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Research Article

High-Throughput Screening of an Octanoic Acid Producer Strain Library Enables Detection of New Targets for Increasing Titers in *Saccharomyces cerevisiae*

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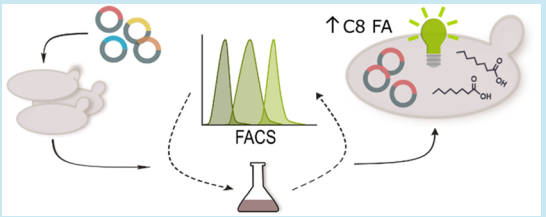
ABSTRACT: Octanoic acid is an industrially relevant compound with applications in antimicrobials or as a precursor for biofuels. Microbial biosynthesis through yeast is a promising alternative to current unsustainable production methods. To increase octanoic acid titers in *Saccharomyces cerevisiae*, we use a previously developed biosensor that is based on the octanoic acid responsive *pPDR12* promotor coupled to GFP. We establish a biosensor strain amenable for high-throughput screening of an octanoic acid producer strain library. Through development, optimization, and execution of a high-throughput screening approach, we were able to detect two new genetic targets, *KCS1* and *FSH2*, which increased octanoic acid titers through combined overexpression by about 55% compared to the parental strain. Neither target has yet been reported to be involved in fatty acid biosynthesis. The presented methodology can be employed to screen any genetic library and thereby more genes involved in improving octanoic acid production can be detected in the future.



KEYWORDS: *octanoic acid, C8 fatty acid, high-throughput screening, overexpression library, FACS, yeast*

INTRODUCTION

Medium-chain fatty acids, such as octanoic acid (C8 fatty acid), are widely used in industry, for example as antimicrobials, or can be converted to fatty-acid derivatives like fatty alcohols, alkanes, or alkenes, thereby widening the application range to biofuels, lubricants and surfactants.[1] The current supply is mostly based on the extraction from oil plants, such as coconut and palm oil plants. However, these vegetable oils mostly consist of long-chain fatty acids with only minor fractions of octanoic and decanoic acid.[2] Furthermore, the cultivation of oil plants oftentimes involves deforestation and extensive land use,[3] making other production methods from renewable substrates desirable. Engineering microbes like yeast to produce octanoic acid is a promising alternative with potentially less environmental impact.

In recent years, several reports have shown the successful engineering of *Saccharomyces cerevisiae* to produce medium-chain (C6−C12) fatty acids.[4−7] Most recently more than 1g/L medium-chain fatty acids were reported to be produced by an engineered *S. cerevisiae* strain.[6] However, producing a mixture of chain lengths reduces the yield of the desired fatty acid, and purification is difficult because different chain lengths have similar physicochemical properties.[1] One way to achieve strains only producing specific fatty acid chain lengths, is by rational engineering of the fatty acid synthase (FAS) genes. Gajewski et al. (2017)[4] introduced an amino acid exchange of arginine to lysine (R1834K) in the MPT domain of *FAS1*, thereby generating an *S. cerevisiae* strain producing mainly octanoic acid. Apart from strategies for chain-length control, engineering efforts to generate higher titers have mostly focused on enzymes involved in precursor routes for increasing cytosolic acetyl-CoA and malonyl-CoA supply or on decreasing fatty acid degradation.[1,8,9] One example to achieve the latter, is knockout of *FAA2*, encoding a medium-chain fatty acyl-CoA synthetase, which activates imported fatty acids in the peroxisomes for degradation via the β-oxidation pathway.[10] Knockout of *FAA2* has previously been demonstrated to increase short- and medium-chain fatty acid titers considerably.[11,12]

Nevertheless, to construct efficient cell factories, targeted engineering strategies are insufficient. Because of the complex cellular network, there are many factors whose direct or indirect involvement in fatty acid biosynthesis, transport, degradation or detoxification mechanisms are unknown so far. An alternative strategy to rational engineering of specific target genes to generate strains with higher product titers is the generation of strain libraries. However, strain evaluation is currently the rate limiting step in such efforts because traditional analytical methods like gas chromatography (GC) are very laborious and time-consuming.[13,14] Biosensors, in

