Looking for the pick of the bunch: high-throughput screening of producing microorganisms with biosensors

Marcus Schallmey, Julia Frunzke, Lothar Eggeling and Jan Marienhagen

The engineering of microbial strains for the production of small molecules of biotechnological interest is a time-consuming, laborious and expensive process. This can be mostly attributed to the fact that good producers cannot be readily obtained by high-throughput screening approaches since increased product formation usually does not confer a clear phenotype to producing strain variants. Recently, advances were made in the design and construction of genetically encoded RNA aptamer-based or transcription factor-based biosensors for detecting small molecules at the single-cell level. The first promising examples for the application of these molecular biosensors in combination with fluorescent-activated cell sorting as a high-throughput screening device demonstrated the value and potential of these new tools for microbial strain development.

Addresses
Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich GmbH, Jülich D-52425, Germany

Corresponding author: Marienhagen, Jan (j.marienhagen@fz-juelich.de)

Introduction

The metabolic power of microorganisms to produce small molecules of biotechnological interest from simple substrates is truly amazing! However, to date, only a few compounds — such as amino acids for applications in food and feed industries — have been microbially produced at scales exceeding 5 000 000 tons/year. Against the background of dwindling crude oil resources, which represent the basis of our contemporary chemical industry, it is expected that many more small molecules will have to be produced by microorganisms from renewable resources in the future. Initially, improvement in the industrial properties of microorganisms was limited to random mutagenesis and selection, but increasing knowledge of the microbial physiology and its regulation, along with the advent of recombinant DNA technology and new DNA sequencing technologies, have dramatically expanded the capabilities to also rationally modify the cellular metabolism for increased synthesis of a specific metabolite. As a result of this development, a tremendous number of different genetic variations of a microbial production strain can be easily constructed by various methods, either *in vivo* or *in vitro*. However, whether strains are randomly mutated or rationally engineered, each genetic variant generally has to be cultivated and evaluated for its productivity individually, employing costly and low-throughput methods such as chromatography or mass spectrometry. The reason for this is that the synthesis of the majority of small molecules of biotechnological interest does not confer a conspicuous phenotype to the producing cells that would allow interfacing with high-throughput (HT) screening technologies. Among the few exceptions are natural chromophores, such as carotenoids [1], or small molecules whose production can be linked to the formation of a colored pigment, as is the case for L-tyrosine, which can be enzymatically converted to the pigment melanin [2]. In such cases, color formation enables direct screening for more intensely colored colonies or cultures using standard assay techniques in a medium-throughput format (microtiter plates or agar plates, respectively).

More powerful in terms of throughput is linking small metabolite production to survival or fitness of the producing microorganism. In a few cases, this can be easily achieved if the target compound is essential for growth of the producer (e.g. by employing classical anti-metabolite selection) or growth of a co-cultivated auxotrophic reporter strain. Other examples include the application of transcriptional regulators that recognize the desired product and respond by expression of a reporter gene, conferring resistance to an antibiotic [3]. The drawback of such strategies relying on survival or improved fitness of a producer is the limited applicability if a production strain, which already produces the small metabolite in question, has to be further improved.

Whereas the laborious and time-consuming screening of microorganisms on agar plates or in microtiter plates practically limits the screenable library size to 10^6 clones, fluorescence-activated cell sorting (FACS) enables the evaluation of very large libraries comprising up to 10^9 clones [4]. Here, tens of thousands of cells can be analyzed per second at the single-cell level for size and

fluorescent signals in response to excitation with laser light of a set wavelength. In principle, FACS can be directly applied for the engineering of microorganisms to produce small molecules, which are either fluorescent or can be stained with fluorescent dyes. For example, FACS was successfully used to isolate improved microbial producers of the fluorescent carotenoid astaxanthin from a library of more than 10^6 clones in only one hour, offering a throughput that was about two orders of magnitude higher and screening that was substantially faster than conventional screenings in microtiter plates [5]. In cases where target compounds are non-fluorescent, dyes detecting physiology-associated changes in response to increased product formation can be applied. This was shown, for example, by staining viable cells accumulating the biodegradable polyester poly-3-hydroxybutyrate with nile red [6] or *Bacillus brevis* producing gramicidin S with fluorescein isothiocyanate [7]. Regrettably, the majority of biotechnologically interesting small molecules are either non-fluorescent or appropriate dyes are not available.

