Displaced by Deceivers: Prevention of Biosensor Cross-Talk Is Pivotal for Successful Biosensor-Based High-Throughput Screening Campaigns

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Supporting Information

ABSTRACT: Transcriptional biosensors emerged as powerful tools for protein and strain engineering as they link inconspicuous production phenotypes to easily measurable output signals such as fluorescence. When combined with fluorescence-activated cell sorting, transcriptional biosensors enable high-throughput screening of vast mutant libraries. Interestingly, even though many published manuscripts describe the construction and characterization of transcriptional biosensors, only very few studies report the successful application of transcriptional biosensors in such high-throughput screening campaigns. Here, we describe construction and characterization of the trans-cinnamic acid responsive transcriptional biosensor pSenCA for Escherichia coli and its application in a FACS based screen. In this context, we focus on essential methodological challenges during the development of such biosensor-guided high-throughput screens such as biosensor cross-talk between producing and nonproducing cells, which could be minimized by optimization of expression and cultivation conditions. The optimized conditions were applied in a five-step FACS campaign and proved suitable to isolate phenylalanine ammonia lyase variants with improved activity in E. coli and in vitro. Findings from this study will help researchers who want to profit from the unmatched throughput of fluorescence-activated cell sorting by using transcriptional biosensors for their enzyme and strain engineering campaigns.

KEYWORDS: transcriptional biosensor, library screening, product sensing, fluorescence-activated cell sorting, protein engineering, directed evolution

Genetically encoded biosensors represent powerful tools in strain and protein engineering as they enable the high-throughput screening of large variant libraries by linking an often inconspicuous production phenotype to a readily detectable output signal.1 In the past years, different biosensor concepts were introduced, namely, transcription factor (TF)-based biosensors, Förster resonance energy transfer (FRET) biosensors, as well as RNA-based biosensors.2 Especially TF-based biosensors received a lot of attention as they are easy to construct and result in a relatively strong fluorescence signal.3 Biosensors of this type take advantage of transcriptional regulator proteins, which specifically bind the molecule of interest and drive or repress expression of a reporter gene (usually encoding for a fluorescent protein or a selection marker). Examples for the application of these TF-based sensor-selector systems include screening campaigns for identifying improved producers from mutant libraries and selecting for suitable synthetic pathway variants.4,5 Additionally, biosensors find application in synthetic sensor-actuator systems to enable the dynamic feedback regulation of heterologous pathways.6–8 In combination with fluorescence activated cell sorting (FACS), such transcriptional biosensors unleash their true potential as they allow for ultrahigh throughput screening on single cell level and isolation of producing single cells from very large libraries.9–13

However, when considering the larger number of biosensors constructed over the past couple of years, studies combining transcriptional biosensors with FACS are rather scarce. For the most part, published biosensor screening applications are limited to agar plate or microtiter plate screenings, or the constructed and characterized biosensors are not put to any use at all. The transition from biosensor construction/characterization to meaningful applications involving FACS could be hampered by several factors. For instance, the engineered organism carrying the biosensor might provide a fluorescence response in cultures, but give only a limited contribution at all. The transition from biosensor construction/characterization to meaningful applications involving FACS could be hampered by several factors. For instance, the engineered organism carrying the biosensor might provide a fluorescence response in cultures, but give only a limited contribution at all. The transition from biosensor construction/characterization to meaningful applications involving FACS could be hampered by several factors. For instance, the engineered organism carrying the biosensor might provide a fluorescence response in cultures, but give only a limited contribution at all.

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prohibiting any FACS-based screening. In addition, diffusion of the target metabolite from strong producing cells to weak or nonproducing strain variants could result in the isolation of mainly false-positive clones, demanding an individual and thus laborious and expensive characterization of many individual clones.

In this manuscript, we present suitable strategies by which these causes of failure can be efficiently tackled to yield robust and reliable biosensor-based FACS-ultrahigh-throughput screenings for protein and metabolic engineering campaigns. In this context, we describe construction and application of a transcriptional phenylpropanoid biosensor, which was used to engineer an ammonia lyase in *E. coli*.

