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The development and application of a single-cell biosensor for the detection of L-methionine and branched-chain amino acids

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ABSTRACT

The detection and quantification of specific metabolites in single bacterial cells is a major goal for industrial biotechnology. We have developed a biosensor based on the transcriptional regulator Lrp that detects intracellular L-methionine and branched-chain amino acids in *Corynebacterium glutamicum*. In assays, fluorescence output showed a linear relationship with cytoplasmic concentrations of the effector amino acids. In increasing order, the affinity of Lrp for the amino acids is L-valine, L-isoleucine, L-leucine and L-methionine. The sensor was applied for online monitoring and analysis of cell-to-cell variability of L-valine production by the pyruvate dehydrogenase-deficient *C. glutamicum* strain Δ*aceE*. Finally, the sensor system was successfully used in a high-throughput (HT) FACS screen for the isolation of amino acid-producing mutants after random mutagenesis of a non-producing wild type strain. These applications illustrate how one of nature's sensor devices – transcriptional regulators – can be used for the analysis, directed evolution and HT screening for microbial strain development.

1. Introduction

Improvements to microbial production of biological molecules represent a key goal for industrial biotechnology. Traditional approaches for strain development include random mutagenesis followed by selection or screening, and the rational design of metabolic pathways to redirect or enhance metabolic flux, optimize precursor supply or tune metabolic regulation (Bailey, 1991). These methods take time, require bulk sampling and do not interface with high-throughput technologies. New techniques that detect and quantify specific metabolites in single cells have the potential to dramatically improve efficiency. They allow the analysis of productivity of a given strain at the single-cell level and offer the potential for high-throughput screening to isolate strains that produce the desired metabolite (Bailey, 1991).

Recent advances in mass spectrometry and next generation sequencing technologies have opened up new ways to analyze and screen production strains. However, they focus mostly on easily accessible phenotypes, while the majority of desirable bacterial products are small, rather inconspicuous molecules that cannot easily be detected at the single-cell level. For such low-profile molecules, genetically-encoded biosensors offer the potential to transform information about a specific metabolite into an optical output. Recently, a range of sensors have been used, including enzymes or multistep enzymatic pathways, inducible expression systems and whole organisms (Dietrich et al., 2010; Michener et al., in press; van der Meer and Belkin, 2010; Zhang and Keasling, 2011). These systems facilitate small-molecule detection through colorimetric assays, fluorescence readout or growth-coupled screening techniques based on auxotrophic growth. However, for the most biotechnology-relevant compounds, notably amino acids, organic acids or polymer precursors, no appropriate optical sensors are currently available.

The Gram-positive soil bacterium *Corynebacterium glutamicum* is an important platform organism for industrial biotechnology (Kinoshita et al., 1957). It is used, for example, for the large-scale production of more than two million tons of L-glutamate and L-lysine per year (Burkovski, 2008; Eggeling and Bott, 2005) and for the production of a few thousand tons each of L-threonine, L-leucine and L-valine, which are essential amino acids for all vertebrates (Eggeling, 2001; Leuchtenberger, 1996). Applications for the branched-chain amino acid L-valine include artificial nutrition and the synthesis of antiviral drugs and herbicides (Bartek et al., 2011; Blombach et al., 2008; Park et al., 2007). The development of strains for the production of L-methionine is of major interest as around 500 kt are produced annually from petrochemicals at present (Ikeda, 2003).

Here, we present a biosensor system that can enhance strain development by detecting intracellular L-methionine and the branched-chain amino acids L-valine, L-leucine and L-isoleucine. The biosensor uses the transcriptional regulator Lrp of *C. glutamicum*, which activates the expression of the *brnFE* operon in the presence of

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increased levels of branched-chain amino acids or L-methionine (Lange et al., 2011). BrnFE, a two-component permease belonging to the LIV-E transporter family, facilitates the export of these amino acids, thus avoiding the inhibitory effects of intracellular amino acid accumulation (Kennerknecht et al., 2002; Trötschel et al., 2005). The export of amino acids is usually tightly regulated to avoid wasting metabolic energy. Lrp binds the intergenic region of *lrp-brnFE* and activates expression in the presence of elevated concentrations of the appropriate amino acids.

We report on the construction of a novel amino acid biosensor based on the transcriptional regulator Lrp, which allows the intracellular detection and quantification of branched-chain amino acids and L-methionine at single-cell resolution. We present this sensor technology as a simple and convenient tool for the analysis of biotechnological production strains and for the establishment of novel high-throughput screening approaches.

2. Material and methods

2.1. Bacterial strains, media and growth conditions

Bacterial strains and plasmids are listed in Table 1. Unless stated otherwise, cultures of *C. glutamicum* were inoculated to an OD_{600} of 1 and grown in CGXII minimal medium with 4% (w/v) glucose as carbon source. For the dipeptide feeding assay, the main culture was inoculated to an OD_{600} of ~3 and cultivated in a BioLector microbioreactor system (Kensy et al., 2009). For random mutagenesis of *C. glutamicum* ATCC 13032 cells were grown to an OD_{600} of 5 in BHIS medium (BHI with 0.5 M sorbitol) and subsequently incubated with N′-methyl-N′-nitro-N-nitrosoguanidine (0.125 mg/ml) for 15 min. After mutagenesis the cells were washed twice with 50 ml 0.9% (w/v) saline and cultivated in BHIS medium for 1 h, after which kanamycin (25 μg ml^{-1}) was added. *Escherichia coli* DH5α was grown aerobically in LB medium on a rotary shaker (120 rpm) or on LB agar plates at 37 °C (Sambrook et al., 2001). Where appropriate, the media contained kanamycin (25 μg ml^{-1} for *C. glutamicum* or 50 μg ml^{-1} for *E. coli*).

