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Screening of an *Escherichia coli* promoter library for a phenylalanine biosensor

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Abstract In recent years, the application of transcription factor-based biosensors for the engineering of microbial production strains opened up new opportunities for industrial biotechnology. However, the design of synthetic regulatory circuits depends on the selection of suitable transcription factor-promoter pairs to convert the concentration of effector molecules into a measureable output. Here, we present an efficient strategy to screen promoter libraries for appropriate parts for biosensor design. To this end, we pooled the strains of the Alon library containing about 2000 different *Escherichia coli* promoter-*gfpmut2* fusions, and enriched galactose- and L-phenylalanine-responsive promoters by toggled rounds of positive and negative selection using fluorescence-activated cell sorting (FACS). For both effectors, responsive promoters were isolated and verified by cultivation in microtiter plates. The promoter of *mtr*, encoding an L-tryptophan-specific transporter, was identified as suitable part for the construction of an L-phenylalanine biosensor. In the following, we performed a comparative analysis of different biosensor constructs based on the *mtr* promoter. The obtained data revealed a strong influence of the biosensor architecture on the performance characteristics. For proof-of-principle, the *mtr* sensor was applied in a FACS high-throughput screening of an *E. coli* MG1655 mutant library for the isolation of L-phenylalanine producers. These results emphasize the developed screening approach as a convenient strategy for the identification of effector-responsive promoters for the design of novel biosensors.

Keywords Biosensor · GFP library · Phenylalanine · Aromatic amino acids · Random mutagenesis · Population heterogeneity

Introduction

During the last years, genetically encoded biosensors have revealed their potential as valuable tools for metabolic strain engineering and for enabling new insights into bioprocesses at single-cell resolution (Delvigne et al. 2014; Liu et al. 2015a; Mahr and Frunzke 2016; Schallmey et al. 2014). By converting the intracellular effector molecule concentration into a measureable output, biosensors are in demand in cases, in which an easily detectable phenotype is not available. Microorganisms are equipped with a variety of metabolite-sensing mechanisms including transcriptional regulators, riboswitches, enzymes, or periplasmic-binding proteins, which can be exploited for the construction of biosensors. Transcription factor (TF)-based biosensors consist of a regulator that is able to sense the level of an intra- or extracellular effector molecule and in turn drives the expression of a reporter gene (e.g., encoding an auto-fluorescent protein, AFP). By this means, accumulation of a certain effector can be converted into a measurable output signal (Mahr and Frunzke 2016). Biosensors based on bacterial TFs have successfully been implemented in high-throughput (HT) screenings of mutant libraries using fluorescence-activated cell sorting (FACS) (Binder et al. 2012; Mustafi et al. 2012; Siedler et al. 2014) or in biosensor-driven adaptive evolution approaches for the improvement of microbial metabolite production (Mahr et al.

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2015). In this context, biosensor-based strategies were shown to enable the identification of novel and non-intuitive targets for engineering and improving of production strains. Furthermore, the efficient screening of feedback-resistant enzymes (Schendzielorz et al. 2014), dynamic pathway regulation (Dahl et al. 2013; Liu et al. 2015b; Xu et al. 2014; Zhang et al. 2012), and the analysis of population heterogeneity at the single-cell level (Hoffmann et al. 2013; Mustafi et al. 2014) demonstrate the broad applicability of biosensors (Liu et al. 2015a; Mahr and Frunzke 2016).

Nature has evolved a broad range of these sensor devices. However, only a few regulators and corresponding target genes have been well characterized to date. Frequently, the plethora of targeted promoters hampers the identification of suitable biosensor candidates. Thus, workflows enabling the rapid identification of responsive promoters are required for efficient sensor design. In this study, we developed a convenient strategy for screening promoter-AFP libraries using FACS. As proof-of-principle, we screened the Alon library consisting of about 2000 different promoter-*gfpmut2* fusions in *Escherichia coli* (Zaslaver et al. 2006) in order to identify biosensor candidates suitable for the detection of L-phenylalanine.

The aromatic amino acid L-phenylalanine is one of the most demanded amino acids besides L-glutamate, L-lysine, and L-methionine and faces an increasing commercial interest (Rodriguez et al. 2014; Sprenger 2007). As precursor for the artificial sweetener aspartame or as building block for pharmaceutical products including infusion fluids or HIV protease inhibitors, L-phenylalanine has played an important role for many years (Sprenger 2006; 2007). Furthermore, the commercially interesting aromatic compounds pinosylvin (van Summeren-Wesenhagen and Marienhagen 2015), cinnamic and *p*-hydroxycinnamic acid (Sariaslani 2007; Vargas-Tah et al. 2015), cinnamaldehyde, and phenylpyruvic acid (Bang et al. 2016; Hou et al. 2015) are synthesized from the precursor L-phenylalanine and serve as flavor enhancers, cosmetical, or pharmaceutical building blocks.

Besides chemical synthesis and hydrolytic cleavage of proteins, microbial fermentation has now become the dominating production process (Bongaerts et al. 2001; Leuchtenberger et al. 2005; Sprenger 2006, 2007). *E. coli* serves as an important platform organism producing L-phenylalanine—as most microorganisms—via the shikimate pathway (Bentley 1990; Sprenger 2007). Rational engineering approaches yielded strains producing final L-phenylalanine titers of up to 50 g/L from glucose (Backman et al. 1990; Rüffer et al. 2004) and 13.4 g/L based on glycerol as substrate (Weiner et al. 2014).

In this study, we established a high-throughput screening approach for the easy and rapid identification of effector-responsive promoters from a GFP library as suitable parts for biosensor design. L-Phenylalanine-responsive promoters were enriched by toggled rounds of selection and counter-selection from the Alon library. This approach revealed the promoter of *mtr*, encoding an L-tryptophan-specific transporter (Pittard et al. 2005), as an appropriate part for sensor design. Subsequently, biosensors based on the *mtr* promoter were characterized and successfully applied during FACS HT-screening of a mutant *E. coli* K-12 MG1655 library for strains with increased phenylalanine production.

Materials and methods

Bacterial strains, media, and growth conditions

In this study, the *E. coli* wild-type strains K-12 MG1655 and DH5α were used (Blattner et al. 1997; Hayashi et al. 2006). All bacterial strains used in this study are listed in Table 1. Unless indicated otherwise, *E. coli* cells were grown on lysogeny broth (LB) agar plates at 37 °C or pre-cultivated in LB medium for 8 h at 37 °C and 170 rpm (Sambrook et al. 2001). Subsequently, a second pre-culture was inoculated in a shake flask containing 20 mL M9 minimal or phenylalanine production medium with 0.5 % glucose, and cells were incubated overnight at 37 °C and 120 rpm. The following day, cells were washed with 0.9 % (*w*/*v*) NaCl solution and adjusted to an optical density (OD_{600}) of 0.5 in 50 mL fresh minimal medium. Further incubation was done at 37 °C and 120 rpm. As minimal medium, M9 medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mL/L trace element solution [3 μM ammonium molybdate, 400 μM H_3BO_3, 30 μM $CoCl_3$, 10 μM $CuSO_4$, 800 μM $MnCl_2$, and 10 μM $ZnSO_4$; filter sterilized]; adjusted to a pH of 7) or an adapted phenylalanine production medium (0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.015 g/L $CaCl_2 \cdot 2H_2O$, 3 g/L KH_2PO_4, 12 g/L K_2HPO_4, 0.1 g/L NaCl, 5 g/L $(NH_4)_2SO_4$, 0.075/1.0 g/L $FeSO_4 \cdot 7H_2O$/Na-citrate, 1.5 mL/L trace element solution [2 g/L $Al_2(SO_4)_3 \cdot 18H_2O$, 0.75 g/L $CoSO_4 \cdot 7H_2O$, 2.5 g/L $CuSO_4 \cdot 5H_2O$, 0.5 g/L H_3BO_3, 24 g/L $MnSO_4 \cdot H_2O$, 3 g/L $Na_2MoO_4 \cdot 2H_2O$, 2.5 g/L $NiSO_4 \cdot 6H_2O$, 15 g/L $ZnSO_4 \cdot 7 H_2O$; dissolved in ddH_2O at pH 1–2], 0.0075 g/L thiamine/HCl; ingredients were dissolved in ddH_2O [pH ~7.2] and filter sterilized) were used (Gerigk et al. 2002; Miller 1972). When necessary, 50 μg/mL kanamycin was added to the medium. All dipeptide stocks were dissolved in the respective minimal medium and stored at −20 °C.

Recombinant DNA work

Cloning techniques including PCR, DNA restriction, and ligation were performed according to standard protocols (Sambrook et al. 2001). Synthesis of oligonucleotides and sequencing of plasmids were performed in subcontracting with Eurofins MWG Operon (Ebersfeld, Germany). All

Table 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Strains or plasmids	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*DM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
E. coli K-12 MG1655	F^- lambda$^-$ *ilvG*$^-$ *rfb*-50 *rph*-1	(Blattner et al. 1997; Hayashi et al. 2006)
Plasmids		
pEKEx2	Kan^R, Amp^R; $oriV_{C.g.}$, $oriV_{E.c.}$, P_{tac}, $lacI^q$, pBL1, pUC18 (*E. coli-C. glutamicum* shuttle vector)	(Eikmanns et al. 1994)
pJC1	Kan^R, Amp^R; $oriV_{C.g.}$, $oriV_{E.c.}$ (*E. coli-C. glutamicum* shuttle vector)	(Cremer et al. 1990)
pJC1-venus-term-BS	pJC1 derivative containing a terminator sequence of *Bacillus subtilis* behind *venus*	Heyer, unpublished
pJC1-*mtr* sensor-type1	pJC1-P_{mtr}-*venus*	This study
pJC1-*mtr* sensor-type2	pJC1-*lacI*-P_{tac}-*tyrR*-P_{mtr}-*venus*	This study
pJC1-*mtr* sensor-type3	pJC1-P_{tac}-*tyrR*-P_{mtr}-*venus*	This study
pJC1-*mtr* sensor-type4	pJC1-P_{tyrR}-*tyrR*-P_{mtr}-*venus*	This study
Oligonucleotides	Sequence (5′–3′)[a]	
*Bam*HI_lacI_fw	CGATCAGCGACGCCGCAGGGGGATCCGCGTTGCG CTCACTGCCC (*Bam*HI)	
*Eco*RV_lacI_rv	GATATCGTCTGAATCTGGTGTATATGGCGAG (*Eco*RV)	
TyrR_inv_fw	CATATACACCAGATTCAGACGATATCCATATTCGCG CTTACTCTTCGTTC	
OL_TyrR_inv_rv	CATCGGCTCGTATAATGTGTGGAGTTCCCATGCGT CTGGAAGTC	
Ptac_fw	GCGAAAGGTTTTGCACCATTCGATGG	
Ptac_rv	TCCACACATTATACGAGCCGATGATTAATTGTC	
OL_200bp_Pmtr_fw	CGAATGGTGCAAAACCTTTCGCGCAGTTACTGGG CGATGCAC	
Pmtr_rv	ATATCTCCTTCTTAAAGTCTATGCATTGCACTGTAC CAGTACAC	
STOP_RBS_venus_fw	TAGACTTTAAGAAGGAGATATATGGTGAGCAAGG GCGAG	
Venus_OL_rv	AAAACGACGGCCAGTACTAGTTACTTGTACAGCTC GTCCATGC (*Spe*I)	
tyrR_OL(*Bam*HI,pJC1)_fw	AGGGCGATCAGCGACGCCGCAGGGGGATCCTTAC TCTTCGTTCTTCTTCTGACTCAGAC (*Bam*HI)	
Pmtr_OL(*Bam*HI, pJC1)_fw	AGGGCGATCAGCGACGCCGCAGGGGGATCCGCAG TTACTGGGCGATGCACAG (*Bam*HI)	
PtyrR_OL(Pmtr)_rv	GCGGCTGTGCATCGCCCAGTAACTGCGGGGATTTC CGTCGTCAGCTTATC	
GFP_int_rv	CAAGAATTGGGACAACTCCAGTG	

[a] Underlined sequences highlight introduced recognition sites for restriction endonucleases (restriction endonucleases indicated in parentheses)

plasmids and oligonucleotides are listed in Table 1. Plasmids were isolated from *E. coli* using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, USA).

