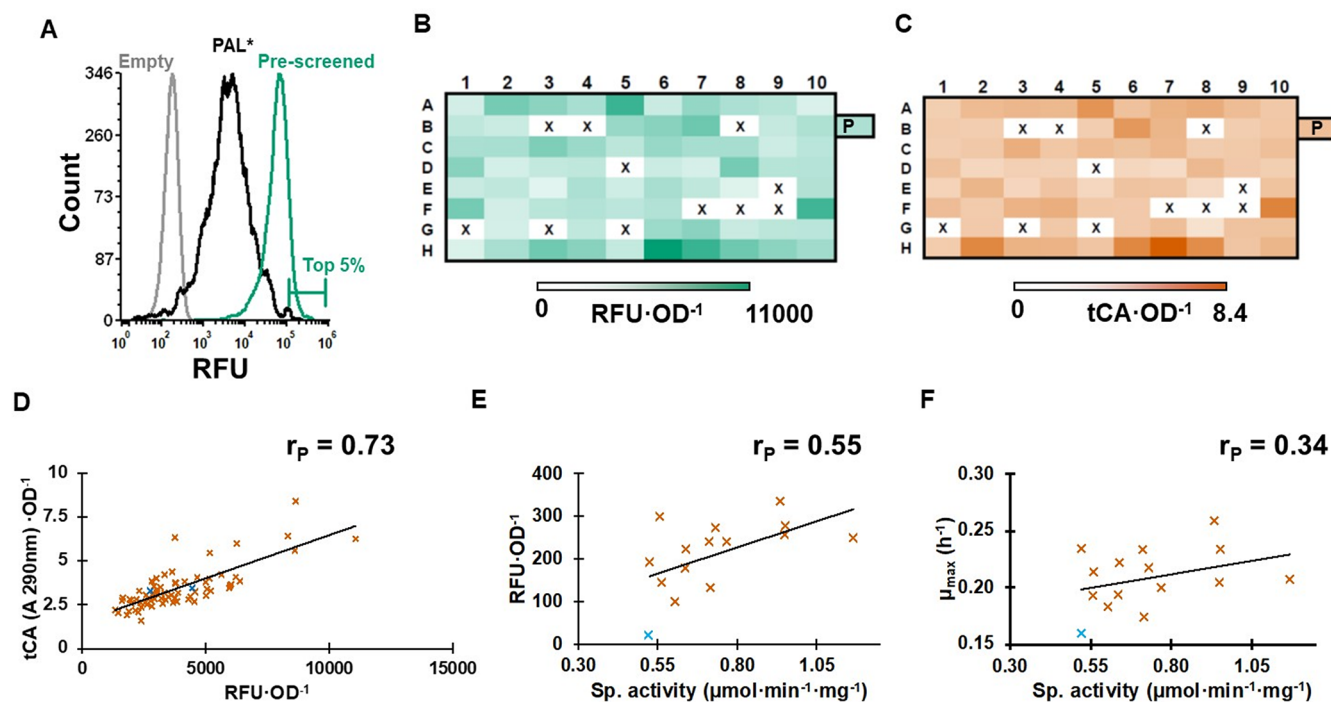


Figure 4. Screening of growth-enriched PAL library. (A) Flow cytometry profile of prescreened library pool transformed into the biosensor strain. (B) Biosensor output ($\text{RFU} \cdot \text{OD}^{-1}$) and (C) whole-cell PAL activity ($\text{tCA} \cdot \text{OD}^{-1}$) of the top 80 variants collected after FACS of prescreened pool. P = parental enzyme. x = variants that did not revive. Correlation of the top 14 variants between (D) cellular tCA production and $\text{RFU} \cdot \text{OD}^{-1}$, (E) $\text{RFU} \cdot \text{OD}^{-1}$ and specific activity of purified variants, and (F) growth rate in minimal ammonium-depleted Phe-supplemented prescreen medium and specific activity of purified variants. Mutants are in brown, whereas wildtype is blue.

validates our hypothesis that false positives are generated only in the presence of PAL^+ (tCA-producing) cells through the import of tCA in PAL^- cells. Thus, the system as is, unsuitable for biocatalyst engineering campaign.