2.2. Recombinant DNA work

Standard methods including PCR, DNA restriction or ligation were carried out according to standard protocols (Sambrook et al., 2001). Oligonucleotides were synthesized by Eurofins MWG Operon (Ebersfeld, Germany) and are listed in Table 1. For construction of the biosensor, the fragment containing the *C. glutamicum lrp* open reading frame, the intergenic region of *lrp-brnF*, the first 30 nucleotides of *brnF,* and the ribosomal binding site of the pET-16b Vector (Novagen, Darmstadt, Germany) were amplified using oligonucleotides Lrp-fw-A-BamHI and Lrp-brnF-rv-I-NdeI introducing a BamHI and NdeI restriction site, respectively. Additionally, *eyfp* was amplified by PCR with oligonucleotides eYFP-fw-H-NdeI and eYFP-rv-D-SalI using the vector pEKEx2-yfp-tetR as template, thereby introducing restriction sites for NdeI and SalI digestion (Frunzke et al., 2008). Both PCR products were cloned into the vector pJC1 (Schäfer et al., 1997), resulting in plasmid pJC1-*lrp-brnF′-eyfp*.

2.3. Fluorescence microscopy and spectroscopy

For phase contrast and fluorescence microscopy, samples were placed on a microscope slide coated with a thin aqueous Poly-L-lysine solution of 0.1% (w/v, Sigma-Aldrich GmbH, Munich, Germany) and covered by a coverslip. Images were taken on a Zeiss Axioplan 2 imaging microscope that was equipped with an AxioCam MRm camera and a Plan-Apochromat 100x, 1.40 Oil DIC oil-Immersion objective. Digital images were acquired and analyzed with AxioVision 4.6 software (Zeiss, Göttingen, Germany).

Fluorescence spectrometry measurements of *C. glutamicum* strains were carried out using a JASCO FP-6500/6600 spectrofluorometer (JASCO GmbH, Gross-Umstadt, Germany) in 3 ml QS fluorescence cells (Hellma GmbH, Müllheim, Germany). Final documentation of the recorded spectra was performed using Spectra-Manager Software (JASCO).

2.4. Adjustment and determination of intracellular amino acid concentrations

To adjust the intracellular amino acid concentration, dipeptides were added to preincubated cells (60 min at 30 °C, 1200 rpm) using different ratios of the corresponding dipeptides at a final concentration of 3 mM. To adjust internal effector levels, the following dipeptides were added: L-methionine: alanyl-methionine (Ala-Met) or methionyl-methionine (Met-Met) with alanyl-alanine (Ala-Ala); L-leucine: alanyl-leucine (Ala-Leu) with Ala-Ala; L-isoleucine: alanyl-isoleucine (Ala-Ile) with Ala-Ala;

Table 1
Bacterial strains, plasmids and oligonucleotides.

Strains or plasmids	Relevant characteristics	Source or reference
Strains		
***C. glutamicum* ATCC 13032**	Biotin-auxotrophic wild type	(Kinoshita et al., 1957)
C. glutamicum* Δ*aceE	In-frame deletion of cg2466	(Schreiner et al., 2005)
***E. coli* DH5α**	*supE44 ΔlacU169* (ϕ80*lacZ*DM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
Plasmids		
pJC1	*E. coli-C. glutamicum* shuttle vector, Kan^R $oriV_{Ec}$ $oriV_{Cg}$	(Schäfer et al., 1997)
pJC1-*lrp-brnF′-eyfp*	Kan^R; pJC1 derivative containing *lrp* (cg0313), the intergenic region of *lrp brnF* (cg0314) and a transcriptional fusion of *brnF* with *eyfp*	This study
pEKEx2-*yfp-tetR*	Kan^R; pEKEx2 derivative containing *eyfp-tetR*, encoding an eYFP-TetR fusion protein under the control of the *tac* promoter	(Frunzke et al., 2008)
Oligonucleotides	**Sequence (5′→3′) and properties[a]**	
Lrp-fw-A-BamHI	GCGCGGATCCTCACACCTGGGGGCGAGCTG (BamHI)	
Lrp-brnF-rv-I-NdeI	GCGCCATATGATATCTCCTTCTTAAAGTTCAGCTTGAATGAATCTCTTGCG (NdeI)	
EYfp-fw-H-NdeI	GCGCCATATGGTGAGCAAGGGCGAGGAG (NdeI)	
EYfp-rv-D-SalI	GCGCGTCGACTTATCTAGACTTGTACAGCTCGTC (SalI)	
aceE-out-fw	CACTTCGGCGTGTCACAATTAGG	
aceE-out-rv	GTCTGACAAGGCGTTCCTCGG	

[a] In some cases oligonucleotides were designed to introduce recognition sites for restriction endonucleases (recognition sites underlined, restriction endonucleases indicated in parentheses).

L-valine: alanyl-valine (Ala-Val) with Ala-Ala (Fig. S1). The internal level of the respective effector amino acid forms a distinct peak within minutes after addition of the dipeptide mixture (Trötschel et al., 2005). This time point was determined to be after 7 min in case of L-methionine and L-leucine, 10 min for L-isoleucine and 20 min for L-valine (see Fig. S1). Intracellular amino acid concentrations were measured in three samples of 200 µl taken from the cell suspension when maximal internal amino acid concentration was reached. The cells were quickly separated from the surrounding medium and extracted in perchloric acid (20%) by silicone oil centrifugation as described previously (Ebbighausen et al., 1989). Amino acids were quantified as their ortho-phthaldialdehyde derivatives using ultra-high pressure liquid chromatography by automatic pre-column derivatization and separation by reversed-phase chromatography on an Agilent 1290 Infinity LC ChemStation (Agilent, Santa Clara, USA) with fluorescence detection. The intracellular volume used to calculate the internal amino acid concentration was 1.7 µl mg (dry weight)$^{-1}$ (Ruffert et al., 1997).