For the construction of the pJC1-*mtr* sensor-type2, the *lacI* fragment was amplified from the pEKEx2 vector using primers *Bam*HI_lacI_fw and *Eco*RV_lacI_rv, the *tyrR* fragment was amplified with primers TyrR_inv_fw and OL_TyrR_inv_rv from genomic DNA of *E. coli* K-12 MG1655, the P_{tac} promoter was amplified from the pEKEx2 vector with primers Ptac_fw and Ptac_rv, 331 bp upstream of the transcriptional start site of the P_{mtr} promoter was amplified from genomic DNA of *E. coli* K-12 MG1655 using primers OL_200bp_Pmtr_fw and Pmtr_rv, and *venus* was amplified from pJC1-venus-term-BS with primers STOP_RBS_venus_fw and Venus_OL_rv. All fragments were pooled to an equimolar concentration and cloned into the *Bam*HI and *Spe*I digested vector pJC1-venus-term-BS by Gibson assembly (Gibson et al. 2009). For the construction of pJC1-*mtr* sensor-type3, the biosensor construct was amplified without

lacI from the pJC1-*mtr* sensor-type2 vector using primers tyrR_OL(*Bam*HI,pJC1)_fw and Venus_OL_rv. For the assembly of pJC1-*mtr* sensor-type4, the fragment P_{tyrR}-*tyrR* was amplified from genomic DNA of *E. coli* using primers tyrR_OL(*Bam*HI,pJC1)_fw and PtyrR_OL(Pmtr)_rv, and P_{mtr} and *venus* were amplified from pJC1-sensor-type2 using primers Pmtr_fw and Venus_OL_rv. The plasmid pJC1-*mtr* sensor-type1 was constructed by amplifying P_{mtr} and *venus* from the pJC1-*mtr* sensor-type2 vector using primers Pmtr_OL(*Bam*HI,pJC1)_fw and Venus_OL_rv. The respective fragments were cloned into the *Bam*HI and *Spe*I digested pJC1-venus-term-BS vector via Gibson assembly (Gibson et al. 2009).

For cloning purposes, *E. coli* DH5α was transformed using the RbCl method (Hanahan 1983). All other *E. coli* strains were transformed using the transformation and storage solution (TSS) procedure (Chung et al. 1989).

Flow cytometry

Flow cytometric (FC) analyses and cell sorting were performed using a FACSAria II flow cytometer (Becton Dickinson, San Jose, USA) equipped with a blue solid-state laser (488 nm excitation). Forward-scatter characteristics (FSC) and side-scatter characteristics (SSC) were detected as small-angle and orthogonal scatters of the 488-nm laser, respectively. GFPmut2 and eYFP fluorescence were detected using a 502-nm long-pass and a 530/30-nm band-pass filter set. FACS-Diva software 6.0 was used to record the measurements. During analyses, thresholding on FSC was applied to remove background noise. As precision mode, four-way purity was used for cell sorting with a threshold rate of up to 10,000 events/s. If not stated differently, cells were sorted on agar plates or on MultiScreen HTS 96-well filter plates (Millipore, Billerica, USA) to separate cells from the FACSFlow™ buffer (Becton Dickinson, San Jose, USA) as already described in Mahr et al. (2015). The filter containing the sorted cells was excised with single-use scalpels (Braun, Melsungen, Germany) and inoculated in fresh medium. For FC analyses, *E. coli* culture samples were diluted to an OD_{600} of 0.05 in FACSFlow™ sheath fluid buffer (BD, Heidelberg, Germany). The analysis software FlowJo v.10.0.8 was used to visualize and evaluate the data (Tree Star, Ashland, USA).

Microplate cultivation

Online monitoring of growth and fluorescence was performed in 48-well microtiter FlowerPlates (MFPs) using the BioLector cultivation system (m2p-labs GmbH, Baesweiler, Germany) (Kensy et al. 2009). If not stated differently, 48-well MPFs were inoculated with *E. coli* cells from a preculture to an OD_{600} of 0.5 in a total volume of 750 μL. In the BioLector instrument, cells were incubated at 37 °C and with agitation of 1200 rpm. While biomass production was recorded as the backscattered light intensity (light wavelength 620 nm; signal gain factor of 20), eYFP fluorescence was measured at an excitation of 510 nm and an emission of 532 nm (signal gain factor of 60). The specific fluorescence for the cells is defined as the eYFP fluorescence per backscattered light intensity (given in arbitrary units, a.u.).

Screening of the Alon library for phenylalanine-responsive promoters

Initially, all strains of the Alon promoter library consisting of about 2000 different promoter-*gfpmut2* fusions in *E. coli* K-12 MG1655 were pooled (Fig. 1a) (Zaslaver et al. 2006). To this end, the single clones stored in 96-well plates (Nunc™ MicroWell™ Plates; Thermo Fisher Scientific, Waltham, MA, USA) were transferred to agar plates (Nunc™ OmniTray™ Single-Well Plates; Thermo Fisher Scientific, Waltham, MA, USA) containing LB with 50 μg/mL kanamycin using a 96-well plate replicator stamp (LabArt, Waldbüttelbrunn, Germany) and incubated at 37 °C for 18 h. The colonies were washed from the agar plates using 10 mL LB and kanamycin and a Drigalski spatula, and collected in a 1-L shake flask. Then, cells were incubated for 1 h at 37 °C and 120 rpm, washed in 1× PBS, and 2 mL of the mixed library was transferred to 100 mL M9 minimal medium containing 0.5 % (*w*/*v*) glucose and kanamycin. The culture was incubated overnight at 37 °C and 120 rpm. The following day, aliquots were prepared of a glycerol cryo-stock containing a final glycerol concentration of 25 % (*v*/*v*) and stored at −80 °C.

For screening, 500 μL of the glycerol stock containing the pooled Alon library was used to inoculate 20 mL M9 minimal medium containing 0.5 % (*w*/*v*) glucose and kanamycin, and incubated at 37 °C and 120 rpm (Fig. 1a). While the desired effector was added to one culture for induction, a second culture without effector was prepared as negative control. Prior to sorting, the cultures were incubated for 3 h. To screen for induced ("ON") promoters, 0.5–1 × 10^6 of the top 35 % cells were sorted on filter plates and the excised filter was incubated in 4 mL LB with kanamycin overnight (Fig. 1a). The following day, cells of 1 mL pre-culture were pelleted, washed with 1× PBS, and used to inoculate 20 mL M9 minimal medium containing 0.5 % (*w*/*v*) glucose and kanamycin. To screen for effector-responsive promoters, which do not show a fluorescent signal under non-induced conditions ("OFF" promoters), the cells were incubated without effector for 4 h (Fig. 1a). Subsequently, 0.5–1 × 10^6 cells from the bottom 35 % gate, which was pre-defined during the first sorting step, were sorted on filter plates and incubated in 4 mL LB with kanamycin. After two toggled rounds of selection, cells were spotted as single clones on agar plates and were re-cultivated in the BioLector system for verification of effector responsiveness