## MATERIALS AND METHODS

### Bacterial Strains, Plasmids, Media, and Growth Conditions

All bacterial strains and plasmids used in this study and their relevant characteristics are listed in Supplementary Table S1. For recombinant DNA work and library construction, *E. coli* DH5α and *E. coli* TOP10 (Thermo Fisher Scientific, Waltham, MA, USA) were used, respectively. For recovery after electroporation, SOC-medium (super optimal broth with catabolite repression) was used (20 g/L tryptone, 5 g/L yeast extract, 0.6 g/L NaCl, 0.2 g/L KCl, 10 mM MgCl₂/MgSO₄, and 20 mM glucose, pH 7). All strains were routinely cultivated at 37 °C on plates or in liquid culture in either Lysogeny broth (LB) medium (10 g/L tryptone, 10 g/L NaCl, 3 g/L KH₂PO₄, and 10 g/L glycerol was added to 900 mL YNB base medium. YNB base preparation of 1 l of YNB medium, 100 mL of ten-times-

*E. coli* DH10B is leucine auxotroph, L-leucine was supplemented to a final concentration of 2 mM for all cultivations. L-arabinose and either 3 mM L-phenylalanine or 3 mM L-tyrosine, respectively. After 16 h of cultivation (25 °C, 900 rpm, 75% humidity, 3 mm throw), product titers were determined.

### Molecular Biology

**Molecular Biology.** Standard Techniques for Molecular Cloning. Polymerase chain reactions, DNA restrictions and ligations were performed according to standard protocols. Enzymes were obtained from Thermo Fisher Scientific (Waltham, MA, USA) and used following the manufacturer’s recommendations. Genes were amplified by PCR using Pfu UltraII polymerase (Agilent, Santa Clara, CA, USA). Cloning of the amplified PCR products was performed using restriction enzyme digestion and subsequent ligation or Gibson assembly. Synthesis of oligonucleotides and sequencing of DNA using Sanger sequencing were performed by Eurofins MWG Operon (Ebersberg, Germany). All oligonucleotides used in this study are listed in Supplementary Table S2.

**Error-Prone PCR and Ammonia Lyase Library Construction.** The *xalT* gene was amplified from plasmid pCB296 using the Clontech Diversify kit (Takara Bio Europe, Saint-Germain-en-Laye, France) incorporating 2.3 or 4.6 mutations/kb and assembled with pBAD plasmid, previously amplified using PCR, using Gibson assembly. The resulting plasmid library was purified and transformed into One Shot TOP10 electrocompetent *E. coli* cells according to the manufacturer’s recommendations. Plasmid preparations were performed using Midi kits according to the manufacturer’s recommendation (Qiagen, Hilden, Germany). The plasmid library was retransformed into *E. coli* DH10B ΔhisaREFCBD pSenCA. Preparation of electrocompetent cells and electroporation of the plasmid library was performed as described elsewhere.

### Chromosomal Deletions

Deletion of chromosomal genes was performed using Lambda (λ)-Red recombineering. Here, the recently published plasmid pSJ8 was used according to the published protocol instead of the original two plasmid approach described by Datsenko and Wanner.

### Fluorescence Activated Cell Sorting (FACS)

Single-cell fluorescence was determined and cell sortings were performed using a BD FACSaria II cell sorter (BD Biosciences, Franklin Lakes, NJ, USA) equipped with a 70 μm nozzle and run with a sheath pressure of 70 psi. A 488 nm blue solid laser was used for excitation. Forward-scatter characteristics (FSC) were recorded as small-angle scatter and side-scatter characteristics (SSC) were recorded as orthogonal scatter of the 488 nm laser. A 502 nm long-pass and 530/30 nm band-pass filter combination enabled EYFP fluorescence detection. Prior to data acquisition, debris and electronic noise were excluded from the analysis by electronic gating in the FSC-H against SSC-H plot. Another gating step was performed on the resulting population in the FSC-H against FSC-W plot to exclude doublets. Fluorescence acquisition was always performed with the population resulting from this two-step gating. For sorting applications, cells were diluted to an OD₆₀₀ below 0.1 where necessary using YNB base buffer, and 200 000 cells were sorted into 5 mL reaction tubes (Eppendorf AG, Hamburg, Germany), prefilled with 3 mL LB medium using an in-house built adapter for 5 mL reaction tubes that was described earlier. To minimize residual sheath fluid in the recovery tube, sorted cells were centrifuged (10 min, 3000g, 4 °C), after removal of 3 mL supernatant and addition of 4.5 mL fresh LB medium, regeneration was performed (16 h, 37 °C, 170 rpm). Sort precision was always set to purity setting and...
the total event rate while sorting never exceeded 16 000 events per second. FACSDiva 7.0.1 (BD Biosciences, San Jose, USA) was used for FACS control and data analysis. FlowJo for Windows 10.4.2 (FlowJo, LLC, Ashland, OR, USA) and Prism 7.04 (GraphPad Software, San Diego, CA, USA) were used to produce high-resolution graphics of FACS data.