2.5. Microfluidic chip fabrication and imaging

Microfluidic chips based on a disposable transparent poly (dimethylsiloxane) (PDMS) (Sylgard 184 Silicone Elastomer, Dow Corning Corp., Midland, MI, USA) were fabricated as described in SI Experimental Procedures (Xia and Whitesides, 1998). For imaging, the device was placed on a fully motorized inverted microscope (Nikon Eclipse Ti, Nikon Instruments Europe). For the study of microcolonies, single *C. glutamicum* Δ*aceE* cells (Schreiner et al., 2005) were seeded into the chip device by flushing the cell suspension (OD_{600} ~4) through the channels with ~25 nl/min (calculated flow in one channel; 300 ml/min for the whole chip device). Once single cells were trapped, CGXII medium with 4% glucose (w/v) and 1.5% (w/v) potassium acetate was infused by a syringe pump with a flow rate of ~25 nl/min. Colony growth was followed by time lapse microscopy for approximately five generations. In order to initiate the L-valine production phase, the medium was switched after 15 h to medium containing 4% glucose (w/v) (Blombach et al., 2007). Images were acquired at 15 min intervals. Fluorescence emission was quantified through mean intensity of every defined region of interest (ROIs), which encompasses the cell area.

2.6. Microtiter plate cultivation

Online monitoring of growth and fluorescence was conducted in 48-well microtiter Flowerplates (MFPs) with the BioLector cultivation system (m2p-labs GmbH, Aachen, Germany). 48-well MFPs containing 750 µl medium per well were inoculated with *C. glutamicum* cells from a preculture (see Section 2.1) and grown at 30 °C, a shaking frequency of 1200 rpm, and a shaking diameter of 3 mm. During cultivation the production of biomass was measured as the backscattered light intensity of sent light with a wavelength of 620 nm (signal gain factor of 10), the eYFP fluorescence of the cultures 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 eYFP fluorescence per scattered light intensity (given in a.u.) (Kensy et al., 2009).

2.7. Flow cytometry

Flow cytometric measurements and sorting were performed on a FACSAria II (Becton Dickinson, San Jose, USA) with 488 nm excitation from a blue solid-state laser. Forward-scatter characteristics (FSC) and side-scatter characteristics (SSC) were detected as small- and large-angle scatters of the 488-nm laser, respectively. EYFP fluorescence was detected using a 502-nm long-pass and a 530/30-nm band-pass filter set. Sorting was performed with four-way purity as the precision mode at a threshold rate up to 10,000 events/s. Cells were sorted on BHI agar plates containing 25 µg ml^{-1} kanamycin or in a tube containing CGXII medium.

2.8. Determination of pyruvate dehydrogenase activity

For enzyme assays, *C. glutamicum* WT and mutant cells were grown in the main culture in CGXII minimal medium with 4% (w/v) glucose, 1.5% (w/v) potassium acetate and 0.1% (w/v) yeast extract and harvested at OD_{600} of ~5. Cells were disrupted by sonication in 0.1 mM TES([*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), pH 7.2, 10 mM $MgCl_2$, 3 mM L-cysteine, and 30% (w/v) glycerol. Crude extract was prepared by subsequent centrifugation and gel-filtrated in the same buffer on PD10 columns (GE Healthcare, Buckinghamshire, UK). Ultracentrifugation of the crude extract and finally the pyruvate dehydrogenase assay were performed as described previously (Hoffelder et al., 2010).

3. Results

3.1. Biosensor construction and proof of principle

To design a biosensor that responds to elevated intracellular concentrations of L-methionine or branched-chain amino acids, we constructed a module based on the transcriptional regulator Lrp (leucine-responsive protein) of *C. glutamicum*. The genomic region containing the open-reading frame of *lrp* and the intergenic region of *lrp-brnF*, which contains the promoter of *brnF*, were cloned in front of *eyfp*, thereby setting *eyfp* expression under the control of P_{brnF} (Fig. 1A). The resulting sensor plasmid was transformed into *C. glutamicum* wild type (WT) strain. In a first set