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combination with a high-throughput detection technology, are an excellent tool to overcome this drawback. Biosensors enable easy and rapid detection of the desired compound, for example, via fluorescence quantification.[13,15−17] Previously, we have developed a whole-cell short- and medium-chain fatty acid biosensor amenable for high-throughput screenings.[18] The biosensor is based on the short- and medium-chain fatty acid responsive promotor *pPDR12* coupled to GFP. *PDR12* encodes an ATP-binding cassette transporter and its expression is regulated by the transcription factor War1p.[19,20] War1p activates GFP expression via *pPDR12* regulation in the presence of inducing molecules, that is, hexanoic, heptanoic, and octanoic acid, in a concentration-dependent manner. For octanoic acid, the linear detection range was between 0.01−0.75 mM (1.4−108 mg/L), and it showed a high dynamic range with up to 10-fold increase in signal after only 2 h of induction.[18]

Here, we modify the biosensor strain and verify its usability in flow cytometry. We aimed at detecting new targets that increase octanoic acid titers in *S. cerevisiae* through a high-throughput screening of a gene overexpression library via fluorescence-activated cell sorting (FACS). This approach enabled us to detect unexpected genes whose overexpression led to increased octanoic acid titers. To our knowledge, this is the first report of the application of an octanoic acid biosensor to identify genes enhancing octanoic acid levels in *S. cerevisiae*.

RESULTS AND DISCUSSION

Adaptation of an Octanoic Acid Biosensor for Use in a FACS-Based Screening. To adapt a previously developed octanoic acid biosensor[18] for screening via FACS, we carried out several modifications. The original biosensor relied on a multicopy plasmid carrying *pPDR12*-GFP. For a single cell screening through flow cytometry, we assumed that GFP expression from a multicopy plasmid would create high single cell variation as a result of differing plasmid copy numbers per cell. This assumption was confirmed in a flow cytometry experiment (Figure S1). To avoid this effect, we considered two options: (1) Normalization of copy numbers through constitutive expression of another fluorescent gene (e.g., mCherry) on the same centromeric plasmid with the biosensor or (2) genomic integration of the biosensor. The first option has been shown to be successful for para-hydroxybenzoic acid (PHBA) detection;[21] however, it is more complex to execute because normalization of each measurement is inevitable. With the second option—a strain with genomically integrated biosensor—we had previously conducted measurements in a monochromator microplate reader. We had observed that the GFP signal was only barely distinguishable from a strain without sensor under octanoic acid supply.[18] To verify the functionality of a genomically integrated biosensor in flow cytometry, we cultured strain LBY27—containing the *pPDR12*-GFP cassette genomically—for 2 h with octanoic acid concentrations ranging between 0 and 100 mg/L. After this induction period, the samples were screened employing flow cytometry and we observed an increase in fluorescence signal with increasing octanoic acid concentrations (Figure 1). Even though the fluorescence values partly overlapped for different concentrations, the mean fluorescence rose in correlation with the octanoic acid concentration. This verified that supplied octanoic acid is taken up by the cells and induces GFP production in a concentration-dependent manner.[12,18] Furthermore, we asserted that the detection in the flow