Various approaches have been used to fine-tune the biosensor response, increase their dynamic range, sensitivity, and prevent crosstalk.¹⁷ However, these approaches are generally designed and tested on a clonal population, often with exogenously added metabolite, which may not always translate when applied to a high-throughput screen of a heterogeneous population such as a biocatalyst library with intracellularly produced metabolite. One approach that seems promising is to alter the expression of the TF⁴² (HcaR, here) or titrating the operator binding sites on promoter driving expression of the reporter protein^{43,44} (in this case, P_{hcaE}). However, this can be laborious and/or time-consuming. Therefore, we sought an alternate approach for the same outcomes by leveraging the native *E. coli* regulatory mechanism, given that the biosensor–promoter pair is native to this bacterium.

Carbon Catabolite Repression Alters the Sensor Response to Suppress Cheater Cell Evolution. HcaR is an activator of *hca* operon,⁴¹ which encodes genes required to catabolize phenolic acids such as tCA. However, as non-preferred substrates, the expression of the *hca* operon (and HcaR) is subject to carbon catabolite repression (CCR) by glucose.⁴⁵ We hypothesized that this CCR could be leveraged to desensitize the biosensor to low intracellular tCA concentrations present in cheater cells that import exogenous tCA (Figure 2A). This creates a threshold for activation that may only be surpassed at high intracellular tCA levels, as expected of cells with active PAL. To test this, we re-created a

mock library of PAL^+ and BFP-tagged PAL^- biosensor cells, as before, and looked for the appearance of cheater cells under different growth conditions—in LB, LB + Phe, glycerol (Gly) + Phe, or glucose (Glc) + Phe. We observed, as expected, that in nonrepressing media (LB, LB + Phe, and Gly + Phe), the percent of cheater cells was high (Figure 2B–D) and vice versa under Glc + Phe condition (Figure 2E). This supports our hypothesis that the native regulatory structure can be leveraged to suppress cheater evolution. To further support our conclusion that Glc-mediated CCR desensitizes the biosensor, enabling activation only at higher intracellular concentrations, we assessed the response of the biosensor to exogenously added tCA in both, Glc and Gly, media (Figure 2G). Although the fluorescence response for both Gly- and Glc-containing media was similar (Figure S2A), Glc condition exhibited higher fold change in fluorescence (150-fold) and larger dynamic range (100–750 μM , Figure 2H). We also observed activation of the tCA sensor in media containing Gly even when not challenged with tCA (Figure S2B), whereas we observed a drop in fluorescence in Glc-containing media (Figure S2B). The EC_{50} observed for the Glc (386 μM) condition is higher than that for Gly (105 μM), indicating decreased sensitivity. This decreased sensitivity appears to be beneficial as it suppresses fluorescence activation in cheater cells that are expected to have a lower intracellular concentration of tCA.

Various alternative approaches could also be used to achieve the goal of decreasing the crosstalk that leads to cheater evolution. For example, during screening of formaldehyde producers, Woolston et al.³⁷ supplemented the media with glutathione to scavenge the free formaldehyde, thus preventing its import in nonproducers. Microencapsulation can also be

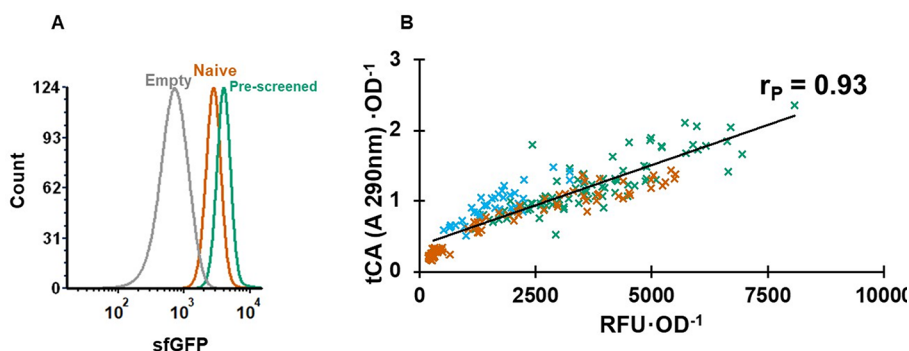


Figure 5. Screening and identification of mutants from a second-generation library. (A) Flow cytometry profile for Ecvdt46 cells carrying empty plasmid (gray), naive library (orange), and growth-enriched passage #3 library (green). Refer to Figure S4 for FACS scatter plots. (B) Correlation between whole-cell PAL activity ($\text{tCA} \cdot \text{OD}^{-1}$) and sfGFP fluorescence ($\text{RFU} \cdot \text{OD}^{-1}$) for variants isolated after FACS of naive (orange) and prescreened (green) libraries. Parental PAL is represented in blue, r_p represents Pearson correlation coefficient across all data points. The sorted naive library shows a fraction of inactive variants, whereas the prescreened library largely comprises variants with higher activity relative to parent.