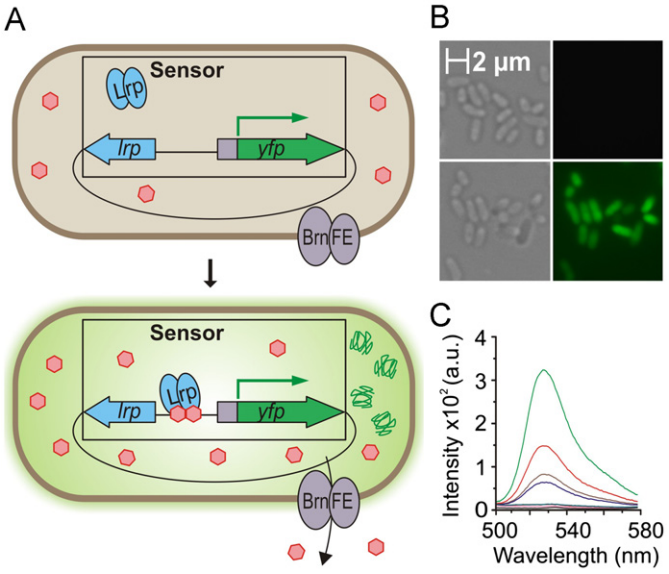

Fig. 1. Construction and verification of the Lrp-based biosensor. (A) A sensor cell with wild type levels of the effector amino acids exhibits background levels of eYFP fluorescence (upper panel). Increased intracellular concentration of the respective amino acids induces *eyfp* expression in sensor cells (lower panel). (B) Phase contrast and fluorescence microscopy images of *C. glutamicum* sensor cells fed with 3 mM Lys-Ala (upper row) or Ala-Met (lower row) dipeptide. (C) Fluorescence spectrometry (488 nm excitation) of *C. glutamicum* sensor cells fed with 3 mM Ala-Met (green), Ala-Leu (red), Ala-Ile (brown), Ala-Val (purple). Controls include CGXII minimal medium (black), *C. glutamicum* WT cells (pink), sensor cells (blue) and sensor cells fed with Lys-Ala (dark red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of experiments, the capability of the system to monitor the intracellular accumulation of the respective amino acids was confirmed by fluorescence microscopy and spectroscopy. To artificially increase the intracellular concentration of specific amino acids, we used the dipeptide feeding strategy described previously (Vrljic et al., 1996). Dipeptides containing the amino acid under study were added to the growth medium; the uptake and subsequent hydrolysis of these dipeptides by cytoplasmic hydrolases leads to an increased pool of the respective amino acids.

C. glutamicum wild type cells transformed with the biosensor showed no discernable fluorescence signal above the autofluorescence of *C. glutamicum* wild type. When the intracellular concentration of L-methionine was increased by the addition of the dipeptide alanyl-methionine (Ala-Met), a significant increase in eYFP fluorescence resulted (Fig. 1B). As expected, elevated intracellular L-leucine, L-isoleucine or L-valine levels, achieved by feeding with Ala-Leu, Ala-Ile or Ala-Val, respectively, also resulted in higher fluorescent output by the biosensor cells, as shown by fluorescence spectroscopy (488 nm excitation). No significant signal was obtained upon addition of Ala-Ala or Ala-Lys or with cells containing the empty vector pJC1. The highest intensity of fluorescence was observed for L-methionine, and decreased from L-leucine to L-isoleucine to L-valine (Fig. 1C). These results demonstrate the functionality of the biosensor: Intracellular detection of elevated levels of L-methionine and branched-chain amino acids is transformed into an optical readout.

3.2. Quantitative characterization of the biosensor response

One prerequisite for the application of biosensors is an accurate description of the relationship between effector input and reporter output. To assess this, we established a competitive dipeptide feeding assay to generate defined intracellular concentrations of amino acids. The approach is based on the principle that a competitor dipeptide, for example, Ala-Ala, competes with an effector-containing dipeptide, for example Ala-Met, for uptake by the cell, allowing internal effector levels to be regulated (see Section 2.4 and SI).

After the addition of dipeptide mixtures, the internal concentrations of amino acids reach distinct maxima within minutes (Fig. S1) (Trötschel et al., 2005). Thus, the approach provides a system to study the correlation between internal amino acid concentration and the resulting reporter output. Effector input and eYFP output was correlated for each Lrp effector amino acid, shown in Fig. 2A. There were linear fluorescence responses to the internal concentration of the amino acids across the concentration ranges tested.

The biosensor displayed highest sensitivity for L-methionine followed by L-leucine, L-isoleucine and L-valine (Fig. 2A, Table 2). Maximal induction in response to L-methionine accumulation was 78-fold, thereby defining the minimal dynamic range of the biosensor. However, due to restrictions in the kinetic properties of the system, such as dipeptide uptake and hydrolysis, the highest internal concentration achieved was 25 mM in *C. glutamicum* wild type. Consequently, the dynamic range and minimal linear detection range given in Table 2 represent minimal values but demonstrate the analog response of the biosensor in the low to medium mM range.

Next, the suitability of the biosensor system for FACS-based screening approaches was tested. Cells fed with dipeptides (containing one of the four Lrp effector amino acids) to the previously determined maximal internal amino acid concentration were sampled two hours after addition of the dipeptides (the time point with the highest fluorescence signal). Then, three or four samples of cells each containing a different internal concentration of a particular amino acid were mixed in a ratio of 1:1:1(:1) and immediately analyzed by flow cytometry. (Fig. 2B–E). Distinct populations of cells with increased internal effector concentrations were found, although these displayed no differences in terms of morphological parameters (side scatter, SSC and forward scatter, FSC, see raw data used for color accentuation in Fig. S2). In the case of L-methionine, concentrations below 1 mM generated a population clearly distinguishable from wild type with respect to eYFP fluorescence. Thus, the biosensor-flow cytometry combination can discriminate subpopulations of cells that exhibit slight differences in the internal accumulation of amino acids in the low to medium mM range – the concentration range relevant to the development of production strains.

3.3. Biosensor-based online monitoring of production strains

To further assess the performance of the biosensor in online monitoring of production strains, we chose the *C. glutamicum* strain Δ*aceE*. This strain lacks the E1p enzyme of the pyruvate dehydrogenase complex and excretes low amounts of L-valine when cultivated in minimal medium with 4% glucose and 1.5% acetate. Production of L-valine *via* the metabolization of glucose has been shown to begin after all of the acetate is consumed (Blombach et al., 2007).

In accordance with published data, *C. glutamicum* Δ*aceE* containing the biosensor grew exponentially without producing L-valine for the first ten hours. Once acetate was depleted, the cells stopped growing and started to produce L-valine. The sensor responded with eYFP fluorescence that correlated with the increased production of L-valine (Fig. 3A). Three hours after the start of L-valine production the signal reached a maximum value, suggesting that a constant internal L-valine concentration is reached and maintained. In contrast, *C. glutamicum* wild type containing the sensor plasmid showed no significant eYFP emission.

3.4. Analysis of L-valine production by C. glutamicum ΔaceE at the single-cell level

As the Lrp-based biosensor allows the visualization of internal amino acid levels in single cells, we analyzed L-valine production by *C. glutamicum* Δ*aceE* cells in isogenic microcolonies. For this purpose, cells were cultivated in PDMS-based microfluidic devices. In contrast to microcolony growth on agar pads, as commonly used, cultivation in microfluidic chips offers the advantage of continuous supply of medium, oxygen, constant temperature and pH, and the ability to switch between different cultivation media.

