Single-cell metabolite sensors for high-throughput strain and enzyme development

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Transcriptional regulators, fluorescent reporters, FACS

Introduction. Bacteria are highly efficient biocatalysts for conversion of renewable carbon sources into a multitude of small molecules of industrial relevance, such as amino acids, organic acids, or vitamins. However, they are not naturally designed for profitable metabolite formation and efficient production strains have to be developed in order to establish a viable industrial process. There are two major approaches for strain development: random mutagenesis of the host strain followed by screening or selection and rational approaches, in which metabolism is altered in a targeted fashion by genetic engineering to optimize the flux from the substrate to the desired product [1]. Both approaches have been successful, however, the rational approach is limited as its targets are exclusively based on existing knowledge, which is incomplete. Moreover, point mutations that e.g. reduce or improve enzyme activities cannot be predicted. In contrast, random approaches target the entire genetic material irrespective of whether there is knowledge on its function. Many production strains used in industry are still based on the random approach. Using next-generation sequencing, it is possible nowadays to search for the relevant mutations in randomly generated producer strains and to combine beneficial mutations in the wild type, an approach termed "genome breeding" [2]. A bottleneck in the random approach is the necessity to screen large libraries of randomly mutagenized cells for those showing the desired phenotype. Unfortunately, many metabolites of industrial interest do not have an easily detectable phenotype, such as color, and therefore screening of clones by complex analytical methods is a laborious and time-consuming process. Here, we describe an approach which can overcome this limitation and allows high-throughput identification and isolation of single bacterial cells overproducing the metabolite of interest.

Results. A novel method for visualizing intracellular metabolite concentrations within single cells of *Escherichia coli* and *Corynebacterium glutamicum* was developed that expedites the screening process of producer strains. It is based on transcriptional regulators, which naturally detect a variety of small ligands, such as amino acids, sugars, sugar phosphates, vitamins etc., but can also be engineered to detect the compound of interest [5-6]. In our proof-of-concept studies, we constructed a single-cell L-lysine sensor and used it to isolate new L-lysine producing mutants of *C. glutamicum* from a large library of randomly mutagenized cells using fluorescence-activated cell sorting (FACS) [3]. The L-lysine sensor is based on the transcriptional regulator LysG, which activates expression of the lysine exporter gene *lysE* when the cytoplasmic L-lysine concentration increases [7]. For construction of the sensor plasmid pSenLys, *lysE* was replaced by *eyfp* encoding enhanced yellow fluorescent protein. When transferred into a series of defined L-lysine producing strains, a linear relationship between the specific fluorescence and the cytoplasmic L-lysine concentration was detected in the range of about 4 – 20 mM. For the isolation of lysine-producing strains, wild-type cells carrying pSenLys were subjected to random mutagenesis and after recovery to FACS analysis. From 6.5 million cells, 120 single cells with increased fluorescence were isolated and the corresponding clones produced 0.2 – 37 mM L-lysine in the supernatant. Genetic analysis of 40 clones revealed known and novel mutations in six genes known to be mutated in L-lysine producer strains, whereas in 16 mutants no mutations were found in these six genes. Genome resequencing of 10 of these clones revealed up to 268 SNPs per genome. Among the mutated genes was *murE*, encoding an enzyme involved in peptidoglycan biosynthesis. Its substrate is meso-diaminopimelate, the precursor of L-lysine. When the mutation *murE*-G81E was introduced into the wild type, it produced >20 mM L-lysine, and increased lysine production was also observed in producer strains carrying the *murE*-G81E mutation. Besides pSenLys, we also constructed sensors for L-methionine and branched-chain amino acids [4], for L-arginine, L-serine, and O-acetyl-serine and further ones are in progress.

Conclusions. The combination of single-cell metabolite sensors with FACS fills the need for HT-methods linking random mutagenesis and genome analysis. The technology has manyfold applications in the screening of mutant libraries carrying mutations in genomes or plasmids and will expedite bacterial producer strain development. Furthermore, such sensors are suitable for single-cell analysis by fluorescence microscopy using microfluidic devices.

Acknowledgements. We thank the Federal Ministry of Education and Research (BMBF) for financial support within the GenoMik-Transfer project "FlexFit".

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