used to prevent the exposure of nonproducers to the target molecule.³⁹ However, this approach requires specialized equipment, which may not be widely available. In addition to these reported approaches, knock-out of transporters responsible for export and/or import of the target molecules and addition of CRP (or other repressor) binding sites in the biosensor promoters can also be potential ways to suppress the cheater enrichment during FACS.

Orthogonal Growth-Coupled Prescreen Further Suppresses Cheater Evolution. Previously, we developed and optimized an enrichment for active PAL variants by linking evolution of ammonium (NH_4^+) to cell growth during deamination of Phe to tCA.⁴⁰ We also recently showed that this screen readily and rapidly eliminates inactive PAL variants from a mutagenized library.⁴⁶ Hence, we posited that using this orthogonal prescreen based on growth can further mitigate cheater cell enrichment during FACS (Figure 3). For this, we created a mock library containing PAL⁺ and BFP-tagged PAL[−], as before (in 1:1 and 1:10 ratios, respectively). This predefined library was subjected to growth-coupled enrichment for three passages in selective minimal medium with Phe as the sole nitrogen source (Figure 3A). At the end of every passage, the samples were analyzed on flow cytometry to detect the presence of cheater cells (Figure 3B–E). We observed a drop in the percent of cheater cells in the population and at the end of passage #3 to <1% (Figure 3F). This indicates that prescreening of library using growth-coupled enrichment can largely eliminate the PAL[−] population and reduce the number of cheater cell events during FACS (Figure 3G).

Biosensor Response is a Predictor of Enzyme Activity. Using the two-step approach, we screened a previously created $\sim 10^5$ member error-prone PCR library of PAL.⁴⁰ After prescreening with three rounds of growth enrichment, we transformed the plasmids into the biosensor strain, Ecvdt46. We noted that >80% of the passage #3 population had higher fluorescence than parental PAL (Figure 4A), further validating the utility of the prescreen. We sorted the library based on fluorescence and collected 80 events from top 5% of the population in a microtiter plate and screened them for tCA production and fluorescence in a spectrophotometer (Figure 4B,C). Of these, 11 variants did not revive. The surviving variants showed good correlation (Pearson correlation coefficient, $r_p = 0.73$) between total cellular PAL activity and biosensor output (Figure 4D). Of these, we picked the top 14 tCA producers for further characterization and

purified them (along with parental PAL) and determined their specific activity in the presence of 30 mM Phe. We observed that biosensor response showed better correlation with PAL specific activity than did growth rate in minimal, ammonium-depleted, Phe medium (Figure 4E,F). This suggests that biosensor-based screen is better to isolate high activity PAL variants compared to the growth-based prescreen. These results also highlight the benefit of the dual approach for directed evolution of PAL where the growth-based prescreen eliminates inactive variants, while the biosensor screen stratifies the remaining library by activity. On sequencing these 14 variants, we found 20 unique mutated positions (Table S2).

Further PAL Engineering Identifies Mutants with Improved Activity. Using these 20 unique positions, we created a new library of $\sim 10^7$ members to identify higher activity variants. To achieve this, we generated a new library by combining three or four mutations/protein ($^{20}\text{C}_{3-4}$, 20 choose up to 3 or 4). This strategy was chosen for diversification to ensure that the library size was small enough to be thoroughly screened while also ensuring comprehensive coverage of all 20 positions and 20 amino acids. In this combinatorial approach, we first performed individual site saturation mutagenesis (SSM) at all the 20 positions as separate reactions. Following assembly into circular plasmids and pooling in equimolar amounts, they served as a template for the second round of individual amplifications using the same SSM primers. Because some of the positions were close together (*viz.* G218, M222), a single SSM primer was generated to span the spatially close sites. Hence, at this stage, each variant contained mutations at two to three out of the 20 positions ($^{20}\text{C}_{2-3}$). This step was repeated once more to obtain three to four mutations out of the 20 positions ($^{20}\text{C}_{3-4}$). This naive pool was prescreened and then transformed into the biosensor strain for FACS (Figure 5A). Passage #3 of the prescreen showed higher median sfGFP fluorescence compared to the naive library, indicating the presence of more active PAL variants. We collected $\sim 10^5$ cells from the top 2% of the population from both the library pools and screened ~ 96 variants from each for fluorescence (using a spectrophotometer) and tCA production (Figure 5B). Variants from both sorts, naive and prescreened, showed good correlation between fluorescence and tCA production. Some variants from the naive library sort showed low activity, indicating continued presence of cheaters. Conversely, all the variants from prescreened and sorted pool displayed similar or higher activity compared to the parental enzyme. Furthermore,