However, in living cells a broad spectrum of different metabolites is detected by an extensive repertoire of natural sensor devices such as riboswitches, transcription factors or enzymes. Recently, genetically encoded biosensors on the basis of such cellular regulatory elements were developed, which specifically translate the intracellular concentration of a target compound into a graded fluorescence output by driving the expression of autofluorescent proteins (AFPs). In combination with FACS, biosensors have the potential to completely change the way microbial production strains will be engineered in the future. This review will summarize the advances made over the last five years in the emerging field of biosensor-based FACS screening of microbial production strains.

RNA-based biosensors

Riboswitches are RNA-based gene regulatory elements that couple an RNA sensing function, encoded in an aptamer, to a gene-regulatory output. Ligand binding to the aptamer directs a structural change within the RNA molecule and thereby modulating the activity of the gene-regulatory domain. By this means, ligand binding can affect translation efficiency (e.g. by modulating the availability of a ribosome binding site), mRNA or protein stability or, in the case of ribozymes, even control the enzymatic activity of the particular RNA molecule itself [8].

For the construction of an RNA-based small-molecule sensor, researchers can either take advantage of nature by transferring a known aptamer structure from its native context into a synthetic circuit, or use molecular evolution and/or computational design to generate new RNA component functions *de novo* [9,10]. The diversity of RNA-based control mechanisms in living cells has inspired many applications of synthetic RNA devices. In one study, Win and Smolke applied a natural xanthine-responsive RNA device controlling expression of *gfp* (green fluorescent protein, GFP) for metabolite detection in single yeast cells [11] (Figure 1a). The endogenous conversion of added xanthosine to xanthine in cells harboring this particular RNA sensor resulted in an increase in fluorescence, which directly correlated with product accumulation as confirmed by LC–MS. The same group used an engineered RNA switch based on a theophylline-responsive aptamer to screen large libraries of mutated caffeine demethylases for increased enzyme activity and product selectivity, employing FACS [12••]. Seven iterative rounds of FACS screening in combination with flow cytometry plate-based screening yielded an enzyme variant with 33-fold increased enzyme activity and 22-fold increased product selectivity. This approach offers a major advantage over conventional directed evolution techniques: the enzyme activity is directly optimized *in vivo*. Although this is an example of biosensor-guided engineering of a single protein, the principle might be directly transferable to the engineering of whole metabolic pathways or host systems.

As early as a decade before the first description of natural riboswitches [13], metabolite-binding aptamers were generated *in vitro* by the 'systematic evolution of ligands by exponential enrichment' (SELEX) method [14,15]. Using SELEX, a pool of about $10^{13–15}$ randomized sequences can be screened for binding to an immobilized metabolite *in vitro*. Immobilization of the metabolite often requires its modification to introduce reactive groups for coupling. Consequently, aptamers selected *in vitro* might not function in the more complex *in vivo* environment.

Recently, RNA aptamers that intracellularly bind fluorophores and thus mimic AFPs were developed [16]. The subsequent fusion of such a fluorophore-binding aptamer (output domain) to an additional aptamer that specifically binds a small molecule (sensing-domain) via a stem sequence (transducer) rendered the fluorescence dependent on the presence of the small molecule [17•] (Figure 1b). These biosensors allow for the imaging of dynamic changes and cell-to-cell variation of intracellular small-molecule levels (e.g. ADP and S-adenosylmethionine) but they have not yet been used for HT screenings.