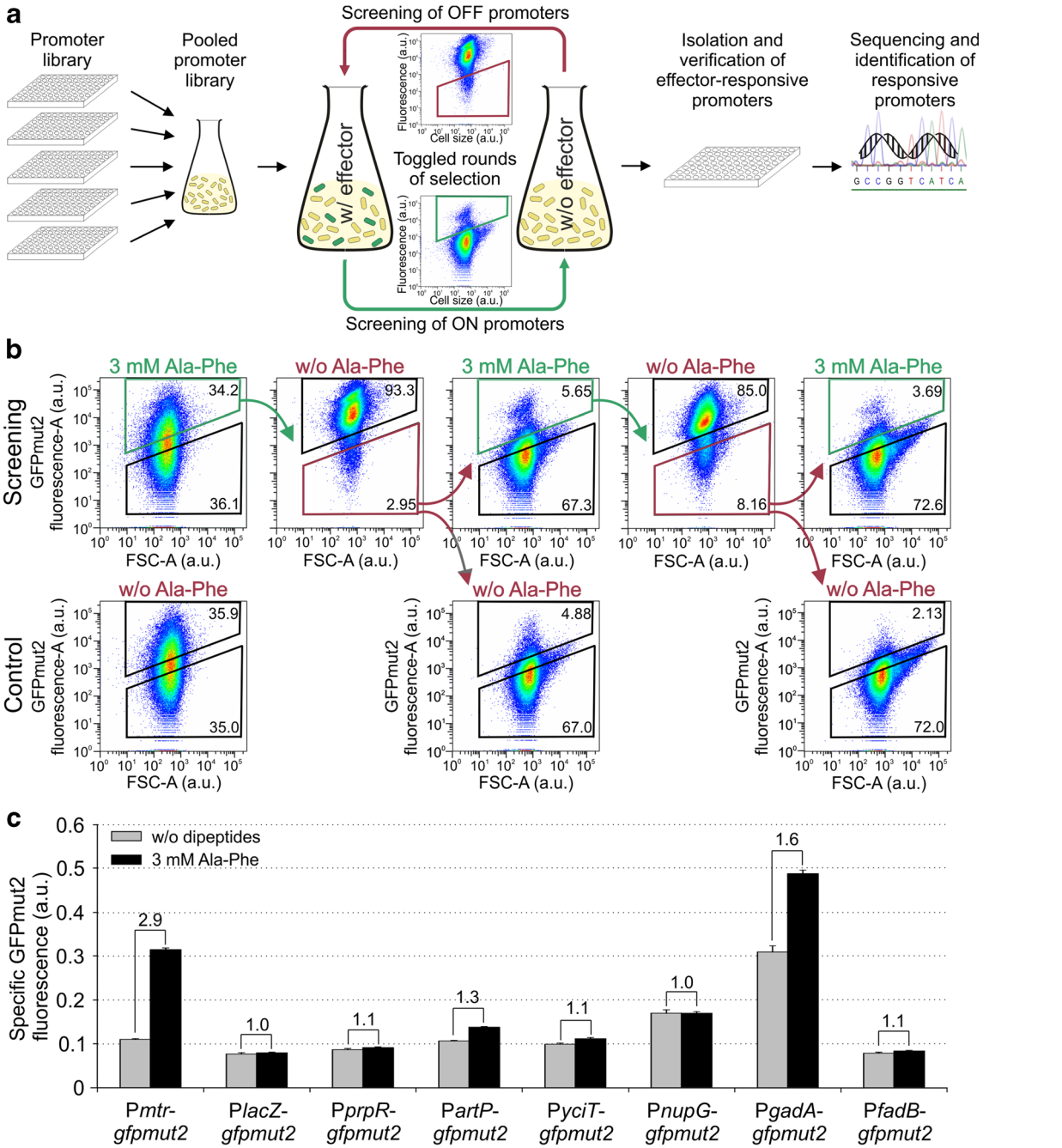

Fig. 1 Screening of L-phenylalanine-responsive promoters. **a** Schematic overview of the screening process. Initially, the Alon library consisting of about 2000 promoter-*gfpmut2* fusions of *E. coli* K-12 MG1655 was pooled and incubated in presence of an effector molecule of interest to induce responsive promoters. In the first step, the top 35 % fluorescent cells were sorted to isolate induced ("ON") promoters (green gate). In the second step, the cells were incubated without effector molecules to separate constitutive "ON" promoters from responsive promoters ("OFF" promoters) by sorting the bottom 35 % fluorescent cells (red gate). After two rounds of toggled selection, effector-responsive promoters were isolated via FACS and cultivated in microtiter plates to verify the screening outcome. Plasmid DNA of positive clones was prepared and sequenced to identify isolated promoters. **b** Screening the Alon library for L-phenylalanine-responsive promoters. For induction, 3 mM of the dipeptide L-alanyl-L-phenylalanine (Ala-Phe) was added to the culture. The rounds of toggled selection are highlighted by the alternation of green ("ON" promoters) and red gates ("OFF" promoters), from which cells were sorted (*green* and *red arrows*). The initially defined 35 % "ON" and "OFF" gates were maintained throughout the screening process for sorting. The *numbers* indicate the percentage of the entire population covered by the respective gate. **c** Specific GFPmut2 fluorescence of selected clones from the Alon library containing promoter-*gfpmut2* fusions that were enriched during the screening for L-phenylalanine-responsive promoters. Cells were cultivated in the presence (*black bar*) or without (*gray bar*) 3 mM L-alanyl-L-phenylalanine (Ala-Phe). *Numbers* above *bars* indicate the minimal dynamic range (fold-change induced versus non-induced). Data represent average values of three independent biological replicates

and determination of the dynamic range (Fig. 1a). From positive clones, plasmid DNA was prepared and sequenced using primer GFP_int_rv to identify isolated promoters.

Fluorescence microscopy

Fluorescence microscopy was performed on a Zeiss Axioplan 2 imaging microscope equipped with an AxioCam MRm camera and a Plan-Apochromat ×100, 1.40 Oil DIC oil-immersion objective. The AxioVision 4.8 software was used to acquire and analyze the images (Zeiss, Göttingen, Germany). Samples were spread on a microscope slide coated with a thin layer of 400 μL 1 % agarose and covered by a cover glass.

Random mutagenesis and FACS screening

Random mutagenesis using the mutagen *N*′-methyl-*N*′-nitro-N-nitrosoguanidine (MNNG) was adapted from the protocol of Harper et al. (2011). Prior to mutagenesis, a pre-culture of *E. coli*/pJC1-*mtr* sensor-type1 cells in LB medium was diluted 1:50 in 20 mL fresh LB medium containing kanamycin. At an OD_{600} of 2, 5 mL of this culture was harvested and washed twice in 2.5 mL 0.1 M citrate buffer (pH 5.5) (2-fold concentration of the cells). Subsequently, the cells were incubated with 50 μg/mL MNNG (dissolved in DMSO) for 30 min at 37 °C and 60 rpm. As control, a second culture was incubated containing the same amount of DMSO. Then, the cells were washed twice with 5 mL 0.9 % (*w*/*v*) saline and cultivated in falcons for 2 h in LB medium containing 4 % glucose at 37 °C and 170 rpm. Mutagenized cells were stored as cryo-stocks in LB medium containing 40 % glycerol (*w*/*v*) at −30 °C for up to one week.

For screening, two LB pre-cultures were inoculated with 1 mL of the cryo-stock containing mutagenized and non-mutagenized cells, respectively. The following day, the pre-cultures were used to inoculate 20 mL phenylalanine production medium containing 0.5 % glucose and kanamycin to an OD_{600} of 0.5. Cells were incubated for 6 h at 37 °C and 120 rpm. Subsequently, 2.5×10^5 cells with a significantly increased fluorescent output compared to the control were sorted on filter plates as already described above ("Flow cytometry" section). The filter with the cells was inoculated in LB medium with kanamycin overnight, and two further enrichment steps sorting 1×10^6 high fluorescent cells using FACS were performed to avoid false positive isolates. Finally, enriched cells were spotted as single cells on agar plates (Nunc™ OmniTray™ Single-Well Plates; Thermo Fisher Scientific, Waltham, MA, USA) containing LB agar with kanamycin. Single cells were picked using a 96-well plate replicator stamp (LabArt, Waldbüttelbrunn, Germany) inoculated in 96-well plates (Nunc™ MicroWell™ Plates; Thermo Fisher Scientific, Waltham, MA, USA) containing LB medium and antibiotic and incubated in the Microtron Pro (Infors HT, Bottmingen, Switzerland) for 8 h. For storage, glycerol at a final concentration of 20 % was added and plates were frozen at −80 °C.

Verification of isolated mutants after FACS HT-screening

Isolated mutants were first inoculated in 48-well MPFs containing 725 μL LB medium and kanamycin plus 25 μL of the thawed glycerol stock, and incubated at 37 °C overnight reaching the stationary phase. Subsequently, 25 μL of this pre-culture were used to inoculate 48-well MFPs containing 725 μL phenylalanine production medium. Incubation and online monitoring of backscatter and fluorescence were performed in the BioLector system. After 28 h of incubation, the supernatant was analyzed for amino acid production using uHPLC.

Quantification of amino acid production

Using ultra-high performance liquid chromatography (uHPLC), amino acids were quantified as *ortho*-phthaldialdehyde derivatives by automatic pre-column derivatization and separation by reverse-phase chromatography on an Agilent 1290 Infinity LC ChemStation (Agilent, Santa Clara, USA) equipped with a fluorescence detector. As eluent for the Zorbax Eclipse AAA 3.5 μm 4.6 × 7.5 mm column (Agilent, Santa Clara, USA), a gradient of Na-borate buffer (10 mM Na_2HPO_4; 10 mM $Na_2B_4O_7$, pH 8.2; adapted to operator's guide) was applied. To determine the concentration of amino acids in the supernatant, culture samples were centrifuged for 10 min at 13,000 rpm and 4 °C and diluted according to the expected concentration.

Results

Proof-of-principle: screening for galactose-responsive promoters

For the straightforward development of novel biosensors with desired sensor properties, HT approaches are required enabling the efficient and rapid identification of suitable parts. In this study, we used an *E. coli* GFP reporter library as a source for the identification of effector-responsive promoters. Therefore, we pooled all *E. coli* K-12 MG1655 strains of the Alon library containing about 2000 different promoter-*gfpmut2* fusions on the plasmids pUA66 or pUA139 (Fig. 1a) (Zaslaver et al. 2006). Toggled rounds of selection and counter-selection were conducted to enrich promoters responsive to the desired effector metabolite (for a detailed description of this workflow, the reader is referred to the "Materials and methods" section). Initially, cells were incubated in the presence of an effector molecule to activate

gfpmut2 expression from inducible promoters (Fig. 1a). Subsequently, 0.5–1 × 10^6 cells of the top 35 % fluorescent cells were isolated using FACS. To get rid of constitutively activated promoters, isolated cells were cultivated without the effector molecule followed by sorting of 0.5–1 × 10^6 cells from a pre-defined bottom 35 % gate. The toggled selection procedure was repeated twice to reduce the amount of false positive clones and to enrich desired promoter-AFP fusions. Finally, selected clones were isolated and verified by monitoring growth and fluorescence using a microplate cultivation system. Plasmid DNA from cells containing metabolite-responsive promoter-*gfpmut2* fusions was prepared and sequenced to identify isolated promoter fusions (Fig. 1a).

For proof-of-principle, we screened for the well-characterized promoters activated by the effector galactose (Fig. S1a). In a first step, the Alon library cultivated without effector molecules was split by FACS in fractions containing cells with high and low fluorescence. To induce responsive promoters of the pooled Alon library, we cultivated the low fluorescent cells in 20 mL M9 minimal containing 0.5 % (*w*/*v*) galactose and kanamycin. For counter-selection and as reference culture (for FACS gating), cells were cultivated in M9 medium with 0.5 % (*w*/*v*) glucose. Prior to sorting, the cells were grown for 6 h at 37 °C and 120 rpm. We enriched cells with a high fluorescent output in the presence of galactose from 3 to 78 % within five steps of toggled selection (the gating strategy is depicted in Fig. S1a). Within two counter-selection steps, constitutively activated or false positive cells were reduced from 25 to 4 %. While 78 % of the cells were induced in presence of galactose, only 9 % of the cells showed an increased fluorescent signal in presence of glucose after five selection steps (Fig. S1a). The plasmid DNA of nine clones showing the strongest activation in the presence of galactose was sequenced: eight clones harbored the P_{galS}-*gfpmut2* fusion and one clone contained the P_{lacZ}-*gfpmut2* fusion. Cultivation in microtiter plates confirmed that both promoter-*gfpmut2* fusions are responsive to galactose by showing an about 2-fold increase (dynamic range) of the reporter output in the presence of 0.5 % galactose (Fig. S1b). Both isolated promoters regulate the expression of well-known genes. While *galS* encodes the galactose isorepressor, which represses the transcription of genes involved in the transport and catabolism of galactose in presence of high galactose concentrations and under glucose limitation (Semsey et al. 2007; Weickert and Adhya 1992), *lacZ* encodes the β-galactosidase representing the first structural gene of the *lac* operon, which was reported to be weakly induced by galactose as well (Williams and Paigen 1968).