no variants from this prescreened library were inactive—again, emphasizing the benefit of employing a screening methodology that minimizes cheater enrichment.

We sequenced the top 11 variants, performed the Michaelis–Menten kinetic characterization using purified enzymes, and determined their whole-cell tCA conversion (Figure 6A,B). Of the sequenced 11 variants, S98Y-T102K-

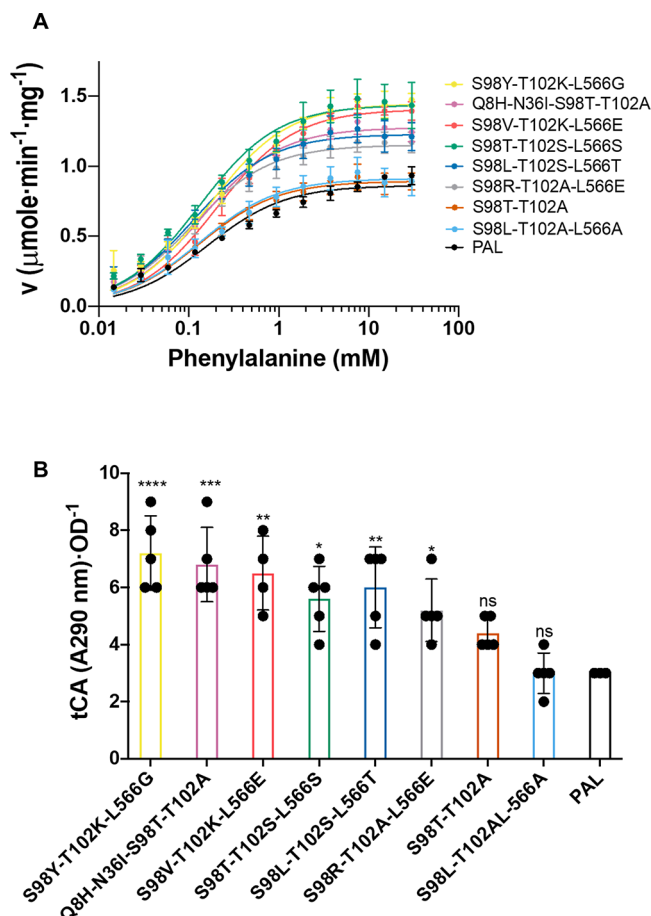


Figure 6. Characterization of variants from passage #3 of second-generation library. (A) Michaelis–Menten profile of variants. (B) Whole-cell tCA production by PAL variants. **** ($p < 0.0001$), *** ($p \leq 0.003$), ** ($p \leq 0.001$), * ($p \leq 0.05$), ns (nonsignificant).

L566G was represented thrice. We did not detect any M222L or G218S variants, which were also well-represented in the hits from the first library (Table S2) and also identified previously.⁴⁰ Thus, in all, we identified eight unique variants and characterized them to determine their kinetic constants

(Figure 6A and Table 1). All the variants displayed typical Michaelis–Menten behavior with S98Y-T102K-L566G showing the highest increase in k_{cat} (~ 1.7 -fold). Except for two variants, S98T-T102A and S98L-T102A-L566A, the rest showed improved k_{cat} compared to the parental enzyme. Correspondingly, we observed the variants with improved activity to exhibit higher whole-cell tCA production (Figure 6B). Notably, most of the variants identified in the current screen had mutations at S98, T102, and L566. Of these, only mutations in T012 have previously been shown to enhance PAL activity.⁴⁶ Furthermore, these positions showed permissiveness to biochemically diverse amino acids. For instance, we observed Tyr, Val, Leu, Thr, and Arg at S98, Ala, Ser, and Lys at T102, and Ala, Gly, Ser, Thr, and Glu at L566. We mapped the sampled positions onto the PAL structure (PDB: 3CZO) to gain insights into their functional significance (Figure S5). We found that S98 and T102 surround the active site pocket of PAL, whereas L566 is present in the region that is not well defined in the crystal structure. Though residues S98 and T102 have not been shown to be catalytically important, it would be interesting to understand their functional significance in future studies.