Initially, single *C. glutamicum* Δ*aceE* sensor cells were tracked in the microfluidic chamber and isogenic microcolonies were grown in minimal medium with 4% glucose and 1.5% acetate. After 15 h the medium was switched to conditions supporting L-valine production and the fluorescent output was measured (Fig. 3B). Significant variations in fluorescence intensity were observed between single cells, indicating different production levels of L-valine. A lineage tree based on the growth of the microcolony revealed another level of heterogeneity, namely variation in cell size and interdivision intervals among single cells (Fig. 3C). Doubling times for single duplication events ranged from 0.5–5 h. In comparison, the parental *C. glutamicum* wild type has an average doubling time of about 70 min in the microfluidic system, with a range of 40–110 min (data not shown).

When an average growth rate was calculated from the lineage tree, the result ($0.24\ h^{-1}$) was comparable to the growth rate of Δ*aceE* in shake flask cultures. We stopped medium flux through the microfluidic device, thereby simulating batch culture

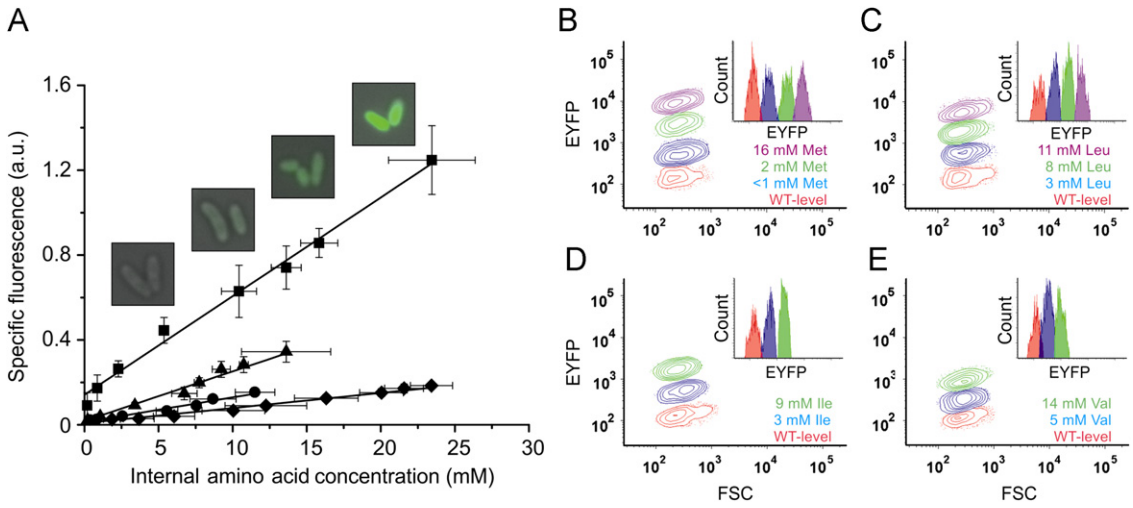

Fig. 2. Characterization of the specificity and affinity of the biosensor. (A) Relationship between intracellular concentrations of effector amino acids L-methionine (■), L-leucine (▲), L-isoleucine (●) and L-valine (♦) and specific fluorescence outputs in *C. glutamicum* cells. Data represent the average values of three independent measurements. The fluorescence microscopy images show *C. glutamicum* sensor cells with increasing internal concentrations of L-methionine (1–20 mM). (B)–(E): FACS-generated contour plots and histograms displaying the FSC signal (forward scatter) and/or the eYFP signal of mixed *C. glutamicum* populations differing in their intracellular amino acid concentrations of (B) Met, (C) Leu, (D) Ile and (E) Val. No variation in terms of morphology parameters (forward scatter (FSC) and side scatter (SSC)) was observed for the populations. For analysis cells fed with dipeptides to the known internal amino acid concentration were mixed and immediately analyzed by flow cytometry. See Fig. S2 for FSC/SSC plots and gating strategy used for color accentuation.

Table 2
Performance parameters for the biosensor ($n=3$).

Effector	Minimal dynamic range (fold increase)	Minimal linear detection range (mM)
L-methionine	> 78	> 0.2–23.5
L-leucine	> 22	> 0.2–13.6
L-isoleucine	> 10	> 0.4–11.5
L-valine	> 12	> 1.5–23.4

conditions in which secreted L-valine accumulates extracellularly. Under these conditions, cell-to-cell variation decreased significantly as cells within the microcolony with low fluorescent signals caught up (Fig. S3). Thus, this sensor-based analysis of an L-valine producer uncovers phenotypic heterogeneity at the single-cell level which is masked by typical bulk measurements.

3.5. Sorting efficiency

In further experiments, the strain Δ*aceE* was used to examine the sensor-based sorting efficiency of the FACS system. Mixtures of *C. glutamicum* wildtype and fluorescing *C. glutamicum* Δ*aceE* cells (1% *vs.* 99% and *vice versa*) were analyzed after 24 h of cultivation by FACS and single cells were sorted on agar plates (1500 cells in total). The two strains could be distinguished by the small colony phenotype of Δ*aceE*. Colony PCR analysis of the sorted cells revealed a > 95% correct sorting efficiency and a survival rate of *ca.* 96%. This demonstrates the suitability of the system for isolating single mutants/cells which exhibit increased amino acid production out of a mixture of non-producing cells.