Transcription factor-based biosensors

Many transcription factors (TFs) control a promoter's transcriptional output in response to binding of a small molecule, thereby reporting on the concentration of the molecule in the cell. Such TFs have long been used to construct whole-cell biosensors for the detection of environmental pollutants, such as arsenite [18] or polychlorinated biphenyls [19], by linking ligand concentrations to the expression of reporter proteins [20]. An early example, employing transcription factors in a non-environmental but biotechnological context was

Figure 1
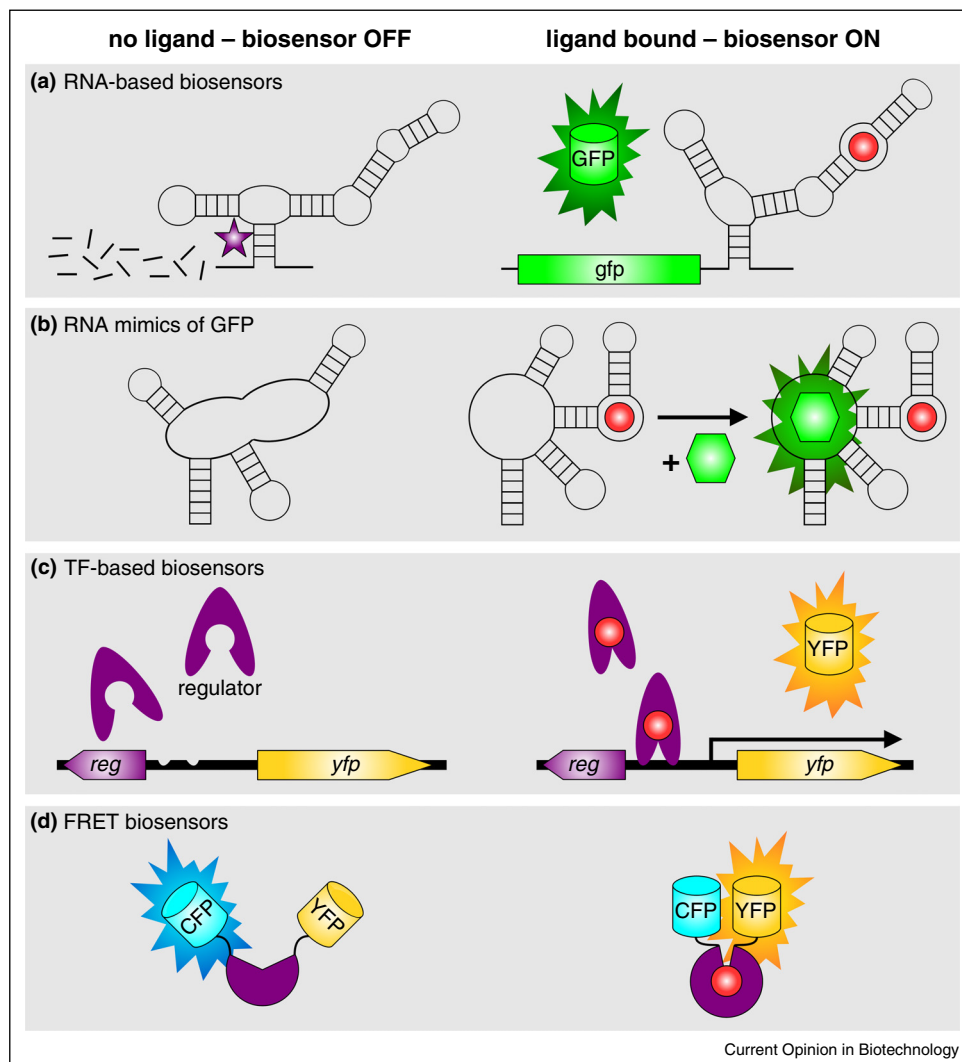
Schematic representation of different biosensors and their respective modes of action. The biosensors are depicted in their OFF state (left) and ON state (right) in the presence of the small metabolite of interest (red dot). **(a)** RNA-based biosensors. In the absence of the small metabolite, a cis-acting ribozyme is active (purple star) and rapidly degrades the mRNA encoding the green fluorescent protein (GFP) (low fluorescence). The presence and binding of the small molecule to an aptamer in the same molecule favors the misfolded and inactive conformation of the ribozyme. In consequence, the mRNA is more stable, resulting in higher *gfp* expression and increased fluorescence. This principle was used to design biosensors for theophylline and tetracycline [11,12]. **(b)** RNA mimics of GFP. A fluorescence signal first requires small-metabolite binding to an aptamer, which then promotes the proper folding of an additional fluorophore-binding aptamer in the same RNA molecule. With biosensors following this design principle, concentrations of various small molecules such as ADP and *S*-adenosylmethionine could be monitored [16,17]. **(c)** TF-based biosensors. In response to the presence of the small metabolite, a transcriptional regulator (encoded by *reg*) binds to the *yfp* promoter and activates expression of the yellow fluorescent protein (YFP). Such TF-based biosensors have been constructed on the basis of the regulators LysG [24••] and Lrp [25•] of *C. glutamicum* to detect elevated intracellular amino acid concentrations. **(d)** FRET biosensors. These biosensors take advantage of the Förster resonance energy transfer (FRET), and are based on autofluorescent protein pairs, such as the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP) which function as FRET donor and acceptor, respectively. Both proteins are separated by a sensory domain, which undergoes conformational rearrangements upon small-metabolite binding and thus modulates the ratio of CFP/YFP fluorescence. As an example, a maltose FRET sensor was developed using the periplasmic maltose binding protein from *E. coli* [33].