### Protein Purification and Enzyme Assays
Selected Xal_f encoding genes (xal_f) were recloned to enable their expression as fusion proteins with an N-terminal hexa histidine (His6)-tag in E. coli DH10B ΔhcaREFCBD. Single colonies were used to inoculate 5 mL LB and grown at 37 °C for 4 h. Afterward, 500 μL culture was used to inoculate 15 mL YNB (0.51% glycerol as carbon source) precultures. After 16 h of cultivation at 37 °C, these precultures were used to inoculate 100 mL YNB (1.2% glycerol as carbon source) cultures to an OD₆₀₀ of 0.2, which were cultivated (2 h, 37 °C, and 130 rpm) prior to the addition of L-arabinose to a final concentration of 1.3 mM. Gene expression was performed (20 h, 25 °C, and 130 rpm) and cells were harvested (15 min, 4000g, 4 °C). After resuspension in 15 mL sonication buffer (50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, pH 7.5) cells were disrupted using an ultrasonic cell disruptor (Branson Ultrasonics Corporation, Danbury, CT, USA, eight sonication cycles of 30 s, 4 °C, duty cycle 34 and output control 8). From this, crude extracts were prepared by centrifugation in an Avanti J25 centrifuge (Beckmann Coulter Life Sciences, Indianapolis, IN, USA. 19 000 rcf, 45 min, 4 °C using a JA25.5 rotor). The supernatant was applied to a gravity flow column filled with Nickel-nitrilotriacetic acid (Ni-NTA) affinity agarose (Qiagen, Hilden, Germany, 1 mL bed volume). Protein loaded onto the column was washed subsequently with 10 mL sonication buffer and 10 mL wash buffer (sonication buffer with 30 mM imidazole) prior to elution with 3 mL elution buffer (sonication buffer with 300 mM imidazole) in 500 μL fractions. Protein containing fractions were pooled and the protein solution was transferred to a hydrated Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific, Schwerte, Germany, molecular cutoff of 10 kDa) and placed in 1 L dialysis buffer/assay buffer (50 mM Tris-HCl, pH 7.5 at 30 °C, with 150 mM NaCl and 10% glycerol). Dialysis was performed (20 h, 4 °C, 30 rpm) and enzyme assays were always performed directly after protein purification.

Enzyme assays were performed in 96 well plates with UV-transparent, flat bottom (Corning, New York, USA) in a Tecan M1000 plate reader (Tecan Group, Maennedorf, Switzerland), by following the increase in absorbance at 276 nm (CA) or 310 nm (pHCA). Twenty μg purified enzyme was transferred to each well and warmed to 30 °C for 60 s. Directly afterward, the substrate was added to a final volume of 200 μL using an E4XLS 100–1200 μL multichannel pipet (Rainin Mettler-Toledo, Giessen, Germany, three mixing steps, 100 μL mixing volume). Product formation was linear in the 80 s used to determine the initial product formation rate and proportional to the protein concentration used. No product formation was detected in absence of substrate or purified enzyme.

### Chemical Analyses
All standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). CA and pHCA concentrations in cell-free cultures were determined using high performance liquid chromatography (HPLC) 1260 Infinity system equipped with an Infinity Diode Array Detector module (Agilent, Santa Clara, CA, USA). For this, 250 μL culture broth were centrifuged in 96 well V-bottom plates (BRAND GMBH + CO KG, Wertheim, Germany) in a Heraeus Multifuge X3 centrifuge (Heraeus, Hanau, Germany, 30 min, 6000g and 8 °C) and 200 μL supernatant was transferred to a new 96 well V-bottom plate and directly applied to the HPLC (8 °C sample chamber temperature). LC separation of 2 μL samples was carried out with a Kinetex 1.7u C18 100-Å-pore-size column (Phenomenex, Torrance, CA, USA, 50 mm by 2.1 mm [internal diameter], 40 °C). For elution, 2% acetic acid (solvent A) and acetonitrile supplemented with 2% acetic acid (solvent B) were used as the mobile phases at a flow rate of 1 mL/min. A gradient was used, where the amount of solvent B was changed over the course of analysis (min 0 to 5, 15% to 90%; minute 5 to 5.5, 90% to 15%). CA and pHCA were detected by determining the absorbance at 295 and 310 nm, respectively, and concentrations were calculated using an appropriate standard curve.