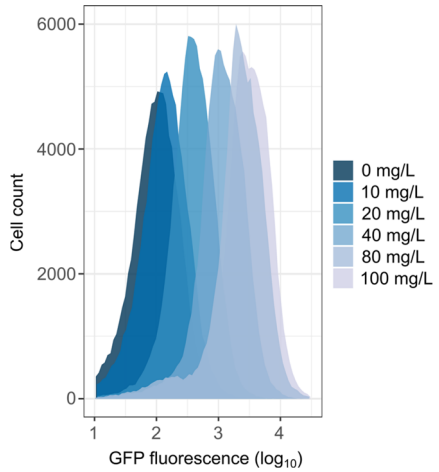

Figure 1. Evaluation of biosensor response to supplementation with octanoic acid. Strain LBY27, which contains a stable genomic integration of the biosensor *pPDR12-GFP*, was inoculated to an OD_{600} = 0.2 and grown in SCD medium for 3 h. The indicated octanoic acid amounts were added and the cultures were grown for another 2 h before measurement of GFP fluorescence in a flow cytometer (common logarithmic transformation of fluorescence area plotted). Results are shown for a single experiment; the experiment was repeated with comparable results (Figure S2).

cytometer is much more sensitive than in the microplate reader confirming that the genomically integrated biosensor is generally suitable for the envisioned screening. With 100 mg/L supplied octanoic acid, the mean fluorescence of the biosensor strain was only slightly higher than with 80 mg/L. Therefore, the system seems to saturate around 80 mg/L of added octanoic acid, posing an upper limit to detection. However, production levels of strains which are used for primary verification of optimization strategies are within this detection range and are therefore suitable to use with this biosensor.

In the next step, we genomically integrated the biosensor in an octanoic acid producer strain. To enable octanoic acid production, the strain contains a mutation in the *FAS1* gene at position 1834 bp, resulting in an amino acid exchange from arginine to lysine ($FAS1^{RK}$).[4] This strain, LBY31, furthermore contains a positive-feedback loop[21] to increase the dynamic range of the sensor. For the positive-feedback loop, the promotor of the transcriptional regulator-encoding gene *WAR1* was replaced with its target promotor *pPDR12*. This was shown to increase the dynamic range of GFP expression in response to PHBA, another activator of *pPDR12*.[21]

When growing LBY31 in buffered SCD medium, we observed that the 35 mg/L octanoic acid produced 24 h after inoculation were reduced to about 50% after 46 h. After 72 h of fermentation, almost no octanoic acid was detectable anymore (Figure 2). To prevent octanoic acid degradation, we deleted *FAA2*, encoding a fatty acyl-CoA synthetase involved in the activation of free medium-chain fatty acids prior to *β*-oxidation.[10] It has previously been shown that deletion of *FAA2* prevents the degradation of medium-chain fatty acids such as octanoic acid.[11,12] The resulting strain LBY39 indeed maintained the octanoic acid titer at the same level even 72 h after inoculation. Therefore, the knockout of *FAA2* proved to be essential to maintain octanoic acid titers stable over several days of fermentation. Stable titers are important to prevent false-positive strains in the screening, which might have varying

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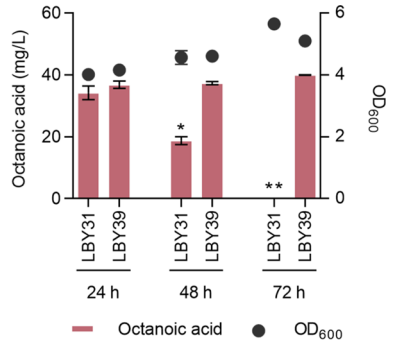

Figure 2. Effect of *FAA2* knockout on octanoic acid titers in biosensor strain. OD_{600} (dots) and octanoic acid titers (bars) of LBY31 and LBY39 fermented in buffered SCD medium over 72 h. LBY31: *pPDR12-GFP*, $FAS1^{RK}$. LBY39: *pPDR12-GFP*, $FAS1^{RK}$, Δ*faa2*. Fatty acids were extracted, methylated, and quantified via GC. $n = 2$, error bars = ± SD. Statistical analysis was performed using two-tailed unpaired *t* test (*$P < 0.05$, **$P < 0.01$).

titers because of the varying degree of intracellular octanoic acid degradation. The resulting biosensor strain LBY39 has a high linear and dynamic range of detection and produced octanoic acid amounts (approximately 35 mg/L) within the detection range of the sensor.