3.6. Biosensor-based FACS high-throughput screen

We next developed a high-throughput-compatible sensor system to screen large mutant libraries in order to detect cells with increased levels of L-methionine or branched-chain amino acids. For a primary screen, wild type cells containing the sensor plasmid were chemically mutagenized by incubation with N′-methyl-N′-nitro-N-nitrosoguanidine, as described in Section 2.1. Subsequent to mutagenesis, *C. glutamicum* cells were incubated in BHIS medium for 6 h and then analyzed and sorted by FACS. As depicted in Fig. 4A, parallel approaches were taken. In screen S1, 150 mutants that exhibited significantly increased fluorescence were sorted out of *ca.* 6 million cells from gate S1-P1 on BHI plates (survival rate: 60%, Fig. 4D). 48 colony forming mutants were used to inoculate a 48-well plate containing CGXII medium with 4% glucose, 1.5% acetate and 0.1% yeast extract. Acetate was added to the screening medium to support growth of L-valine producing mutants with decreased pyruvate dehydrogenase activity. After 48 h of cultivation in microtiter plates, amino acid concentrations in the supernatant were assayed by uHPLC. This approach (S1 in Fig. 4A) yielded 23% positive clones (11 out of 48). *Via* the chosen screening conditions especially L-valine-excreting mutants were obtained – all positive clones produced significant amounts of this amino acid. 10% of the mutants also produced up to 2 mM L-leucine and L-isoleucine (for an overview of all screened mutants, see Table S1). An iterative approach was developed (S2) to screen for clones that do not have significant growth defects under the chosen conditions. In screen S2, cells were cultivated for 24 h after mutagenesis and mutants with increased eYFP emission were sorted into liquid BHIS complex medium and grown at 30 °C. After 24 h 10,000 cells with most enhanced fluorescence emission were again sorted into a BHIS containing tube and cultivated for 24 h. This procedure of isolating and cultivating the upper 10,000 fluorescence cells was repeated three times.

The iterative screen resulted in an up-shift of eYFP fluorescence in the whole population (Fig. 4C). Due to the wide distribution of the fluorescence signal, the cells were subdivided into three populations: S2-P1 (highest fluorescence), S2-P2 (medium fluorescence) and S2-P3 (low fluorescence). A clear pattern was observed in these subpopulations: in the groups of cells exhibiting middle (S2-P2) and low levels of fluorescence (S2-P3), 6% and 2% of the tested clones excreted significant amounts of L-valine, respectively; within the subpopulation that produced high levels of fluorescence (S2-P1), 25% excreted significant amounts of L-valine (Fig. 4D). The survival rate of the sorted cells increased significantly from 59% (S1) to almost 96% on the fourth day of the iterative screen (S2).

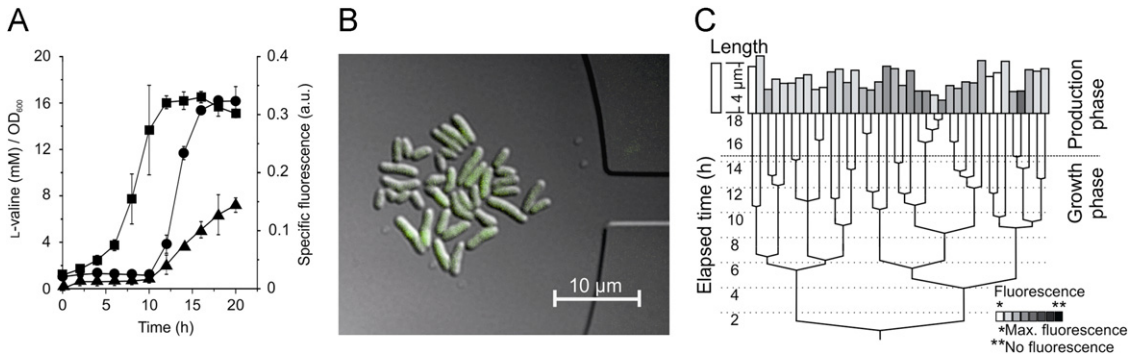

Fig. 3. Biosensor-based analysis of *C. glutamicum* Δ*aceE*. (A) Growth (■), eYFP fluorescence (●) and L-valine production (▲) of *C. glutamicum* Δ*aceE* cultivated in minimal medium containing 4% glucose and 1.5% acetate. Data represent average values of three independent cultivations. (B) A microcolony and (C) respective lineage tree of *C. glutamicum* Δ*aceE* containing the biosensor plasmid grown in a microfluidic chamber, illustrating variation at the single-cell level. Growth phase: minimal medium containing 4% glucose and 1.5% acetate. Production phase: minimal medium with 4% glucose. EYFP fluorescence was quantified in single cells after 18 h. The length of the boxes corresponds to the length of the cells at the time of fluorescence measurement. The lineage tree is based on Video S1 (growth phase).