### Bioinformatic Methods
Dose–response data of the pSenCA biosensor construct was fit using the [Agonist] vs response function (variable slope) of Prism 7.04 (GraphPad Software, San Diego, CA, USA) with a constraint of the minimal fold induction (μₘᵟᵣₐₓ) to 1.

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\mu(l) = \mu_{\text{min}} + \frac{I^h \times (\mu_{\text{max}} - \mu_{\text{min}})}{(I^h + EC50^h)}
\]

μᵢ, fold induction (also referred to as induction factor); Iᵢ, inducer concentration; hᵢ, Hill coefficient/Hill slope; EC50ᵢ, inducer concentration resulting in an induction of 50% μᵢₘᵟᵣᵢₓ.

For the DNA sequence analysis of isolated xal_f variants, all DNA sequences obtained from Eurofins MWG Operon were aligned with the original xal_f sequence for identifying single nucleotide polymorphisms using the Clonemanager Professional software, version 9.51 (Scientific & Educational Software, Denver, CO, USA).

### RESULTS

**Construction and Characterization of the trans-Cinnamic Acid Biosensor pSenCA.** In bacteria, plants and fungi, ammonia lyases catalyze the nonoxidative deamination of the aromatic amino acids l-Phe (then best referred to as phenylalanine ammonia lyases, PALs; EC 4.3.1.24) and l-Tyr (then best referred to as tyrosine ammonia lyases, TALs; EC 4.3.1.23) yielding the phenylpropanoids trans-cinnamic acid (CA) or p-coumaric acid (pHCA), respectively. This reaction represents the committed step in the biosynthesis of biotechnologically and pharmaceutically interesting polyphenols such as flavonoids, stilbenes, and lignans. In bacteria, plants and fungi, ammonia lyases catalyze the nonoxidative deamination of the aromatic amino acids l-Phe (then best referred to as phenylalanine ammonia lyases, PALs; EC 4.3.1.24) and l-Tyr (then best referred to as tyrosine ammonia lyases, TALs; EC 4.3.1.23) yielding the phenylpropanoids trans-cinnamic acid (CA) or p-coumaric acid (pHCA), respectively. This reaction represents the committed step in the biosynthesis of biotechnologically and pharmaceutically interesting polyphenols such as flavonoids, stilbenes, and lignans. In the context of engineering microorganisms for this purpose, generally low PAL- and TAL-activities were identified to be limiting the overall performance of the heterologous pathway in the respective microbial hosts.

Driven by the motivation to engineer a PAL/TAL-enzyme toward increased activity for future applications in microbial plant polyphenol production, a biosensor for trans-cinnamic acid (CA) was designed and constructed. E. coli can catabolize a broad range of aromatic compounds including phenylpropanic acid (PP) and phenylpropanoids such as CA, via a dioxygenolytic pathway, which is partly encoded by the hca gene cluster. Transcription of the hca cluster is induced by HcaR, which is a LysR type transcriptional regulator (LTTR),...
in the presence of PP or CA. Therefore, HcaR and its target promoter, P_{hcaE} were selected for the construction of the plasmid-based CA biosensor pSenCA. The biosensor pSenCA harbors the regulator gene hcaR, under control of its native promoter, P_{hcaR}, the target promoter of HcaR, P_{hcaE}, and the first 45 bp of hcaE (hcaE') transcriptionally fused to the eyfp-gene encoding for the enhanced yellow fluorescent protein (EYFP) (Figure 1A). This translational fusion was designed and constructed, because previous experiments with other regulator/promoter combinations showed that the interplay between the promoter and the 5'-end of the original open reading frame, which has been fine-tuned by evolution, enhance the overall biosensor response. Earlier work on the hca operon showed strongly reduced expression of hcaR in cultivations with glucose as carbon and energy source, as the expression of hcaR is subject to catabolite repression. Consequently, glycerol was used as sole carbon and energy source, resulting in a strong and homogeneous fluorescence response upon CA supplementation. To circumvent degradation of CA over the course of cultivation, the hca operon was subsequently deleted in E. coli DH10B, resulting in strain E. coli DH10B ΔhcaREFCBD. Cultures of E. coli DH10B ΔhcaREFCBD pSenCA (hereafter referred to as E. coli pSenCA), were supplemented with CA and also pHCA at different concentrations ranging from 1 μM to 1000 μM. The dose–response curve for CA was sigmoidal, with an operational range stretching from 3 μM CA to 300 μM CA thus spanning 2 orders of magnitude (Figure 1B). The inducer concentrations resulting in 5% (EC5) and 95% (EC95) of the maximal fold induction are 7.5 μM CA and 126 μM CA, respectively. The maximal fold induction determined in specific EYFP fluorescence was 120-fold. In contrast, presence of pHCA in the culture medium triggered a minor fluorescence response (2-fold) of pSenCA, showing that this biosensor is indeed CA-specific.