We, then, sought to test whether the octanoic acid producer strain LBY39 can be distinguished from the nonproducer LBY27 in flow cytometer analysis, which we performed 4, 24, and 48 h after inoculation (Figure 3). After only 4 h, LBY39 showed an increase in the mean fluorescence compared to the nonproducer. Since octanoic acid production mainly occurs after glucose depletion (approximately 18 h), this difference was much more pronounced after 24 h. After 48 h, producer and nonproducer populations become clearly distinct with median values differing by a factor of about 10^2. This suggests that the higher intracellular octanoic acid levels in LBY39 after 48 h also led to higher GFP expression despite loss of octanoic acid to the extracellular environment.

High-Throughput Screening for Genes Enhancing Octanoic Acid Levels. Our general procedure for a high-throughput screening is depicted in Figure 4. To find genes that when overexpressed lead to an increase in octanoic acid titers, we combined the sensor strain LBY39 with a gene overexpression library. Such libraries allow for detection of genes that when overexpressed (through the presence of high plasmid copy numbers) will have a positive effect on the titer of the produced compound.[22−24] We used a library[25] containing approximately 10 kb fragments of the entire yeast genome cloned on multicopy (2μ) vectors with each vector comprising roughly 3−10 ORFs flanked by endogenous up- and downstream sequences. To prepare the library from *Escherichia coli* glycerol stocks, we grew the 1588 strains individually in 96-deepwell plates ensuring thereby representation of all plasmids. Through transformation of LBY39 with a mixture of all library plasmids, about 22 000 colonies were generated—resulting in a theoretical coverage of the library of almost 14 times, assuming equal transformation probability of all plasmids. We inoculated eight shake flasks with colonies from 3 to 4 transformation plates each and grew them in buffered SCD^{-leu} medium (pH 6.5). At pH 6.5, octanoic acid is mostly present in its dissociated form and hence unable to cross the plasma membrane of the cell for re-entry after secretion. By choosing this pH, we aimed at minimizing octanoic acid uptake from the medium, thereby avoiding cross-exchange of octanoic acid between cells.

The first sampling was performed 48 h after inoculation to favor enrichment of cells with high octanoic acid titers and simultaneously good growth. Our high-throughput screening design was based on the attempt to decrease false positive results by integrating three subsequent enrichment steps (E1−E3 sorting). Fluorescence signals of the sorting steps are shown in Figures S3−S5. Iterative screening rounds are essential to account for the variation of production on a single cell level. To avoid too strict cut-offs, we slowly decreased the percentage of highest fluorescent events sorted from E1 to E3. In the first round (Figure S3), we collected the highest 10% of fluorescent events into fresh liquid medium from each of the 8 flasks separately (E1 sorting) and let them grow for 66 h. After this time period, we conducted another flow cytometry measurement. Here, the populations from flasks 1−6 showed a low fluorescence close to the negative control strain LBY27 (Figure S4). We, therefore, solely continued with flasks 7 and 8, collecting the top 7.5% fluorescent events of each (E2 sorting).