Screening for L-phenylalanine-responsive promoters

In the next step, we screened for L-phenylalanine-responsive promoters. Therefore, the pooled Alon library was cultivated in 20 mL M9 minimal medium containing 0.5 % (*w*/*v*) glucose and kanamycin (Fig. 1b). After 1 h of incubation, the dipeptide L-alanyl-L-phenylalanine (Ala-Phe, final concentration 3 mM) was added as effector molecule. After 3 h of induction, activated cells falling within an upper 35 % gate were sorted using FACS (Fig. 1b). For counter-selection, the screening medium was prepared without Ala-Phe. We used dipeptides for screening, as previous characterizations of biosensors featured an improved uptake of short peptides—the intracellular hydrolysis of which leads to an increase of the respective effector amino acids in the cytoplasm (Mustafi et al. 2012; Payne 1977; Simmonds and Griffith 1961). Within five steps of toggled selection, no significant enrichment of clones falling within the respective gate was observed. However, a distinct subpopulation of cells displaying an increased reporter output became visible in the second and third round of induction. This population was not detected in the non-induced control sample. After five toggled selection steps, the fraction of cells in the induced state was about 2-fold higher in comparison to the control sample (3.7 and 2.1 %, respectively, Fig. 1b). Plasmid DNA of 23 isolated strains (from gate P1) was sequenced and the following promoter fusions were identified: *mtr* (12 clones; L-tryptophan/indole:H^+ symporter), *lacZ* (three clones; β-galactosidase), *prpR* (one clone; 2-methylcitrate DNA-binding transcriptional regulator), *artP* (one clone; L-arginine ABC transporter-ATP binding subunit), *yciT* (one clone; DNA-binding transcriptional regulator), *nupG* (one clone; nucleoside:H^+ symporter), *gadA* (one clone; glutamate decarboxylase A), *fadB* (one clone; fatty acid oxidation complex, α component), *yihU* (one clone; 3-sulfolactaldehyde reductase), and *dinJ* (one clone; antitoxin of YafQ-DinJ toxin-antitoxin system and DNA-binding transcriptional repressor). Verification of the isolated strains via microplate cultivation revealed a significant response of the *mtr* promoter to the addition of the effector containing dipeptide Ala-Phe (minimal dynamic range 2.9). The promoters *gadA* and *artP* were also activated in the presence of L-phenylalanine probably due to pleiotropic effects and featured a low dynamic range (1.6 and 1.3, respectively, Fig. 1c). For this reason, these promoters were not included in the further evaluation. The other isolated promoter fusions did not show an increase of reporter output in the presence of Ala-Phe.

Characterization of the *mtr* promoter fusion

The expression of the *mtr* gene, encoding an L-tryptophan-specific transporter, is regulated by the two transcriptional regulators TrpR (tryptophan repressor) and TyrR (tyrosine regulator) (Fig. 2a) (Heatwole and Somerville 1991; Pittard et al. 2005; Whipp and Pittard 1977). As reported in previous studies, the expression of *mtr* is induced in the presence of phenylalanine and tyrosine, and fully repressed in presence of tryptophan (Whipp and Pittard 1977). In order to verify the

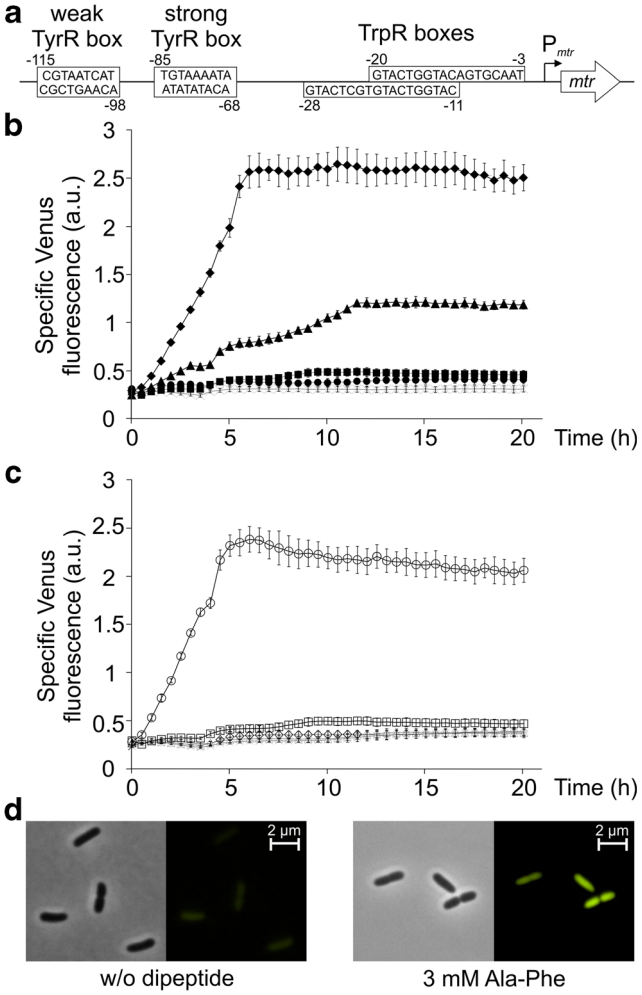

Fig. 2 Isolation of the *mtr* promoter as suitable part for the design of an L-phenylalanine biosensor. **a** The *mtr* promoter consists of a weak TyrR box (centered at −107 bp relative to the transcriptional start site) and a strong TyrR box (centered at −77 bp) as well as two TrpR binding sites located between −3 and −20 bp, and between −11 and −28 bp downstream of the transcriptional start site (*arrow*) (http://ecocyc.org) (Keseler et al. 2013; Pittard 1996; Pittard et al. 2005). Phenylalanine-dependent gene expression is promoted by binding of TyrR to the strong TyrR box and to the C-terminal domain of the α-subunit of RNA polymerase, while for tyrosine-mediated expression, additional binding of TyrR to the weak TyrR box is required. The transcription is completely blocked by binding of the TrpR repressor in presence of L-tryptophan. **b** Specific Venus fluorescence of *E. coli* K-12 MG1655/pJC1-*mtr* sensor-type1 cultivated without dipeptides (*filled squares*) and in the presence of 3 mM Ala-Ala (*filled circles*), Ala-Phe (*filled diamonds*), Ala-Trp (×), and Ala-Tyr (*filled triangles*). **c** Specific Venus fluorescence of the same strain cultivated without dipeptides (*open squares*) and in the presence of equimolar mixtures (1.5 mM) of Ala-Phe and Ala-Tyr (*open circles*), Ala-Phe and Ala-Trp (*open diamonds*), Ala-Trp and Ala-Tyr (*open triangles*), and Ala-Phe, Ala-Tyr, and Ala-Trp (×). Cultivation was performed in microtiter plates at 37 °C and 1200 rpm in M9 minimal medium containing 0.5 % (*w/v*) glucose and kanamycin. The data represent average values from three independent biological replicates. **d** Phase contrast and fluorescence microscopy images of *E. coli* K-12 MG1655/pJC1-*mtr* sensor-type1 without (*left panels*) and in the presence of 3 mM Ala-Phe (*right panels*)

mtr promoter as suitable part for biosensor design, the respective promoter region (including 331 bp upstream of the transcriptional start site) was transcriptionally fused to the gene encoding the yellow fluorescent protein Venus by adding AAGAAG as ribosomal binding site (RBS) seven base pairs upstream of the start codon. The fragment covering the biosensor circuit was cloned into the vector pJC1 resulting in pJC1-*mtr* sensor-type1 (Fig. 3a). In the presence of the dipeptides Ala-Tyr and Ala-Phe, the biosensor featured minimal dynamic ranges of 3.7 and 7.8, respectively (Fig. 2b). However, under all tested conditions, the sensor strain exhibited a low basal fluorescent signal. This result was confirmed by fluorescence microscopy showing low Venus fluorescence in the absence of dipeptides and a strong signal in the presence of 3 mM Ala-Phe (Fig. 2d). While the combination of Ala-Tyr and Ala-Phe led to an about 8-fold increase of the sensor output, induction was counteracted by the addition of Ala-Trp to the dipeptide mixture (Fig. 2c). This is in line with previous findings that repression by TrpR in the presence of L-tryptophan dominates the regulation of *mtr* expression.

Quantitative characterization of different biosensor designs based on the promoter of *mtr*

In the following, we aimed to analyze the impact of variation in the biosensor design on functionality. Therefore, four different biosensors designs based on the *mtr* promoter were constructed and characterized (Fig. 3a, b). All biosensors consist of the transcriptional fusion of the *mtr* promoter and *venus* as described above (Fig. 3a). Additionally, biosensor types two to four include *tyrR* under the control of P_{tac} (types two and three) or the native promoter (type four), respectively. To control expression from P_{tac}, we included the *lacI* gene in sensor construct type two (Fig. 3a).

The application of biosensors for FACS HT-screenings requires a detailed quantitative characterization of the biosensor response in terms of specificity, sensitivity, and dynamic range using FACS. A competitive dipeptide feeding strategy was used to describe the relationship between effector input concentrations and the biosensor output (Mustafi et al. 2012). More precisely, different ratios of effector containing dipeptides (Ala-Phe) and non-effector dipeptides (Ala-Ala) competitive for dipeptide uptake systems were added to adjust the intracellular concentration of effector molecules (Payne 1977; Vrljic et al. 1996).