**Optimization of Heterologous Gene Expression Enables CA Production and Biosensor-Mediated Product Detection in E. coli.** Subsequently, a codon-optimized synthetic gene for the aromatic amino acid ammonia lyase XalTc, originating from Trichosporon cutaneum, was introduced into E. coli pSenCA. In a previous study, this enzyme stood out

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**Figure 1.** trans-Cinnamic acid biosensor pSenCA. (A) Schematic of the sensor principle. Upon binding of supplemented CA, the HcaR regulator undergoes conformational changes that enable binding to the target promoter P_{hcaE} and activation of hcaE' and eyfp expression. (B) Dose–response plot, CA (circles) or PHCA (filled circles) were supplemented extracellularly in eight different concentrations ranging from 1 to 1000 μM. The biosensor response after 24 h is shown as fold change in specific EYFP fluorescence in comparison to the background fluorescence (no inducer). Error bars represent standard deviations calculated from three biological replicates. CA, trans-cinnamic acid; pHCA, p-coumaric acid; ECS, inducer concentration that results in 5% of maximal fold induction; EC95, inducer concentration resulting in 95% of maximal fold induction.

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**Figure 2.** Influence of induction of heterologous xalTc expression with 13 μM L-Ara or 130 μM L-Ara on biosensor response and CA production in E. coli pSenCA pBAD-xalTc. After cultivation for 3 h in the presence of different L-Ara concentrations, either 3 mM L-Phe (circles and triangles) or 3 mM CA (squares) was added. Samples were taken at four time points depicted by dotted lines (A) Specific fluorescence is shown (EYFP fluorescence × biomass formation−1, arbitrary units). (B) CA concentration in E. coli culture supernatants. (C) FACS measurement of EYFP fluorescence of 62,000 representative single cells in the histogram representation. Abbreviations: CA, trans-cinnamic acid; L-Ara, L-arabinose; and L-Phe, L-phenylalanine.
Prevention of Biosensor Cross-Talk Is a Prerequisite

**Figure 3.** EYFP fluorescence of various mixed cultures of trans- and nonproducing E. coli strains expressing PSEnCA pBAD-. For any FACS applications using pSenCA, the cell population of interest has to be identified using FACS screens. The cell population fraction of interest is thereby marked by a genetically encoded fluorescent marker. The fluorescence signal of the entire cell population is determined using a microtiter plate reader. In contrast, single-cell FACS analysis is performed using a flow cytometer. The fluorescent signal of the individual cells is measured and subsequently used for further analysis.

**Figure 2A.** Wild type E. coli BL21(DE3) harboring pBAD xalTc plasmid. After induction with 13 μM L-Ara, a titer of 12,000-fold increase in xalTc activity over the background in a P. fluorescens xalTc extract could be observed.

**Figure 2B.** Wild type E. coli BL21(DE3) harboring pBAD xalTc plasmid. After induction with 13 μM L-Ara, a titer of 12,000-fold increase in xalTc activity over the background in a P. fluorescens xalTc extract could be observed.
production in these otherwise genetically homogeneous cultures. In combination with the biosensor cross-talk, the observed heterogeneity provides an explanation for the homogeneous looking population at an iOD$_{600}$ of 0.5 as the increasing number of CA-producers among the 100% CA$^+$ cells also produced more CA, which was then taken up by the nonproducing cells. Consequently, with a decreasing share of CA$^+$ cells at different CA$^+$/CA$^-$ ratios and decreasing iOD$_{600}$ tested, the number of CA-producing cells was more and more reduced, resulting in a decreased median fluorescence of the respective cultures, but also a more pronounced small population of highly fluorescent CA-producing cells (Figure 3). This indicates that a reduced inoculum reduces the biosensor cross-talk between CA$^+$ and CA$^-$ cells presumably enabling for the efficient biosensor-guided isolation of CA$^+$ cells by FACS. Subsequently performed FACS experiments in which only the top 5% fluorescent cells of the 20% CA$^+$/80% CA$^-$ and 50% CA$^+$/50% CA$^-$ cultures with an iOD$_{600}$ of 0.02 or 0.004 were isolated and characterized, confirmed this assumption as all isolated cells were CA$^+$ cells (Supplementary Figure S2B–E).