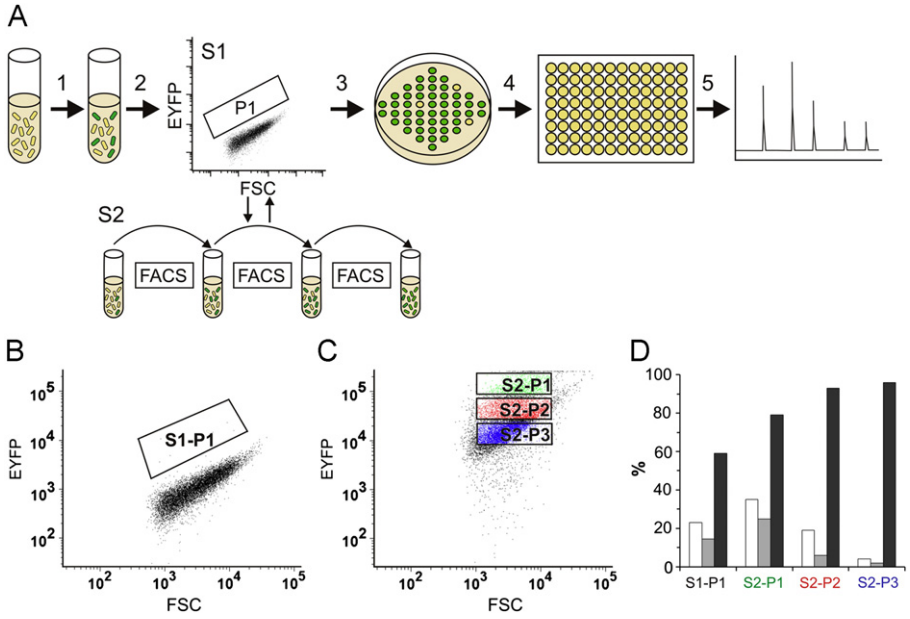

Fig. 4. Biosensor-based FACS high-throughput screening. (A) Schematic diagram of FACS screening: (1) Mutagenesis, (2) Screening approach S1: fluorescent cells were sorted by FACS, Screening approach S2: The 10,000 mutants with highest levels of eYFP emission were sorted in liquid medium, isolated and re-cultivated; this procedure of sorting and re-cultivation was repeated three times. (3) Sorting on plates, (4) cultivation in microtiter plates (48 h) and (5) uHPLC analysis of supernatant. (B) Dot plot displaying the FCS signal against the eYFP signal of mutagenized *C. glutamicum* cells six hours after mutagenesis and subsequent incubation in BHIS medium. Cells were sorted from gate S1-P1. (C) Dot plot (FSC/eYFP) of mutagenized cells on the fourth day of the iterative screen. Cells were sorted on BHI agar plates from gates S2-P1, S2-P2, and S2-P3. (D) The percentage of positive clones (white bars), those excreting >2 mM effector amino acids (gray bars) and viability of clones after sorting (black bars).

Overall, the top five mutants obtained from FACS screening (S1 and S2) produced up to 8 mM L-valine, up to 2 mM of L-isoleucine and up to1 mM L-isoleucine after 48 h in minimal medium with 4% glucose, 1.5% acetate and 0.1% yeast extract (Table 3). Growth and sensor output of the top five mutants are shown in Fig. S4. In two of the top five mutants PDH activity was about twofold decreased in comparison to the WT (mutant 2: 0.049 ± 0.001 U/mg, mutant 4: 0.043 ± 0.007 U/mg, WT: 0.082 ± 0.01 U/mg), which could be a reason for L-valine excretion. However, no mutation in the gene sequence of *aceE* could be identified in these mutants.

Table 3
Top five mutants excreting branched-chain amino acids ($n=2$).

Mutant no.	L-valine (mM)	L-leucine (mM)	L-isoleucine (mM)
1	8.7	1.3	0.9
2	5.1	1.0	1.3
3	4.8	1.0	1.0
4	4.6	n.d.	2.1
5	4.6	1.2	1.2

4. Discussion

Nature has evolved versatile sensor devices for small molecules, metabolites and physical parameters in the form of proteins, RNA and DNA. We harnessed the functionality of one of these – the transcriptional regulator Lrp – to report on *in vivo* amino acid production in *C. glutamicum*, an organism of considerable importance to the biotechnology industry. This Lrp-based biosensor detects intracellular L-methionine and the branched-chain amino acids L-leucine, L-isoleucine and L-valine by transforming the information into a fluorescent output signal.

Screening and analysis of production strains using a biosensor requires that the relationship between the effector molecule input and reporter output is well understood. To achieve this,

we favoured an *in vivo* determination of the sensor transfer curve. Although the dipeptide feeding approach that we used was restricted by the kinetics of peptide uptake and hydrolysis, the minimal dynamic range and linear detection range (described in Table 2) covers the low to medium mM range. This range makes the system suitable for the analysis and screening of production strains as it allows the discrimination of strains with significantly elevated intracellular amino acid levels (mM range) from wild type level (nM to high μM range). A few studies report on the intracellular concentration of amino acids in production strains, but the high external accumulation of the respective metabolite often makes an exact determination difficult. Eggeling and co-workers reported an accumulation of up to 110 mM isoleucine in the isoleucine-producing strain *C. glutamicum* SM13 (Morbach et al., 1996). In-house measurements of the internal L-lysine concentration of several lysine production strains revealed a concentration ranging from 5–30 mM L-lysine, depending on the strain background (Eggeling, unpublished). In the case of the PDHC-deficient L-valine producers *C. glutamicum* Δ*aceE* and Δ*aceE*(pJC4-ilvBNCE), the internal concentration of the L-valine precursor pyruvate amounts to 25 and 2.3 mM, respectively (Blombach et al., 2007). If the assessment of higher accumulations of internal amino acids with the described sensor system is desired, exporter deletion strains could be used, which have been reported to accumulate up to 0.5–1 M of the respective amino acids (Kennerknecht et al., 2002; Vrljic et al., 1996). However, this approach seems inappropriate, since comparability to the wild type strain in regard to the formation of a distinct internal amino acid peak is hardly given.

The sensor technology was used in an ultra high-throughput FACS screen (10,000 cells per second) to isolate cells with increased intracellular concentrations of the effector amino acids following random mutagenesis. Our first screening approach resulted in the isolation of about 40 mutants that produced branched-chain amino acids, out of 192 screened by uHPLC (Fig. 4, Table S1). Among these, the top five mutants produced up to 8 mM L-valine, up to 2 mM of L-leucine and up to 1 mM L-isoleucine. As known from previous studies, the activity of the PDH complex is crucial for the production of L-valine (Bartek et al., 2011; Blombach et al., 2007). Indeed, two of the top five mutants showed a reduced PDH activity, but sequencing of the *aceE* revealed wild type situation in all of the top five mutants. Further reasons for a reduced PDH activity might be for example an impaired cofactor biosynthesis or mutations in genes encoding regulatory proteins (Bartek et al., 2010). For detailed insights genomic analysis of promising mutants is required for identification of novel and unexpected targets for strain improvement. For example, a melanin-based screen for L-tyrosine led to the identification of mutants in unknown proteins which could not have been predicted by rational strain design (Santos and Stephanopoulos, 2008).