Biosensor types two and four, including the *tyrR* gene, featured minimal dynamic ranges of 4.3 and 4.5, respectively. Both constructs displayed considerable high basal specific fluorescent outputs without effector molecules or in the presence of 3 mM Ala-Ala (Fig. 3b). Biosensor types one and three displayed minimal dynamic ranges of 3.2 and 4.1 showing also a lower level of basal specific fluorescence. Biosensors expressing *tyrR* featured a saturated signal at

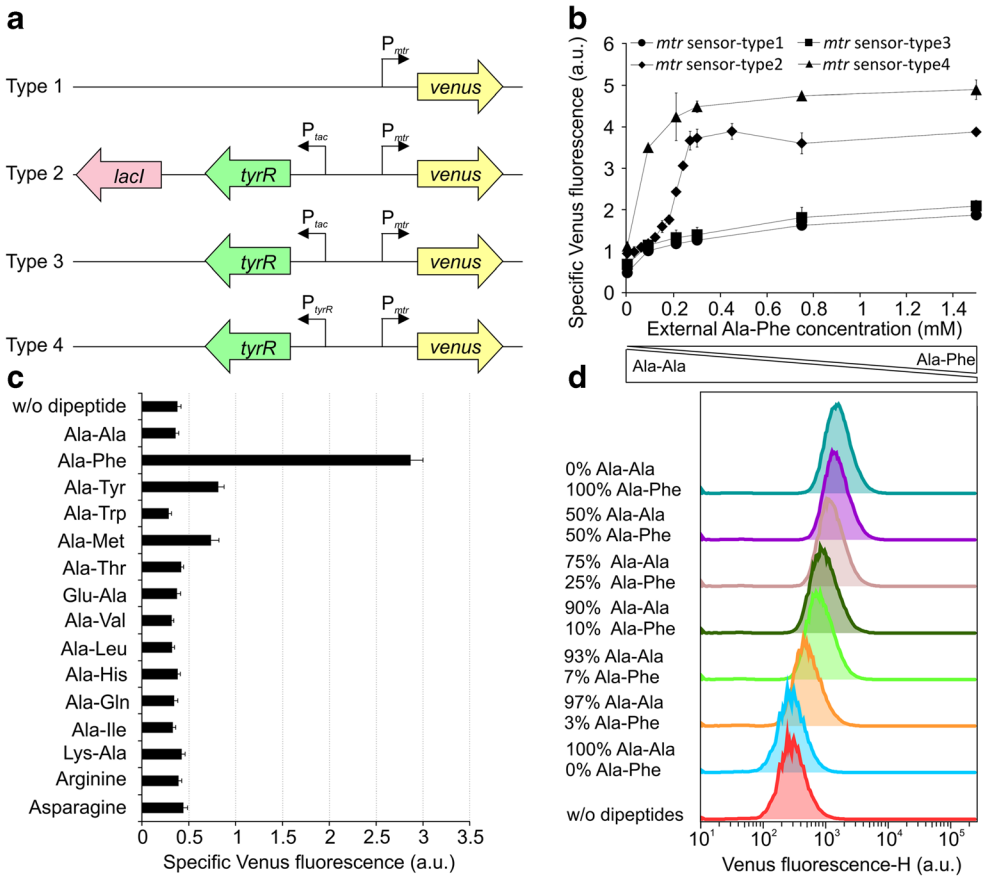

Fig. 3 Comparative analysis of different biosensor designs. **a** For all *mtr* biosensor constructs, *venus* was cloned under the control of the *mtr* promoter. Sensor types two to four encode additionally *tyrR* under the control of P_{tac} (types two and three) or the native P_{tyrR} promoter (type four). For the regulation of P_{tac}, *lacI* was included in biosensor type two. **b** Relationship between the added extracellular concentration of L-phenylalanine and the specific Venus fluorescence of the *mtr* biosensor types one (*filled circles*), two (*filled diamonds*), three (*filled squares*), and four (*filled triangles*) in *E. coli* K-12 MG1655/pJC1-*mtr* sensor-type1. IPTG (25 μM) was additionally added to cells containing the *mtr* biosensor type two. **c** The specificity of the *mtr* biosensor was tested in the BioLector system using *E. coli* K-12 MG1655/pJC1-*mtr* sensor-type1 cells. The different amino acids and dipeptides were added to a final concentration of 3 mM. The specific Venus fluorescence was recorded after 6 h of cultivation (**b**, **c**). Represented data show average values from three independent biological replicates. **d** FACS histograms showing the Venus fluorescent signal of *E. coli* pJC1-*mtr* sensor-type1 after 7 h of cultivation in the BioLector in the presence of different dipeptide ratios (3 mM in total). **b**, **d** To adjust the intracellular amino acid level, different ratios of the dipeptide containing the effector amino acid and the competing dipeptide Ala-Ala were added to a final concentration of 3 mM

extracellular concentrations above 0.3 μM Ala-Phe, while the lack of *tyrR* expression resulted in a saturated signal at 1.5 mM (Fig. 3b).

To verify the specificity of the *mtr* sensor for aromatic amino acids, *E. coli* K-12 MG1655/pJC1-*mtr* sensor-type1 cells were cultivated in the presence of 13 different dipeptides and two amino acids (Fig. 3c). We observed a significant response to dipeptides containing the aromatic amino acids L-phenylalanine and L-tyrosine. However, we observed that the biosensor also responds to 3 mM Ala-Met with a minimal dynamic range of 1.9. To our knowledge, the activation of the *mtr* promoter in the presence of L-methionine has not been described previously.

For testing the suitability of the biosensors for FACS HT-screening, *E. coli* K-12 MG1655/pJC1-*mtr* sensor-type1 cells were incubated in the presence of different ratios of Ala-Ala and Ala-Phe, and analyzed via flow cytometry (Fig. 3d, Fig. S2). We determined a significant up-shift of the population in the presence of increased Ala-Phe levels suggesting the applicability of the biosensor for FC analyses and screening.

Biosensor-based high-throughput screening of randomly mutagenized strains

To verify the applicability of the *mtr* biosensor for FACS HT-screening of phenylalanine producing cells, the strain *E. coli* K-12 MG1655/pJC1-*mtr* sensor-type1 was chemically mutagenized using *N*′-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (Fig. 4a). Mutant cells exhibiting an increased biosensor output were spotted on agar plates using FACS.

Notably, also the analysis of non-mutagenized sensor cells revealed about 0.6 % high fluorescent cells under all tested

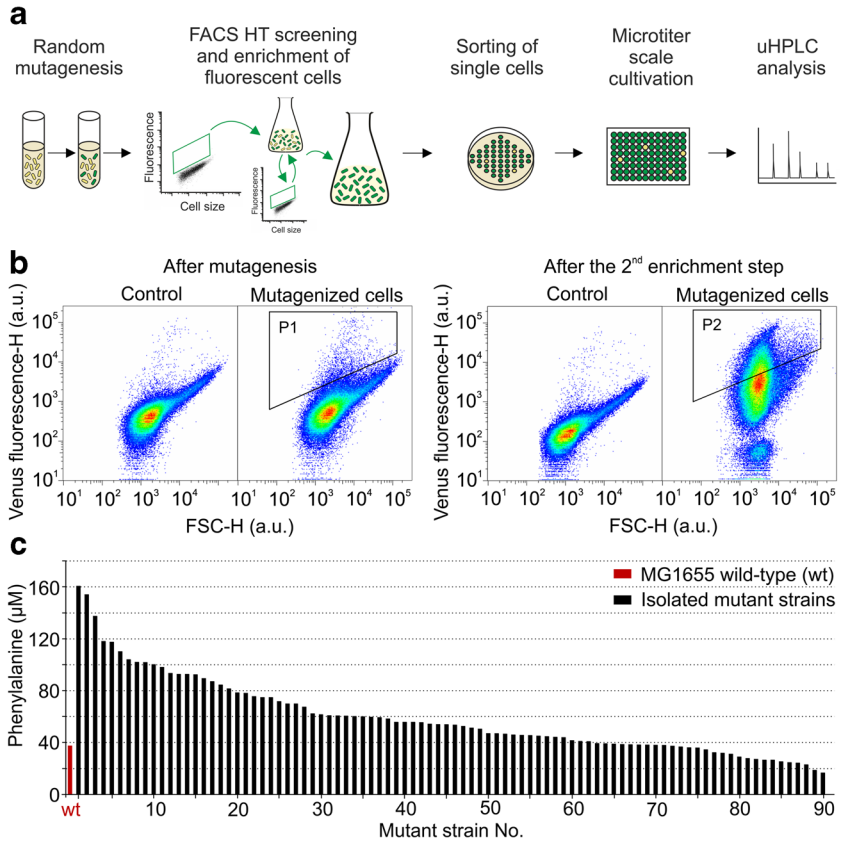

Fig. 4 Biosensor-based FACS HT-screening. **a** Schematic overview of the screening process. Initially, *E. coli* K-12 MG1655/pJC1-*mtr* sensor-type1 cells were mutagenized using the chemical mutagen MNNG. Mutagenized cells were analyzed by flow cytometry (FC), and 2.5×10^5 to 1×10^6 mutants with the top fluorescent output were sorted and re-cultivated. To enrich positive mutants, this step was repeated twice. Subsequently, single cells were spotted on agar plates, re-cultivated as isolated clones in the BioLector for 28 h in phenylalanine production medium, and amino acid production in the supernatant was determined by uHPLC. **b** The dot plots display the Venus fluorescence against the forward scatter (FSC) of control (non-mutagenized) and mutagenized cells upon treatment with MNNG and after two enrichment steps. Mutagenized cells were sorted from gates P1 and P2, respectively. As control, cells of the entire population were sorted. **c** L-Phenylalanine production of 90 isolated mutant clones (*black bars*) in comparison to the non-mutagenized *E. coli* K-12 MG1655/pJC1-*mtr* sensor-type1 strain (*red bars*). The cells were cultivated in the BioLector in phenylalanine production medium containing 0.5 % (*w*/*v*) glucose and kanamycin at 37 °C and 1200 rpm, and phenylalanine levels in the supernatant were determined by uHPLC after 28 h of incubation

conditions (Fig. S4). This observed phenotypic heterogeneity was either evoked spontaneously by the expression of the biosensor or reflects variability of the metabolic state of bacterial cells within the population. To discriminate between phenotypic heterogeneity and the rise of spontaneous (sensor) mutations, we isolated 2×10^5 spontaneously fluorescent cells (gate P1, 0.66 %) and cells from the entire population as control (gate P2) using FACS (Fig. S4). Isolated cells were re-analyzed by FC after 8 h of cultivation in minimal medium. Remarkably, both cultures showed a comparable fraction of spontaneously fluorescent cells (0.6 % of gate P1, 0.84 % of gate P2). This suggests that the observed spontaneous fluorescence did not originate from genomic modifications or contaminations, but might reflect phenotypic heterogeneity in terms of the intracellular amino acid level (Fig. S4).

To get rid of these spontaneous cells and in order to enrich positive mutants, we pursued an iterative selection strategy. Therefore, mutant cells were sorted and re-cultivated again before a second sorting step was conducted (Fig. 4b) (Mahr et al. 2015). The two enrichment steps resulted in an increase of top fluorescent cells from 2 % (gate P1) to 53.8 % (gate P2, Fig. 4b). As control, the entire population of non-mutagenized cells was sorted without revealing an increase in the fluorescent output. From gate P2, about 500 single cells exhibiting a significantly increased fluorescent output were spotted on agar plates (Fig. 4b). Ninety randomly selected mutants were cultivated in microtiter plates and the supernatant was analyzed after 28 h for amino acid production using uHPLC (Fig. 4c, Fig. S3). The majority of clones displayed increased biomass formation and significantly increased specific fluorescent signals compared to the non-mutagenized control strain (Fig. S3). Out of 90 randomly selected clones, 71 featured higher L-phenylalanine production than the wild-type strain *E. coli* K-12 MG1655/pJC1-*mtr* sensor-type1 (40 ± 1.7 μM). Twenty-seven percent of the analyzed mutants depicted a more than 2-fold increased L-

phenylalanine production. The best mutant produced 4.3-fold increased L-phenylalanine levels (160 μM) compared to the wild-type strain (Fig. 4c).