Directed Evolution of an Ammonia Lyase by Multistep FACS Screening. The previously established and optimized gene expression and cultivation conditions were subsequently used to screen a diverse XAL$_{Tr}$ library for isolating enzyme variants with an improved activity in E. coli. For this purpose, a randomly mutated xal$_{Tr}$ library of 2.3 × 10$^{6}$ variants was constructed by subcloning of xal$_{Tr}$ error-prone PCR products into the pBAD vector, and transformation into electrocompeent E. coli pSenCA cells.

A multistep FACS enrichment strategy was performed in which always the top 5% fluorescent cells were collected and recultivated for the next enrichment step (Figure 4). During this campaign, the average CA production of the recovered cells in the culture was determined by HPLC after every step to judge successful enrichment of CA producing cells. The first FACS enrichment steps were performed under strong xal$_{Tr}$-induction conditions with supplementation of 130 μM l-Ara to reliably enrich CA producing variants. In parallel, during each round of enrichment, the respective E. coli cultures were also analyzed by FACS under weak xal$_{Tr}$-induction conditions (13 μM l-Ara) for comparison and also without any induction of xal$_{Tr}$ expression (no l-Ara) or addition of the Xal$_{Tr}$ substrate l-Phe for identifying false positive variants bearing spontaneous mutations leading to constitutive eyfp expression. In the course of the enrichments under strong xal$_{Tr}$-induction conditions, the CA titer increased from 232 to 651 μM (1st FACS-enrichment step), from 651 to 706 μM (2nd FACS-enrichment step), and from 706 to 734 μM (3rd FACS-enrichment step), respectively, without a detectable increase in fluorescence in the false positive controls (Figure 4). A subsequent fourth enrichment step was performed with lower induction of heterologous gene expression (13 μM l-Ara) for identifying the best CA producers in the enrichments. The strategy was changed as the third step with high induction of gene expression resulted only in a small increase of the CA titer by 28 μM. Presumably this was the case because most remaining cells in the third enrichment were already CA producers, promoting strong fluorescence under high induction of heterologous gene expression (130 μM l-Ara). Surprisingly, this fourth enrichment step in the presence of 13 μM l-Ara resulted in the occurrence of a pronounced fluorescent population as a shoulder in the histogram of the control FACS experiment without l-Ara or l-Phe, indicating that false positive variants were enriched under these conditions. Hence, an additional control experiment was performed, in which a fifth positive FACS screening of the library in the presence of 13 μM l-Ara (positive sort under weak induction conditions) was conducted (Figure 4, dotted line). As expected, a decreasing average CA-titer (688 μM CA)

Figure 4. Stepwise enrichment of improved CA producers from a xal$_{Tr}$ library using biosensor-based FACS-screening. The continuous orange graph depicts the development of the CA titer of the xal$_{Tr}$ library at the culture level relative to the starting variant E. coli pSenCA pBAD-xal$_{Tr}$ (black line). The dashed orange graph depicts the CA titer development in a control experiment, in which another positive sorting (fluorescence) instead of the negative sorting (no fluorescence) was performed. The dark gray shading depicts the CA-titer range of one standard deviation from the Xal$_{Tr}$ starting variant, whereas light gray shading depicts the CA-titer range of three standard deviations. Histograms show the fluorescence distribution of the cultures of each cultivation step without induction of heterologous gene expression (no l-Ara) or substrate addition (no l-Phe) (histogram without pattern), with supplementation of 13 μM l-Ara and 3 mM l-Phe (vertical lines) and 130 μM l-Ara and 3 mM l-Phe added (horizontal lines). Conditions of each step leading to the eventually isolated xal$_{Tr}$ variants are highlighted in bright orange relative to the conditions only used as controls that are shown in dim orange.
of the enriched culture was determined as the false positives variants were further enriched. The solution was a final negative FACS-step (no L-Ara and L-Phe supplementation) and sorting of the 5% least fluorescing cells to exclude false positive variants. This increased the average CA-titer of the library to 850 μM upon recultivation.