CONCLUSIONS

Our work outlines a methodology to successfully overcome enrichment of false positives during strain/protein engineering campaigns that utilize biosensors to screen combinatorial libraries. We used the tCA biosensor based on the *E. coli* *hca* operon (including the tCA-binding HcaR TF, its native promoter, as well as its target promoter, P_{hcaE}) and a PAL enzyme as a model system. We demonstrate that false positives (or cheaters) enrich due to cross-feeding of tCA—the PAL reaction product and biosensor activator. We hypothesized that because these cheaters likely have lower intracellular tCA concentration, decreasing the sensitivity of the biosensor could, at least partially, mitigate cheater enrichment. We leverage the intrinsic CCR on the *hca* operon by glucose to increase the LOD of the system (decreasing its sensitivity) and demonstrate that this suppresses the relative enrichment of cheaters. While this would also inhibit the catabolism of tCA by *E. coli*, which could have been beneficial in mitigating cross-feeding, we believe that the benefits of desensitizing the biosensor likely outweigh any potential benefit of tCA metabolism. Next, we incorporate our previously developed growth-based enrichment to further repress cheater enrichment by promoting growth of cells carrying active PAL. While the growth-based prescreen was effective at eliminating inactive PAL variants from the pool, it was only modestly able to link cell growth to tCA productivity. Conversely, we

Table 1. Kinetic Constants for PAL Variants

variant	v_{max} ($\mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1}\cdot\text{M}^{-1}$)	fold increase k_{cat}
PAL (parental)	0.86 ± 0.02	160 ± 17	0.90	5.6	1.00
S98Y-T102K-L566G	1.45 ± 0.02	168 ± 14	1.51	9.0	1.68
Q8H-N36I-S98T-T102A	1.28 ± 0.01	141 ± 08	1.33	9.4	1.48
S98V-T102K-L566E	1.41 ± 0.02	205 ± 16	1.46	7.2	1.63
S98T-T102S-L566S	1.44 ± 0.03	131 ± 13	1.50	11.4	1.67
S98L-T102S-L566T	1.23 ± 0.02	112 ± 10	1.28	11.4	1.42
S98R-T102A-L566E	1.15 ± 0.02	108 ± 09	1.20	11.1	1.33
S98T-T102A	0.89 ± 0.02	131 ± 12	0.93	7.1	1.04
S98L-T102A-L566A	0.91 ± 0.02	131 ± 16	0.95	7.2	1.06

found that the desensitized biosensor itself was better at quantitatively linking PAL activity to the reporter output. Thus, a combination of the two screens was able to optimally identify high activity variants from large, mutagenized libraries. Although the variants isolated here were not as active as those identified via a more thorough deep mutational scanning guided engineering approach,⁴⁶ the outcomes are more successful than the previously described approach to engineering PAL activity using the same biosensor.³⁴ Furthermore, this work required only a single round of FACS sorting at each step, compared to the previous approach, where five rounds were needed. We expect that a similar analysis and approach could be broadly beneficial to other biosensor-guided engineering workflows that suffer from ligand cross-feeding.

MATERIALS AND METHODS

General Molecular Biology and Microbiological Techniques. Error-prone PCR on *A. variabilis* PAL was performed as described previously.⁴⁰ PCR was performed using Phusion DNA polymerase or Platinum SuperFi II Green PCR Master Mix (ThermoFisher Scientific). *E. coli* NEB5 α (New England Biolabs) was used for plasmid propagation and *E. coli* MG1655 *rph*⁺ was used for the screening of libraries and purification of recombinant PAL and its mutants. Sequences of constructed plasmids were confirmed through DNA sequencing (Genewiz). PAL was expressed under constitutive T5 promoter from plasmid pBAV1k carrying chloramphenicol resistance. BFP-tagged PAL[−] strain was constructed by knocking-in BFP under IPTG-inducible T7 promoter at *araC* locus using lambda-red recombineering. During the flow cytometry experiments, BFP was induced by adding IPTG (500 μ M).