Discussion

In this study, we developed an efficient strategy based on FACS to screen promoter-GFP libraries for metabolite-responsive promoters, which can be applied as suitable parts for the construction of genetically encoded biosensors. By toggled rounds of selection under induced and non-induced conditions, metabolite-responsive promoters were enriched from an *E. coli* promoter library consisting of about 2000 different promoter-*gfpmut2* fusions (more than 75 % of all *E. coli* promoters) (Fig. 1, Fig. S1) (Zaslaver et al. 2006). For proof-of-principle, we screened for galactose- and phenylalanine-responsive promoters. We successfully enriched *galS* and *lacZ* promoter-*gfpmut2* fusions, whereof the regulation by galactose was well studied (Fig. S1) (Williams and Paigen 1968). The screening for L-phenylalanine-responsive promoters yielded the *mtr* promoter, which was subsequently integrated and characterized in different biosensor circuits.

The control of biosynthesis and transport of aromatic amino acids underlies a complex hierarchy of regulatory processes (Sprenger 2007) including feedback inhibition of pacemaker enzymes (Ogino et al. 1982), transcriptional control by the regulators TyrR and TrpR (Herrmann and Weaver 1999; Pittard 1996), as well as attenuation mechanisms at the transcriptional/translational interface (Chen and Yanofsky 2003). This challenges strain development for the production of L-phenylalanine. Thus, the design of suitable biosensors would provide a convenient tool for strain analysis and screening, and might be especially useful for the identification of non-intuitive targets for strain improvement (Binder et al. 2012; Mahr et al. 2015; Mustafi et al. 2012). However, our study revealed that several parameters require careful consideration to set up a screening procedure for effector-responsive promoters, including the adaption to growth medium, the uptake and catabolism of effector molecules (Luo et al. 2014; Payne 1977), the activation of the gene expression machinery (Bintu et al. 2005b; Carey et al. 2013; Fritz et al. 2014; Mäkelä et al. 2013), the maturation of fluorescent proteins (Craggs 2009; Iizuka et al. 2011), and last but not least, the composition of the particular growth medium. Here, we used M9 minimal medium due to its lack of other amino acids and carbon source and its very low auto-fluorescence (Miller 1972). Furthermore, the gating strategy during FACS has of course a prominent impact on the outcome of the screening. In this study, we have chosen an upper and lower 35 % gate for the entire library, which excludes 30 % of the cells with the mean fluorescent output (Fig. 1b). The clearly separated sorting gates eliminate cells with a constitutively mean fluorescent output by maintaining a high level of clonal diversity of sorted cells. To get rid of constitutively activated promoters, we established the toggled rounds of selecting promoters with a high fluorescent output under induced conditions and promoters with a low fluorescent output under non-induced conditions. Using this strategy, we successfully enriched galactose- and L-phenylalanine-responsive promoters (Fig. 1, Fig. S1). Our screening process was developed in a way that promoters activated in response to effector molecules were enriched. The selection of negatively regulated promoters is proposed to be achieved by selecting low fluorescent promoters in the presence of the effector of interest and by selecting high fluorescent promoters in the absence of the same. Repressible promoters may also be turned into a positive output in the presence of the effector by linking them to a strong repressor in the particular biosensor design (Mustafi et al. 2015; Ohlendorf et al. 2012).

The existence of a library is prerequisite for the presented approach. However, genomic plasmid or promoter libraries have been constructed for a variety of different model species, including the *E. coli* library of this study (Zaslaver et al. 2006), *Salmonella Thyphimurium* (Freed et al. 2008), *Staphylococcus aureus* (Schneider et al. 2002), and *Saccaromyces cerevisiae* (Newman et al. 2006). In a similar effort, Uchiyama and co-workers developed the substrate-induced gene expression (SIGEX) strategy to identify catabolic genes expressed in response to a relevant substrate, by inserting metagenomics fragments into an operon-trap vector (Uchiyama et al. 2005; Uchiyama and Watanabe 2008). The presented approach can therefore likewise be used for metagenomics libraries or for the isolation of functional parts after random/saturation mutagenesis of promoter or regulator sequences. Alternative approaches for screening promoter libraries mostly rely on the cultivation and characterization of single clones one by one, which appears cost-intensive and laborious (Bjarnason et al. 2003; Keren et al. 2013; Robijns et al. 2014; Zaslaver et al. 2006; Zeevi et al. 2011). Here, the implementation of FACS pre-screening significantly reduces the time- and cost-intensive screening process.

During screening for L-phenylalanine-responsive promoters, the promoter of *mtr* encoding a high-affinity L-tryptophan-specific permease (Heatwole and Somerville 1991; Hiraga et al. 1968) was enriched. Besides *mtr*, the expression of the gene encoding the low-affinity L-tyrosine transporter TyrP is also known to be activated by L-phenylalanine (Kasian and Pittard 1984; Pittard and Davidson 1991; Pittard et al. 2005). However, this promoter was not enriched during the screening process due to the 10-fold lower expression level in the presence of L-phenylalanine (Pittard et al. 2005). Heatwole and Somerville described the activation of *mtr* gene

expression by L-phenylalanine and L-tyrosine via TyrR and the superior repression of *mtr* by L-tryptophan via TrpR. This regulatory hierarchy enables the cell to modulate the intracellular ratio of the three aromatic amino acids by increasing the cellular level of L-tryptophan in the presence of high L-phenylalanine and L-tyrosine concentrations or by reducing the uptake of same by repressing *mtr* in the presence of high L-tryptophan levels (Heatwole and Somerville 1991; Sarsero and Pittard 1991). At the molecular level, TyrR dimerizes in the presence of L-phenylalanine, binds to the strong TyrR box, and promotes the recruitment of RNA-polymerase, while hexamerization of TyrR and the additional binding to the weak TyrR box in the presence of L-tyrosine is required for tyrosine-mediated gene expression (Fig. 2xa) (Pittard 1996; Pittard et al. 2005). L-Tryptophan-mediated binding of TrpR completely hinders RNA polymerase recruitment and thereby counteracts activation by TyrR (Heatwole and Somerville 1991; Pittard et al. 2005). This differential expression of the *mtr* gene was likewise observed during biosensor characterization (Fig. 2). For the construction of the *mtr* biosensor, the promoter includes both TrpR and TyrR binding sites. The presence of the TrpR boxes, however, implies consequently the disturbance of biosensor signaling under increased L-tryptophan concentrations. Hence, high intracellular concentrations of L-phenylalanine and L-tyrosine would be masked by high L-tryptophan concentrations. Here, the sensitivity of the biosensor could be reduced by mutation of TrpR-binding sites for candidates with reduced affinity towards TrpR.

The analysis of different biosensor designs revealed a significant influence of the circuit architecture on the performance characteristics in terms of sensitivity and dynamic range (Fig. 3a, b). The additional *tyrR* expression evoked a high basal fluorescence and a saturated biosensor signal at already low L-phenylalanine concentrations (up to 0.21 mM external Ala-Phe). In contrast, native TyrR levels led to similar dynamic ranges, but a reduced basal fluorescence and decreased sensitivity towards L-phenylalanine (Fig. 3b, d). Plasmid-based expression of *tyrR* results in significantly increased levels of the transcriptional regulator TyrR compared to native expression. Conventional gene expression analyses usually rely on simple promoter-reporter fusions (Keren et al. 2013; Robijns et al. 2014; Zaslaver et al. 2006). For the construction of biosensors, however, the corresponding TF is typically added not only to express heterologous genes but also to avoid titration effects of the native regulator. The reason for this design is, for example, obvious in the case of biosensors based on transcriptional regulators controlling a single target operon, such as in the case of Lrp of *Corynebacterium glutamicum* (Lange et al. 2012; Mustafi et al. 2012). Here, an increased number of TF binding sites (due to plasmid-based biosensor expression) generates titration effects due to the naturally tight regulation of the transcription factor to a low number of binding sites (Brewster et al. 2014). Global regulators like TyrR controlling a complex regulon with multiple binding sites within the host genome, however, are not necessarily affected by a few additional TF binding sites due to their natural high protein abundance. Here, the additional expression of the transcriptional regulator might significantly interfere with the host gene regulatory networks. Furthermore, the rate of occupation of a promoter by transcription factors recruiting the RNA polymerase through protein-protein interaction determines the rate of gene expression—hence, the sensitivity and dynamic range of the biosensor (Bintu et al. 2005a; b; Tabor et al. 2009). Thus, the higher the level of the transcriptional regulator, the more effector molecules can bind simultaneously resulting in a highly sensitive response and saturated operator sites at low concentrations. Likewise, the introduction of a second operator site or the modification of transcription factors were used to increase the sensitivity and dynamic range of sensor constructs in different studies (Lutz and Bujard 1997; Mahr and Frunzke 2016; Silva-Rocha and de Lorenzo 2012; Tabor et al. 2009). The present study highlights the impact of the architecture of a biosensor on the sensor transfer curve and, thus, illustrates the necessity of comparative sensor design studies as prerequisite for an application in strain development or single-cell analysis.

Based on the observed biosensor performance characteristics, we chose the *mtr* sensor type one for an implementation in a FACS HT-screening of an *E. coli* K-12 MG1655 mutant library upon random chemical mutagenesis (Fig. 4). To reduce the amount of false positive isolates, we included two iterative enrichment steps in the FACS HT-screening (Fig. 4a) (Mahr et al. 2015). Remarkably, about 30 % of 90 isolated and characterized mutant strains showed at least 2- to 4-fold increased L-phenylalanine titers (Fig. 4c), which is in line with the successful isolation of productive clones in previous studies (Binder et al. 2012; Mustafi et al. 2012; Santos and Stephanopoulos 2008). However, the best clone isolated displays still a considerably low production of phenylalanine (160 μM), which likely results from the high affinity of TyrR for the ligand phenylalanine (K_d = 260 μM) (Wilson et al. 1995). This limitation might be overcome by further engineering of the sensor towards a higher K_d value and a lowered sensitivity. Furthermore, the negative impact of L-phenylalanine on cellular growth may represent a disadvantage, especially in the case of iterative screening rounds (Grinter 1998). At external L-phenylalanine concentrations of 5 g/L, the growth rate of *E. coli* wild-type cells was found to be reduced by half (Polen et al. 2005). The iterative enrichment of high fluorescent cells, however, selects against false positives but also cells exhibiting a reduced growth rate at the same time. This might hinder the isolation of growth-defective mutants with high internal L-phenylalanine titers—an aspect which needs to be considered in future studies.