During the FACS campaign, the stepwise enrichment resulted in a culture of E. coli variants, which accumulated up to 20% more CA in the supernatant in comparison to a culture of the starting variant E. coli pSenCA pBAD-xalTc that accumulates 713 μM CA. For comparison, prior to any FACS enrichment, the randomly mutated and unsorted XalTc-library accumulated 232 μM CA, which is 70% less CA compared to the starting variant.

The E. coli cells recovered after the fifth enrichment step were spread on LB agar plates and 182 single colonies were individually cultivated and characterized with regard to their respective CA production capabilities (Figure S5A). These experiments revealed that 8% of these variants accumulated less CA compared to the starting variant, whereas 16% could not be distinguished from this control. In contrast, 76% of the strains accumulated significantly (>10%) more CA, with the upper 20% accumulating 30% to 50% more CA compared to the starting variant E. coli pSenCA pBAD-xalTc.

Subsequently, the pBAD-xalTc plasmids of the 15 best variants and five randomly selected variants with significantly higher CA accumulation compared to the starting variant were isolated and retransformed into E. coli pSenCA to exclude the potential influence of undesired random genomic mutations (Figure S5A). The resulting strains were compared to E. coli pSenCA pBAD-xalTc regarding their CA and pHCA accumulation from supplemented 1-Phe and 1-Tyr, respectively (Figure S5 B). In parallel, the DNA sequence of all 20 xalTc variants was determined. All variants accumulated 10% to 60% more CA or pHCA in the supernatant when L-Phe or L-Tyr were supplemented, respectively. Interestingly, DNA sequencing revealed that 6 strains have mutations in the P_{araB}-promoter upstream of xalTc, whereas 14 variants exclusively carry mutations in the xalTc open reading frame (Supplementary table S3).

Nonetheless, the increased CA titer of these 14 strains could be also due to improved heterologous expression of xalTc, and thus higher XalTc abundance and does not necessarily have to relate to altered enzyme kinetics of the enzyme. With the aim, to preclude any undesired expression effects, an in vitro characterization of seven XalTc variants with purified proteins was performed. Seven muteins, including XalTc-F167L-K602N, XalTc-N464S/V523E, XalTc-F167L/N588H, XalTc-1552N, XalTc-V523E/K602T, XalTc-I552T/K602N, and XalTc-V274A were selected to determine enzyme kinetics in vitro. These variants accumulated the highest CA concentrations in the in vivo experiments and did not carry mutations in the araC gene or in the P_{araBAD} promoter (except for XalTc-I552N comprising P_{araBAD}^{a10g}). For the in vitro enzyme assays, all genes were

Figure 5. (A) trans-Cinnamic acid production of 183 FACS-isolated XalTc variants. Presented data are means of three cultivations and error bars depict standard deviations. The dark gray shading depicts the CA-titer range of one standard deviation from the XalTc starting variant, whereas light gray shading depicts the CA-titer range of three standard deviations. (WT cultivated as biological triplicates). Variants selected for a more detailed analysis are highlighted in orange. (B), CA (left) and pHCA (right) production of 19 strains selected during the initial characterization (Figure 5A), after retransformation of the respective pBADxalTcEc plasmid. All cultivations were performed in biological triplicates, error bars depict the standard deviation. Abbreviations: CA, trans-cinnamic acid; pHCA, p-coumaric acid.
Interestingly, mutein XalTc-V274A does have a reduced deamination of L-Phe in comparison to the wild-type enzyme. F167L/K602N and XalTc-F167L/N588H show the lowest metabolism. Lrp activates the expression of the brnF operon, encoding for the branched-chain amino acid exporter BrnFE, which is responsible for secretion of excess amino acids to avoid cytotoxic effects of elevated intracellular amino acid concentrations. HcaR, in contrast, activates the expression of the hca gene cluster involved in the catabolism of aromatic acids, which can serve as valuable carbon and energy sources in absence of other more preferred substrates. A strong expression at low inducer concentrations is therefore beneficial to compete with other microorganisms for such valuable resources.