Enzyme Assay, Purification, and Kinetic Characterization. PAL activity was monitored by measuring the production of tCA at 290 nm over time. Briefly, 200 μ L reaction as performed by 1 μ g of purified enzyme to prewarmed (37 °C) 1 $\times$ PBS containing 30 mM Phe. The assay was performed in a 96-well F-bottom UVStar (Greiner Bio-One, Kremsmünster, Austria) microtiter plate, and absorbance at 290 nm was measured every 15 s at 37 °C using a SpectraMax M3 (Molecular Devices) plate reader.

The enzyme was purified from a 25 mL culture. The pellet was washed once with 1 $\times$ PBS and resuspended in 500 μ L of the same. This cell suspension was sonicated on ice using a Sonifier SFX 150 (Branson Ultrasonics, Danbury, CT) (10 s ON; 1 min OFF; 2 min; 40%), and cell debris was separated from the lysate by centrifuging at 20,000g for 10 min at 4 °C. As each construct included a N-term His₆-tag, the enzyme was purified via immobilized metal affinity chromatography (IMAC). Briefly, the lysate was loaded onto HisPur Ni-NTA Spin Plates (Thermo Fisher Scientific) and incubated for 2 min. After being washed five times with equilibration buffer, pure protein was then eluted using 200 μ L of Elution buffer (300 mM NaCl, 50 mM NaH₂PO₄, 500 mM imidazole, pH 8.0). Elution fractions were then dialyzed using Tube-O-Dialyser tubes (1 kDa MWCO, Geno-Tech). Protein concentration was estimated by Bradford reagent (VWR) using bovine serum albumin as the standard. For kinetic analysis, PALs were purified and assayed as described above. The activity was measured at 12 concentrations of Phe ranging from 15 μ M to 30 mM in PBS at 37 °C. A Michaelis–Menten curve was fit in GraphPad Prism software using the initial rate at each Phe concentration.

Flow Cytometry. Flow cytometry analysis was performed using Attune NxT flow cytometer. Relevant flow cytometer settings: 12.5 μ L/min flow rate, 20,000 events collected per sample, FSC: 120, SSC: 240, BL1: 300V, and VL1: 250 V. The FCS files were analyzed using FSC software v6.

Microscopy. Microscopy was performed using a DMI8 automated inverted microscope (Leica Microsystems, #11889113) equipped with a CCD camera (Leica Microsystems, #DFC300 G) and a LED405 (Ex 375–435 nm, Em 450–490 nm, exposure time—10 ms, gain 2) and a YFP (Ex 490–510 nm, Em 520–550 nm, exposure time—5 ms, gain 1) filter cube. 1 μ L of cultures were spotted on agarose pads (2% w/v, 1 mm thick).

Fluorescence-Activated Cell Sorting. Cell sorting was performed on a Bio-Rad S3e Cell Sorter. Sorting gates were drawn on dot plots with FL1 and FL4 on the axes. Sorting was performed on the events in the FL1 green emission channel. After sorting, some of the cells were plated on LB + Cm (25 μ g·mL^{−1}) + Amp (100 μ g·mL^{−1}) agar plates, and the remainder was recovered in LB + Cm + Amp liquid medium and frozen at −80 °C for long-term storage.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.1c00506>.

Flow cytometry profile of Figure 2G; response of tCA sensor strain (Ecvdt46) in glycerol and glucose; flow cytometry profile of mock library containing PAL⁺/PAL[−] in a 1:1 ratio and enriched in glucose + Phe (30 mM); FACS profile of ²⁰C_{3/4} library; mutants identified in the present study mapped onto the structure of PAL (PDB 3CZO); flow cytometry data of PAL⁺/PAL[−] in 1:1, 1:10, and 1:100 in LB, LB + Phe (30 mM), glycerol + Phe (30 mM), and glucose + Phe (30 mM); and mutants isolated from the orthogonal screen (PDF)

Mutants identified in the present study mapped onto the structure of PAL (MP4)

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Author Contributions

N.U.N., T.C.C., K.M., Z.J.S.M., and V.D.T. conceived the idea, designed the research project, analyzed the data, and co-wrote the manuscript. T.C.C., K.M., Z.J.S.M., and V.D.T. performed the experiments. All the authors have reviewed the manuscript and approved it for submission.

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Notes

The authors declare the following competing financial interest(s): V.D.T., T.C.C., and N.U.N. are co-founders of Enrich Bio, LLC. Tufts University and the authors have applied for a patent on some of the methods and mutations described.

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