published by Witholt and co-workers. They utilized a mutant of the transcriptional activator NahR from *Pseudomonas putida* for the specific detection of benzoate and 2-hydroxybenzoate production in *Escherichia coli* [3]. Here, the NahR mutant controlled the expression of reporter genes, such as the tetracycline resistance gene

tetA, which allowed for the selection of clones capable of reducing benzaldehyde and 2-hydroxybenzaldehyde. Clones with improved reduction of added benzaldehyde and 2-hydroxybenzaldehyde could be selected on tetracycline-containing agar plates at a ratio of 10^{-6} from a large library of 10^7. More recently, this approach was

extended towards the HT screening of microorganisms producing small molecules at the single-cell level by employing TFs that induce the product-dependent expression of AFPs in combination with FACS. Product-induced gene expression (PIGEX) was one of the first examples to exploit a TF for product-dependent *gfp* expression in a FACS-based HT screening of large libraries [21]. For this purpose, metagenomic libraries from activated sludge were screened in *E. coli* for the expression of functional benzamidases, which hydrolyze benzamide to benzoate, the latter being an effector for the transcriptional regulator BenR from *P. putida*. In another example, a biosensor based on a variant of the transcriptional activator AraC was used in *E. coli* to detect clones with increased intracellular mevalonate concentrations, an important precursor for the production of isoprenoids [22•]. Interestingly, AraC does not naturally recognize mevalonate and first had to be engineered to induce gene expression in the presence of this molecule. In a subsequent study, AraC was engineered to recognize triacetic acid lactone, an important intermediate in organic synthesis and involved in polyketide synthesis [23]. This TF-based biosensor might also be suitable for the HT screening for improved microbial triacetic acid lactone producers.

TF-based biosensors have been intensively used to engineer the industrial amino acid producer *Corynebacterium glutamicum* towards increased productivities. Eggeling and co-workers described the construction and application of an L-lysine biosensor on the basis of the TF LysG, which activates expression of the basic amino acid exporter LysE in response to elevated intracellular levels of basic amino acids [24••] (Figure 1c). The plasmid-based biosensor comprising the gene for LysG and the promoter sequence of *lysE* in front of the enhanced yellow fluorescent protein gene *eyfp* as reporter gene was used to screen a library of more than 10^7 mutagenized *C. glutamicum* cells by FACS for increased fluorescence, yielding numerous L-lysine producers (Figure 2). The genome sequencing of several clones that do not harbor mutations in genes known to contribute to L-lysine synthesis identified novel genetic targets for the metabolic engineering of L-lysine overproducers. Similarly, Frunzke and co-workers used the L-leucine-responsive transcription factor Lrp of *C. glutamicum* to detect intracellular accumulation of L-methionine and branched-chain amino acids L-leucine, L-isoleucine and L-valine [25•]. The biosensor is composed of a transcriptional fusion of the promoter of *brnFE* to *eyfp*. In its native context, the operon *brnFE* encodes an amino acid permease, which facilitates the export of these amino acids. FACS was successfully employed to isolate producers of L-valine and other branched-chain amino acids after random mutagenesis of *C. glutamicum*. In general, the construction of biosensors on the basis of TFs controlling specific small-molecule exporter proteins seems to be a promising strategy, since several other sensors, for example for