However, despite a low operational range, a biosensor can be used in FACS screenings if screening conditions are selected, in which the expected product concentrations match with the biosensor characteristics. In the case of the directed XalTc evolution performed in this study, only the two lowest inducer concentrations for heterologous xalTc-expression enabled pSenCA-guided FACS screening. As alternative for optimizing cultivation conditions, the biosensor itself can be adapted. For example, alteration of the operational range of a transcriptional biosensor can be achieved by engineering the transcriptional regulator toward reduced affinity to the ligand, thereby enabling a graded fluorescence output for higher ligand concentrations. This approach was followed recently to optimize the operational range of a whole-cell biosensor for the detection and quantification of the macrodilide antibiotic pamamycin produced by *Streptomyces alboniger.* By rational engineering of the binding pocket of the PamR2 repressor, a mutein with reduced affinity for pamamycin could be constructed, extending the upper detection limit of the sensor system from 1 mg/L to more than 5 mg/L.

Additionally, given that the constructed biosensor does not provide sufficient dynamic range, often because of incompatibility between the host strain and heterologous regulatory elements, the target promoter of the regulator incorporated in the sensor or the promoter of the regulator gene can be mutated. For example, a 3,4-dihydroxy benzoate responsive biosensor based on the pcaU gene under control of its promoter P_pcad from *Acinetobacter sp ADP1* was applied in *E. coli.* By random mutagenesis, a library of 33 000 promoter mutants was constructed and subsequently screened using FACS. Applying positive and negative screening, three variants with improved dynamic range were isolated. Another example is the a biosensor comprised of PadR, a repressor specific for p-coumaric acid from *Bacillus subtilis,* and its cognate promoter in *E. coli.* A sensor variant with improved dynamic range was constructed by screening a set of P_padR mutants for variants with reduced expression of padR. In other cases where neither optimization of cultivation conditions nor adaptation of the biosensor is an option, identification of the most suitable time-point for the FACS screening during cultivation presents a possible solution to prevent saturation of the sensor system.

Cross-talk between producers and nonproducing cells as it could be observed in this study impedes any biosensor-based FACS screening if the metabolite in question can diffuse of biological membranes or is readily taken up by the microorganism. Dilution to a starting OD<sub>600</sub> of 0.004 and short cultivation times (<8 h) successfully suppressed cross-talk in our case, even with an excess of producing cells in the culture.
A possible alternative to dilution for preventing cross-talk is compartmentalization of individual strain variants in solution, e.g., by emulsion droplets, which can be sorted in microfluidic devices. By coencapsulation of an E. coli strain carrying a p-coumaric acid-responsive biosensor and p-coumaric acid producing Saccharomyces cerevisiae, yeast cells with elevated p-coumaric acid production capabilities could be isolated from mixtures of different producer strains. However, design and construction of droplet-based screening assays is most likely more laborious, and the sorting speed in microfluidic devices is usually limited to less than 500 cells per second when reasonable sort efficiencies are desired. Recently, successful sorting of double emulsion droplets in a FACS device could be demonstrated, increasing the throughput of droplet sorting to 1000 cells per second. When performing dilution assays in this study, a pronounced heterogeneity with respect to the fluorescent response could be detected, which was presumably caused by heterogeneous expression of the xalT gene. This heterogeneous expression in the presence of subsaturating L-Ara concentrations was described earlier and could be demonstrated, increasing the throughput of droplet sorting to reasonable sort efficiencies.

### CONCLUSIONS

In this study, a transcriptional biosensor for the phenylpropanoid trans-cinnamic acid could be successfully designed, constructed, and applied in a high-throughput FACS screening campaign. Key to success was a detailed characterization of the biosensor in combination with fine-tuning of cultivation and screening conditions to overcome hurdles such as biosensor cross-talk, which impede the successful application of more biosensors in the field of protein engineering or strain development. We believe that the strategies outlined in this article will help others to also develop elegant biosensor-based screening campaigns using the high-throughput capabilities of FACS.

### ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.9b00149.

Table S1: Strains and plasmids used in this study; Table S2: Oligonucleotides used in this study; Table S3: Overview of all xalT-variants isolated in the FACS campaign, which were characterized in detail; Figure S1: Performance of the E. coli pSenCA pBAD-xalT system; Figure S2: Influence of the inoculum size (OD600) on producer isolation efficiency; Figure S3: SDS-PAGE analysis of a typical XalT purification (PDF).

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**Author Contributions**

L.K.F. designed the experiments, L.K.F. and S.S. conducted the experiments. L.K.F. and J.M. wrote the manuscript.

**Notes**

The authors declare no competing financial interest.

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