As far as we are aware, this is the first biosensor-based FACS screen for the *in vivo* detection of inconspicuous, biotechnologically-relevant products, such as amino acids, in single bacterial cells (Dietrich et al., 2010). Previously, targets for FACS screenings have included metabolites that are natural or synthetic photophores, such as carotenoids (Albrecht et al., 2000; An et al., 1991), the antibiotic gramicidin S (Azuma et al., 1992) or polyhydroxyalkanoates (Vidal-Mas et al., 2001). A common problem of FACS screenings is the high rate of false positives, in some cases beyond 99% (Sitnikov et al., 1995). In our Lrp biosensor-based screening, secondary screening revealed that 20–30% (depending on the gating strategy) of the isolated mutants excreted significant amounts of the effector amino acids. Among so-called "false-positive" clones we will certainly find several mutants which intracellularly accumulate high amounts of the respective amino acids, but might have a defect in export of those (*e.g.* mutation of *brnFE* itself). Thus, we emphasize this system also for the identification of transport systems. This could, for example, easily be realized by screening transposon-mutagenized sensor cells for cells with significantly increased fluorescent output.

In these experiments, the screening conditions led to isolation of mutants producing branched-chain amino acids. However, the outcome of screens can be influenced by several factors, including cultivation conditions, the original strain used for mutagenesis and the gating strategy used in FACS. As L-methionine is currently of major interest for biotechnological strain development, future work will adapt the screening procedure for the isolation of L-methionine-producing mutants. Prevention of reuptake (Δ*metNI* and Δ*metP*) (Trötschel et al., 2008), derepression of L-methionine biosynthesis genes (Rey et al., 2003) and the addition of reduced sulfur compounds such as methanethiol or dimethyldisulfide, might be important prerequisites for the successful isolation of L-methionine producing mutants in future screening approaches (Bolten et al., 2010).

The intracellular, rather than extracellular, detection of a specific metabolite is a prerequisite for a biosensor system compatible with high-throughput FACS screening or single cell analysis of production strains. Systems, such as the one described in this study, allow the rapid isolation of mutations, but cannot provide information with respect to the excretion of the particular metabolite. Thus, a quantitative analysis of the extracellular metabolite concentrations by uHPLC or mass spectrometry is required to determine whether the isolated mutants excrete the respective metabolite in the supernatant. As the described biosensor system depends on the use of a transcriptional regulator (Lrp) as the sensor device, specificity and dynamic range of the respective system depends on the biochemical characteristics of the chosen regulator. These can, in principle, be optimized by protein engineering and/or evolution. However, in many cases more than one specific effector metabolite influences the activity of a given regulator; in case of Lrp four amino acids (L-methionine and the branched-chain amino acids) have been described (Lange et al., 2011). Thus, the intracellular detection of more than one effector metabolite as well as the typically high rate of false positive clones in FACS screenings clearly necessitates secondary screening, for example, uHPLC, for reliable mutant analysis. The use of GFP-based autofluorescent proteins as reporter proteins also have some pros and cons. The stable, non-cytotoxic expression of *gfp* and its derivatives, plus their outstanding brightness, make them a favorable option (Chudakov et al., 2005; Frommer et al., 2009; Giepmans et al., 2006). However, signal delay due to chromophor maturation or the dependency on molecular oxygen are drawbacks (Shaner et al., 2005; Tsien, 1998).

It has long been known that clonal populations of microorganisms display substantial variation in phenotypic traits. Reasons for this population heterogeneity include micro-environmental differences, variations in cell age, stages in the cell cycle and stochastic effects (Elowitz et al., 2002; Lidstrom and Konopka, 2010). Cell-to-cell variation with regard to promoter activity or protein abundance is easily detected using autofluorescent proteins but there has been little study of single-cell quantification of metabolites (Heinemann and Zenobi, 2011). Biosensors, in the form of FRET sensors, and regulatory circuits do provide powerful tools for single-cell resolution (Deuschle et al., 2005; Michener et al., in press; van der Meer and Belkin, 2010; Zhang and Keasling, 2011). In this study, we achieved a first glimpse of variation in productivity using the PDHC-deficient L-valine producer *C. glutamicum* Δ*aceE*. Our analysis of microcolonies grown from one single cell revealed variations in doubling time, cell size and, most interestingly, single-cell fluorescence. Such effects could not be detected in shake flask experiments where L-valine

accumulates in the supernatant and in turn influences internal levels of L-valine. Indeed, stopping the flow of medium through the microfluidic chamber almost completely masked cell-to-cell variation (Fig. S3). The origin of the variability in internal L-valine accumulation among Δ*aceE* cells will be assessed in future studies. Factors reported to influence L-valine production in *C. glutamicum* include the expression level of L-valine biosynthesic genes, the activity of the PDHC by-pass pyruvate:quinone oxidoreductase, as well as the supply of NADH, depending on the flux through the pentose phosphate pathway (Bartek et al., 2010; Blombach et al., 2008).

5. Conclusions

Biosensors based on transcriptional regulators have a successful history in bioengineering, where they are used to detect environmental pollutants including antibiotics, carcinogens and heavy metals (van der Meer and Belkin, 2010; Zhang and Keasling, 2011). However, the great potential of transcription factor-based sensor technologies for strain development and screening strategies in industrial biotechnology has not been exploited at all so far. The technology described here initiatives that process: it can be applied to an extensive repertoire of natural sensor devices for the design of biosensors that detect a broad spectrum of important biotechnological metabolites.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2012.02.002.

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