Figure 2
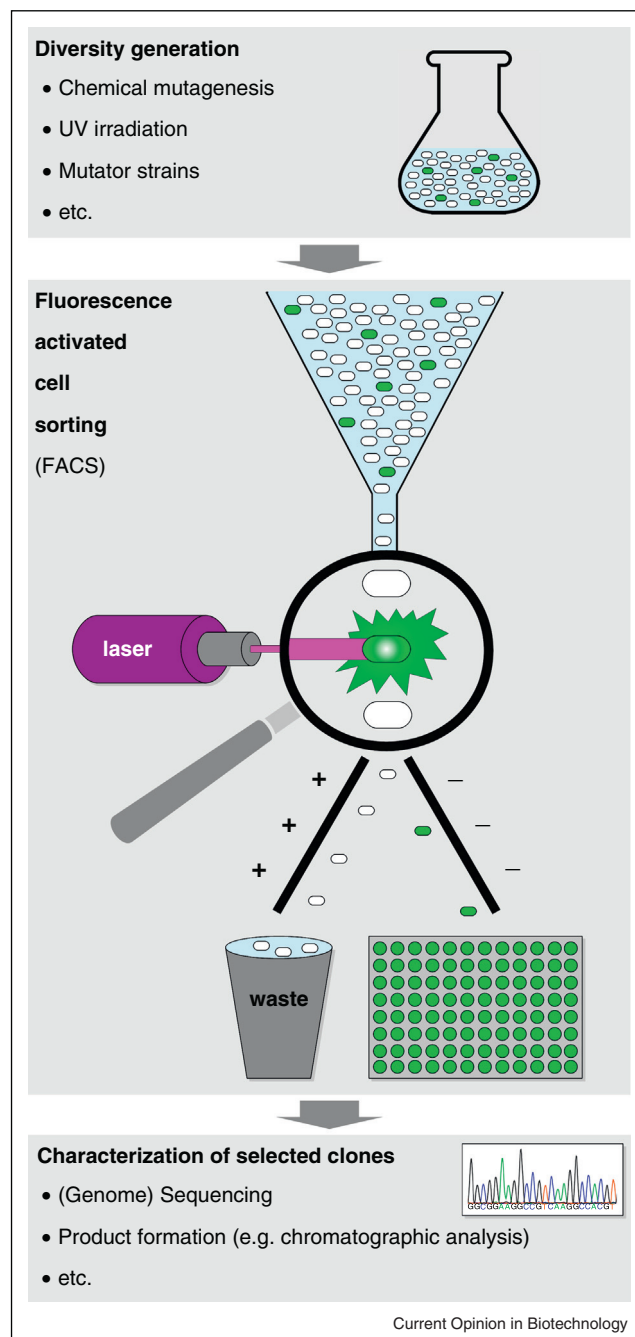
Schematic representation of biosensor-based FACS screening of microorganisms producing small metabolites. First, a genetically diverse culture of microorganisms harboring a biosensor is generated. After cultivation, cells showing fluorescence in response to elevated intracellular metabolite concentrations are sorted by FACS and collected (e.g. in microtiter plates). Subsequently, selected cells can be characterized in detail in terms of product formation or underlying (genomic) mutations [24,25•].

L-serine or *O*-acetyl-L-serine sensors were constructed following the same design principle [24••]. This can be explained by the fact that TFs have evolved to report on intracellular amino acid accumulation with a binding constant typically in the low millimolar range. Consequently, they can be easily used for the construction of biosensors suitable for discriminating microbial productions with significantly elevated intracellular amino acid levels (mM range) from wild-type cells (intracellular amino acid levels ranging from nM to high μM).

Additional examples of TF-based biosensors for detecting biotechnologically relevant small molecules in combination with flow cytometry include sensors for fatty acid CoA thioesters, 2-hydroxybenzoate, adipate and succinate, which might be suitable for FACS screening [26,27]. Of special interest is a 1-butanol sensor on the basis of the TF BmoR from *P. putida* because 1-butanol can be detected in a linear dynamic range from 0.01 mM to 40 mM in *E. coli* [27]. The drawback of this biosensor is the broad specificity for other short (branched) alcohol ligands such as 2-methyl-1-butanol and 1-hexanol, rendering it less feasible for the specific detection of 1-butanol. Very recently, a hybrid promoter-regulator system for malonyl-CoA sensing in *E. coli* was constructed, which could potentially control and optimize carbon flux leading to robust biosynthetic pathways for the production of malonyl-CoA-derived compounds [28].