Genetically encoded biosensors have enlarged the repertoire of tools for engineering and monitoring of biotechnological production strains. Although nature provides a plethora of sensor devices, the identification of suitable candidates requires the deep knowledge of underlying regulatory mechanisms and intensive research. Efficient and automatized workflows are required, which enable the rapid identification of novel and suitable parts for biosensor design. This study emphasizes the screening of promoter-AFP libraries using FACS for the efficient identification of effector-responsive promoters. Furthermore, we were able to demonstrate the significant influence of the biosensor design on the sensor's performance characteristics. Taken together, these efforts contribute to the exploitation and development of custom-made biosensors according to the researcher's purpose.

Compliance with ethical standards

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Conflict of interest Julia Frunzke declares that she has no conflict of interest. Regina Mahr declares that she has no conflict of interest. Raphael Freiherr von Boeselager declares that he has no conflict of interest. Johanna Wiechert declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Backman K, O'Connor MJ, Maruya A, Rudd E, McKay D, Balakrishnan R, Radjai M, DiPasquantonio V, Shoda D, Hatch R, Venkatasubramanian K (1990) Genetic engineering of metabolic pathways applied to the production of phenylalanine. Ann N Y Acad Sci 589:16–24. doi:10.1111/j.1749-6632.1990.tb24231.x

Bang HB, Lee YH, Kim SC, Sung CK, Jeong KJ (2016) Metabolic engineering of *Escherichia coli* for the production of cinnamaldehyde. Microb Cell Factories 15:16. doi:10.1186/s12934-016-0415-9

Bentley R (1990) The shikimate pathway—a metabolic tree with many branches. CRC Crit Rev Biochem Mol Biol 25:307–384. doi:10.3109/10409239009090615

Binder S, Schendzielorz G, Stäbler N, Krumbach K, Hoffmann K, Bott M, Eggeling L (2012) A high-throughput approach to identify genomic variants of bacterial metabolite producers at the single-cell level. Genome Biol 13:R40. doi:10.1186/gb-2012-13-5-r40

Bintu L, Buchler NE, Garcia HG, Gerland U, Hwa T, Kondev J, Kuhlman T, Phillips R (2005a) Transcriptional regulation by the numbers: applications. Curr Opin Genet Dev 15:125–135. doi:10.1016/j.gde.2005.02.006

Bintu L, Buchler NE, Garcia HG, Gerland U, Hwa T, Kondev J, Phillips R (2005b) Transcriptional regulation by the numbers: models. Curr Opin Genet Dev 15:116–124. doi:10.1016/j.gde.2005.02.007

Bjarnason J, Southward CM, Surette MG (2003) Genomic profiling of iron-responsive genes in *Salmonella enterica* serovar *typhimurium* by high-throughput screening of a random promoter library. J Bacteriol 185:4973–4982. doi:10.1128/JB.185.16.4973-4982.2003

Blattner FR, Plunkett G, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997) The complete genome sequence of *Escherichia coli* K-12. Science 277:1453–1462. doi:10.1126/science.277.5331.1453

Bongaerts J, Krämer M, Müller U, Raeven L, Wubbolts M (2001) Metabolic engineering for microbial production of aromatic amino acids and derived compounds. Metab Eng 3:289–300. doi:10.1006/mben.2001.0196

Brewster RC, Weinert FM, Garcia HG, Song D, Rydenfelt M, Phillips R (2014) The transcription factor titration effect dictates level of gene expression. Cell 156:1312–1323. doi:10.1016/j.cell.2014.02.022

Carey LB, van Dijk D, Sloot PM, Kaandorp JA, Segal E (2013) Promoter sequence determines the relationship between expression level and noise. PLoS Biol 11:e1001528. doi:10.1371/journal.pbio.1001528

Chen G, Yanofsky C (2003) Tandem transcription and translation regulatory sensing of uncharged tryptophan tRNA. Science 301:211–213. doi:10.1126/science.1084902

Chung CT, Niemela SL, Miller RH (1989) One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci U S A 86:2172–2175

Craggs TD (2009) Green fluorescent protein: structure, folding and chromophore maturation. Chem Soc Rev 38:2865–2875. doi:10.1039/b903641p

Cremer J, Eggeling L, Sahm H (1990) Cloning the *dapA dapB* cluster of the lysine-secreting bacterium *Corynebacterium glutamicum*. MGG Mol Gen Genet 220:478–480

Dahl RH, Zhang F, Alonso-Gutierrez J, Baidoo E, Batth TS, Redding-Johanson AM, Petzold CJ, Mukhopadhyay A, Lee TS, Adams PD, Keasling JD (2013) Engineering dynamic pathway regulation using stress-response promoters. Nat Biotechnol 31:1039–1046. doi:10.1038/nbt.2689

Delvigne F, Zune Q, Lara AR, Al-Soud W, Sorensen SJ (2014) Metabolic variability in bioprocessing: implications of microbial phenotypic heterogeneity. Trends Biotechnol 32:608–616. doi:10.1016/j.tibtech.2014.10.002

Eikmanns BJ, Thum-Schmitz N, Eggeling L, Ludtke KU, Sahm H (1994) Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. Microbiology 140(Pt 8):1817–1828. doi:10.1099/13500872-140-8-1817

Freed NE, Silander OK, Stecher B, Böhm A, Hardt WD, Ackermann M (2008) A simple screen to identify promoters conferring high levels of phenotypic noise. PLoS Genet 4:e1000307. doi:10.1371/journal.pgen.1000307

Fritz G, Megerle JA, Westermayer SA, Brick D, Heermann R, Jung K, Radler JO, Gerland U (2014) Single cell kinetics of phenotypic switching in the arabinose utilization system of *E. coli*. PLoS ONE 9:e89532. doi:10.1371/journal.pone.0089532

Gerigk M, Bujnicki R, Ganpo-Nkwenkwa E, Bongaerts J, Sprenger G, Takors R (2002) Process control for enhanced L-phenylalanine production using different recombinant *Escherichia coli* strains. Biotechnol Bioeng 80:746–754. doi:10.1002/bit.10428

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA 3rd, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6:343–345. doi:10.1038/nmeth.1318

Grinter NJ (1998) Developing an L-phenylalanine process. Chem Tech: 33–35

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557–580. doi:10.1016/S0022-2836(83)80284-8

Harper MA, Chen Z, Toy T, Machado IM, Nelson SF, Liao JC, Lee CJ (2011) Phenotype sequencing: identifying the genes that cause a

phenotype directly from pooled sequencing of independent mutants. PLoS ONE 6:e16517. doi:10.1371/journal.pone.0016517

Hayashi K, Morooka N, Yamamoto Y, Fujita K, Isono K, Choi S, Ohtsubo E, Baba T, Wanner BL, Mori H, Horiuchi T (2006) Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. Mol Syst Biol 2(2006):0007. doi:10.1038/msb4100049

Heatwole VM, Somerville RL (1991) The tryptophan-specific permease gene, *mtr*, is differentially regulated by the tryptophan and tyrosine repressors in *Escherichia coli* K-12. J Bacteriol 173:3601–3604

Herrmann KM, Weaver LM (1999) The shikimate pathway. Annu Rev Plant Physiol Plant Mol Biol 50:473–503. doi:10.1146/annurev.arplant.50.1.473

Hiraga S, Ito K, Matsuyama T, Ozaki H, Yura T (1968) 5-methyltryptophan-resistant mutations linked with the arginine G marker in *Escherichia coli*. J Bacteriol 96:1880–1881

Hoffmann K, Grünberger A, Lausberg F, Bott M, Eggeling L (2013) Visualization of imbalances in sulfur assimilation and synthesis of sulfur-containing amino acids at the single-cell level. Appl Environ Microbiol 79:6730–6736. doi:10.1128/AEM.01804-13

Hou Y, Hossain GS, Li J, Shin HD, Liu L, Du G (2015) Production of phenylpyruvic acid from L-phenylalanine using an L-amino acid deaminase from *Proteus mirabilis*: comparison of enzymatic and whole-cell biotransformation approaches. Appl Microbiol Biotechnol 99:8391–8402. doi:10.1007/s00253-015-6757-0

Iizuka R, Yamagishi-Shirasaki M, Funatsu T (2011) Kinetic study of *de novo* chromophore maturation of fluorescent proteins. Anal Biochem 414:173–178. doi:10.1016/j.ab.2011.03.036

Kasian PA, Pittard J (1984) Construction of a *tyrP-lac* operon fusion strain and its use in the isolation and analysis of mutants derepressed for *tyrP* expression. J Bacteriol 160:175–183

Kensy F, Zang E, Faulhammer C, Tan RK, Büchs J (2009) Validation of a high-throughput fermentation system based on online monitoring of biomass and fluorescence in continuously shaken microtiter plates. Microb Cell Factories 8:31. doi:10.1186/1475-2859-8-31

Keren L, Zackay O, Lotan-Pompan M, Barenholz U, Dekel E, Sasson V, Aidelberg G, Bren A, Zeevi D, Weinberger A, Alon U, Milo R, Segal E (2013) Promoters maintain their relative activity levels under different growth conditions. Mol Syst Biol 9:701. doi:10.1038/msb.2013.59

Keseler IM, Mackie A, Peralta-Gil M, Santos-Zavaleta A, Gama-Castro S, Bonavides-Martinez C, Fulcher C, Huerta AM, Kothari A, Krummenacker M, Latendresse M, Muniz-Rascado L, Ong Q, Paley S, Schroder I, Shearer AG, Subhraveti P, Travers M, Weerasinghe D, Weiss V, Collado-Vides J, Gunsalus RP, Paulsen I, Karp PD (2013) EcoCyc: fusing model organism databases with systems biology. Nucleic Acids Res 41:D605–D612. doi:10.1093/nar/gks1027

Lange C, Mustafi N, Frunzke J, Kennerknecht N, Wessel M, Bott M, Wendisch VF (2012) Lrp of *Corynebacterium glutamicum* controls expression of the *brnFE* operon encoding the export system for L-methionine and branched-chain amino acids. J Biotechnol 158:231–241. doi:10.1016/j.jbiotec.2011.06.003

Leuchtenberger W, Huthmacher K, Drauz K (2005) Biotechnological production of amino acids and derivatives: current status and prospects. Appl Microbiol Biotechnol 69:1–8. doi:10.1007/s00253-005-0155-y

Liu D, Evans T, Zhang F (2015a) Applications and advances of metabolite biosensors for metabolic engineering. Metab Eng 31:35–43. doi:10.1016/j.ymben.2015.06.008

Liu D, Xiao Y, Evans BS, Zhang F (2015b) Negative feedback regulation of fatty acid production based on a malonyl-CoA sensor-actuator. ACS Synth Biol 4:132–140. doi:10.1021/sb400158w

Luo Y, Zhang T, Wu H (2014) The transport and mediation mechanisms of the common sugars in *Escherichia coli*. Biotechnol Adv 32:905–919. doi:10.1016/j.biotechadv.2014.04.009

Lutz R, Bujard H (1997) Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. Nucleic Acids Res 25:1203–1210. doi:10.1093/nar/25.6.1203

Mahr R, Frunzke J (2016) Transcription factor-based biosensors in biotechnology: current state and future prospects. Appl Microbiol Biotechnol 100:79–90. doi:10.1007/s00253-015-7090-3

Mahr R, Gätgens C, Gätgens J, Polen T, Kalinowski J, Frunzke J (2015) Biosensor-driven adaptive laboratory evolution of L-valine production in *Corynebacterium glutamicum*. Metab Eng 32:184–194. doi:10.1016/j.ymben.2015.09.017

Mäkelä J, Kandhavelu M, Oliveira SM, Chandraseelan JG, Lloyd-Price J, Peltonen J, Yli-Harja O, Ribeiro AS (2013) *In vivo* single-molecule kinetics of activation and subsequent activity of the arabinose promoter. Nucleic Acids Res 41:6544–6552. doi:10.1093/nar/gkt350

Miller, J. H., 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory.