The combination of TF-based biosensors with FACS also opens up new possibilities for the HT genome engineering of microbial producers at the single-cell level. As a first example, Eggeling and co-workers used oligonucleotide recombineering for the site saturation of a key residue in the *murE* gene involved in cell wall synthesis and previously identified to be beneficial for increased L-lysine production in *C. glutamicum* [29]. By using their aforementioned LysG-based L-lysine biosensor and FACS screening, several *murE* mutants could be isolated which accumulate significantly more L-lysine than the ancestor strains assayed.

FRET biosensors
Intracellular small molecule concentrations can also be determined by fluorescent biosensors which make use of Förster resonance energy transfer (FRET) induced upon small-molecule binding [30–32]. These biosensors are usually based on AFP pairs, such as the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP), which function as FRET donor and acceptor, respectively (Figure 1d). Both proteins are separated by a sensory domain, which undergoes conformational rearrangement after ligand binding. In the ligand-free state, CFP fluorescence dominates. The binding of a ligand results in structural changes of the sensory domain, which reduces the distance between CFP and YFP, and, as a consequence, leads to increased YFP fluorescence due to FRET. The

large family of periplasmic-binding proteins, in particular, have been successfully exploited as ligand-binding domains due to their Venus-flytrap structural reorganization upon the binding of ligands such as maltose [33].

While FRET biosensors are superior over TF-based biosensors in terms of short signal response, as they do not require the immanent time lag between the induction of gene expression and the AFP signal, FRET biosensing principles have not been considered for the HT screening of small-molecule producers to date. The reasons for this might be relatively low absolute signals, quantified as emission ratios of FRET acceptor over donor. Furthermore, FRET biosensors monitor changes in ligand concentrations in the nanomolar to micromolar range, making them unfeasible for the detection of higher concentrations usually encountered when engineering microbial strains for the production of small molecules. In fact, only a few FRET biosensors have been developed for biotechnologically interesting products, e.g. L-tryptophan [34] or L-glutamate [35], but none have been used in HT screens to date.

Conclusions
The metabolic engineering of microorganisms for the production of small molecules is a highly complex process, and product yields are dictated by a host of parameters, which are still difficult to predict. Therefore, HT screening that goes beyond the screening capabilities of microtiter plates is highly desired. Genetically encoded biosensors, which transform the presence of otherwise inconspicuous small molecules into an optical output at the single-cell level, in combination with FACS meet this demand, and will prove to be a valuable tool in metabolic engineering. Currently, such biosensors are usually constructed on the basis of natural genetic circuits, taking advantage of the ligand specificities of either RNA aptamers or TFs. Over the last few years, great progress has been made in this emerging field, and many more FACS-compatible biosensors are expected to become available in the near future.

However, several significant challenges still remain, representing opportunities for the development of a new generation of biosensors. Since current biosensors rely on natural genetic circuits, they can only recognize a very limited number of cellular metabolites, and are unable to detect compounds not naturally synthesized by the cell. Nonetheless, these compounds are of special interest, as a myriad of small molecules (polymer building blocks, fine chemicals, etc.) currently produced from crude oil by chemical means have to be microbially produced from renewable resources in the future. Hence, biosensors have to be engineered to also specifically detect such heterologous compounds in microbes. These custom-made biosensors should be very specific for the respective target molecule and should exhibit a large dynamic range to make

them useful tools for metabolic engineering. Furthermore, the application of biosensors in combination with FACS is currently limited to a few microorganisms, such as *E. coli, S. cerevisiae* and *C. glutamicum.* Therefore, expansion of this screening concept to other platform organisms of industrial biotechnology is highly desired.

It should also be noted that the application of the genetically encoded biosensors described here is not limited to HT screening by FACS. They can be also used for the online monitoring of metabolite production by live cell imaging using microfluidic lab-on-a-chip devices [36], and they could represent suitable tools for the monitoring of population dynamics [37].

Conflict of interest
The authors declare that there is no conflict of interest.

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