Mustafi N, Bott M, Frunzke J (2015) Genetically-encoded biosensors for strain development and single cell analysis of *Corynebacterium glutamicum*. In: Burkovski A (ed) *Corynebacterium glutamicum*: from systems biology to biotechnological applications. Caister Academic Press, Norfolk, p. 190

Mustafi N, Grünberger A, Kohlheyer D, Bott M, Frunzke J (2012) The development and application of a single-cell biosensor for the detection of L-methionine and branched-chain amino acids. Metab Eng 14:449–457. doi:10.1016/j.ymben.2012.02.002

Mustafi N, Grünberger A, Mahr R, Helfrich S, Noh K, Blombach B, Kohlheyer D, Frunzke J (2014) Application of a genetically encoded biosensor for live cell imaging of L-valine production in pyruvate dehydrogenase complex-deficient *Corynebacterium glutamicum* strains. PLoS ONE 9:e85731. doi:10.1371/journal.pone.0085731

Newman JR, Ghaemmaghami S, Ihmels J, Breslow DK, Noble M, DeRisi JL, Weissman JS (2006) Single-cell proteomic analysis of *S. cerevisiae* reveals the architecture of biological noise. Nature 441:840–846. doi:10.1038/nature04785

Ogino T, Garner C, Markley JL, Herrmann KM (1982) Biosynthesis of aromatic compounds: ^{13}C NMR spectroscopy of whole *Escherichia coli* cells. Proc Natl Acad Sci U S A 79:5828–5832. doi:10.1073/pnas.79.19.5828

Ohlendorf R, Vidavski RR, Eldar A, Moffat K, Möglich A (2012) From dusk till dawn: one-plasmid systems for light-regulated gene expression. J Mol Biol 416:534–542. doi:10.1016/j.jmb.2012.01.001

Payne JW (1977) Transport and hydrolysis of peptides by microorganisms. CIBA Found Symp:305–334

Pittard AJ, Davidson BE (1991) TyrR protein of *Escherichia coli* and its role as repressor and activator. Mol Microbiol 5:1585–1592

Pittard J (1996) The various strategies within the TyrR regulation of *Escherichia coli* to modulate gene expression. Genes Cells 1:717–725. doi:10.1111/j.1365-2443.1996.tb00012.x

Pittard J, Camakaris H, Yang J (2005) The TyrR regulon. Mol Microbiol 55:16–26. doi:10.1111/j.1365-2958.2004.04385.x

Polen T, Krämer M, Bongaerts J, Wubbolts M, Wendisch VF (2005) The global gene expression response of *Escherichia coli* to L-phenylalanine. J Biotechnol 115:221–237. doi:10.1016/j.jbiotec.2004.08.017

Robijns SC, Roberfroid S, Van Puyvelde S, De Pauw B, Uceda Santamaria E, De Weerdt A, De Coster D, Hermans K, De Keersmaecker SC, Vanderleyden J, Steenackers HP (2014) A GFP promoter fusion library for the study of *Salmonella* biofilm formation and the mode of action of biofilm inhibitors. Biofouling 30:605–625. doi:10.1080/08927014.2014.907401

Rodriguez A, Martinez JA, Flores N, Escalante A, Gosset G, Bolivar F (2014) Engineering Escherichia coli to overproduce aromatic amino acids and derived compounds. Microb Cell Factories:13. doi:10.1186/s12934-014-0126-z

Rüffer N, Heidersdorf U, Kretzers I, Sprenger GA, Raeven L, Takors R (2004) Fully integrated L-phenylalanine separation and concentration using reactive-extraction with liquid-liquid centrifuges in a fed-batch process with *E. coli*. Bioprocess Biosyst Eng 26:239–248. doi:10.1007/s00449-004-0354-4

Sambrook J, MacCallum P, Russell D (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York

Santos CN, Stephanopoulos G (2008) Melanin-based high-throughput screen for L-tyrosine production in *Escherichia coli*. Appl Environ Microbiol 74:1190–1197. doi:10.1128/AEM.02448-07

Sariaslani FS (2007) Development of a combined biological and chemical process for production of industrial aromatics from renewable resources. Annu Rev Microbiol 61:51–69. doi:10.1146/annurev.micro.61.080706.093248

Sarsero JP, Pittard AJ (1991) Molecular analysis of the TyrR protein-mediated activation of *mtr* gene expression in *Escherichia coli* K-12. J Bacteriol 173:7701–7704

Schallmey M, Frunzke J, Eggeling L, Marienhagen J (2014) Looking for the pick of the bunch: high-throughput screening of producing microorganisms with biosensors. Curr Opin Biotechnol 26:148–154. doi:10.1016/j.copbio.2014.01.005

Schendzielorz G, Dippong M, Grünberger A, Kohlheyer D, Yoshida A, Binder S, Nishiyama C, Nishiyama M, Bott M, Eggeling L (2014) Taking control over control: use of product sensing in single cells to remove flux control at key enzymes in biosynthesis pathways. ACS Synth Biol 3:21–29. doi:10.1021/sb400059y

Schneider WP, Ho SK, Christine J, Yao M, Marra A, Hromockyj AE (2002) Virulence gene identification by differential fluorescence induction analysis of *Staphylococcus aureus* gene expression during infection-simulating culture. Infect Immun 70:1326–1333

Semsey S, Krishna S, Sneppen K, Adhya S (2007) Signal integration in the galactose network of *Escherichia coli*. Mol Microbiol 65:465–476. doi:10.1111/j.1365-2958.2007.05798.x

Siedler S, Stahlhut SG, Malla S, Maury J, Neves AR (2014) Novel biosensors based on flavonoid-responsive transcriptional regulators introduced into *Escherichia coli*. Metab Eng 21:2–8. doi:10.1016/j.ymben.2013.10.011

Silva-Rocha R, de Lorenzo V (2012) Broadening the signal specificity of prokaryotic promoters by modifying cis-regulatory elements associated with a single transcription factor. Mol BioSyst 8:1950–1957. doi:10.1039/c2mb25030f

Simmonds S, Griffith DD (1961) Metabolism of phenylalanine-containing peptide amides in *Escherichia coli*. J Bacteriol 83:256–263

Sprenger GA (2006) Aromatic amino acids. In: Steinbüchel A (ed) Microbiology monographs. vol. Amino acid biosynthesis—pathways, regulation and metabolic engineering. Springer-Verlag, Berlin Heidelberg

Sprenger GA (2007) From scratch to value: engineering *Escherichia coli* wild type cells to the production of L-phenylalanine and other fine chemicals derived from chorismate. Appl Microbiol Biotechnol 75:739–749. doi:10.1007/s00253-007-0931-y

Tabor, J. J., Groban, E. S., Voigt, C. A., 2009, Performance characteristics for sensors and circuits used to program *E. coli*. In: Lee, S. Y., (Ed.), Systems biology and biotechnology of *Escherichia coli*. Springer Science & Business Media B.V.

Uchiyama T, Abe T, Ikemura T, Watanabe K (2005) Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. Nat Biotechnol 23:88–93. doi:10.1038/nbt1048

Uchiyama T, Watanabe K (2008) Substrate-induced gene expression (SIGEX) screening of metagenome libraries. Nat Protoc 3:1202–1212. doi:10.1038/nprot.2008.96

van Summeren-Wesenhagen PV, Marienhagen J (2015) Metabolic engineering of *Escherichia coli* for the synthesis of the plant polyphenol pinosylvin. Appl Environ Microbiol 81:840–849. doi:10.1128/AEM.02966-14

Vargas-Tah A, Martinez LM, Hernandez-Chavez G, Rocha M, Martinez A, Bolivar F, Gosset G (2015) Production of cinnamic and p-hydroxycinnamic acid from sugar mixtures with engineered *Escherichia coli*. Microb Cell Factories 14:6. doi:10.1186/s12934-014-0185-1

Vrljic M, Sahm H, Eggeling L (1996) A new type of transporter with a new type of cellular function: L-lysine export from *Corynebacterium glutamicum*. Mol Microbiol 22:815–826. doi:10.1046/j.1365-2958.1996.01527.x

Weickert MJ, Adhya S (1992) Isorepressor of the *gal* regulon in *Escherichia coli*. J Mol Biol 226:69–83. doi:10.1016/0022-2836(92)90125-4

Weiner M, Albermann C, Gottlieb K, Sprenger GA, Weuster-Botz D (2014) Fed-batch production of L-phenylalanine from glycerol and ammonia with recombinant *Escherichia coli*. Biochem Eng J 83:62–69. doi:10.1016/j.bej.2013.12.001

Whipp MJ, Pittard AJ (1977) Regulation of aromatic amino acid transport systems in *Escherichia coli* K-12. J Bacteriol 132:453–461

Williams B, Paigen K (1968) Paradoxical effect of weak inducers on the *lac* operon of *Escherichia coli*. J Bacteriol 96:1774–1782

Wilson TJ, Argaet VP, Howlett GJ, Davidson BE (1995) Evidence for two aromatic amino acid-binding sites, one ATP-dependent and the other ATP-independent, in the *Escherichia coli* regulatory protein TyrR. Mol Microbiol 17:483–492

Xu P, Li L, Zhang F, Stephanopoulos G, Koffas M (2014) Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. Proc Natl Acad Sci U S A 111:11299–11304. doi:10.1073/pnas.1406401111

Zaslaver A, Bren A, Ronen M, Itzkovitz S, Kikoin I, Shavit S, Liebermeister W, Surette MG, Alon U (2006) A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. Nat Methods 3:623–628. doi:10.1038/nmeth895

Zeevi D, Sharon E, Lotan-Pompan M, Lubling Y, Shipony Z, Raveh-Sadka T, Keren L, Levo M, Weinberger A, Segal E (2011) Compensation for differences in gene copy number among yeast ribosomal proteins is encoded within their promoters. Genome Res 21:2114–2128. doi:10.1101/gr.119669.110

Zhang F, Carothers JM, Keasling JD (2012) Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. Nat Biotechnol 30:354–359. doi:10.1038/nbt.2149