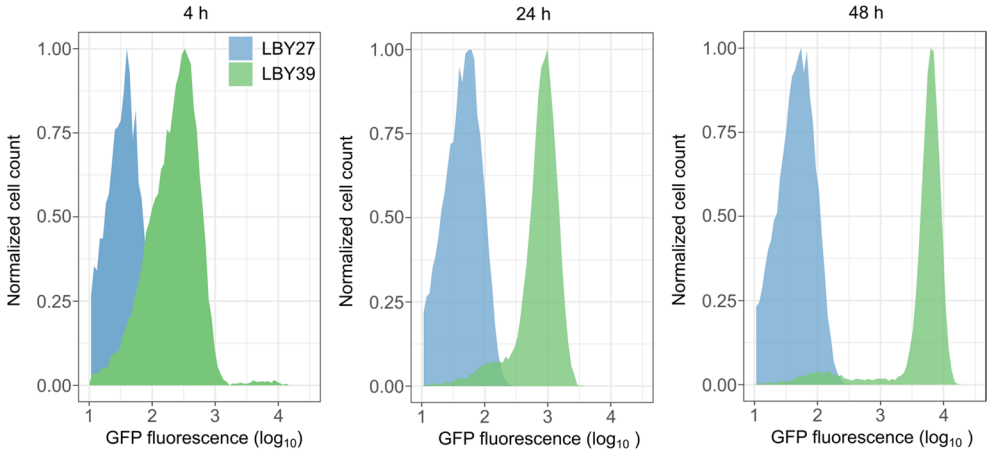

Figure 3. Fluorescence signal of the genomic octanoic acid sensor in producer and nonproducer strain. Strains LBY27 and LBY39 contain a stable integration of the biosensor *pPDR12-GFP*.Strains were inoculated to $OD_{600} = 0.1$ and grown in buffered SCD medium. Aliquots were taken after 4, 24, and 48 h for GFP fluorescence measurement in a flow cytometer (common logarithmic transformation of fluorescence area plotted). LBY39: $FAS1^{RK}$, Δ*faa2*.

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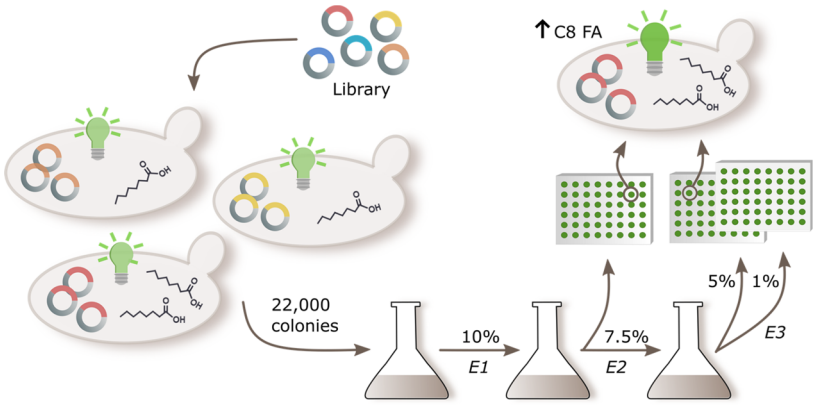

Figure 4. High-throughput screening of an octanoic acid producer strain library. The producer and biosensor strain LBY39 was transformed with a multicopy yeast genomic library resulting in roughly 22 000 colonies. These were analyzed in three consecutive enrichment steps (E1−E3) and highest fluorescent events were sorted via flow cytometry. Random colonies of enriched cultures were analyzed further and strains that increase octanoic acid (C8 FA) titers could be detected.

Table 1. Identification of Library Plasmids after High-Throughput Screening[a]

Flask	Enrichment	Name	Genes contained on library plasmid								
F7	E2 (top 7.5 %)	F7-E2.1	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					
		F7-E2.2	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					
		F7-E2.4	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					
		F7-E2.5	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					
		F7-E2.8	*[YBL046W]&*	*COR1*	*YBL044W*	*ECM13*	*FUI1*	*[PRE7]**			
		F7-E2.11	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					
	E3 (top 5 %)	F7-E3.1	*[ESC1]&*	*ERG8*	*FMP42*	*FSH2*	*UBP8*	*MRE11*	*MRLP44*	*YMR226C*	*TAF7* *[MTF1]**
F8	E2 (top 7.5 %)	F8-E2.2	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					
		F8-E2.3	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					
		F8-E2.4	*GDH3*	*[YAL061W]**							
		F8-E2.5	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					
		F8-E2.6	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					
		F8-E2.7	*[PSF1]*	*RAD61*	*YDR015C*	*DAD1*	*[KCS1]*				
		F8-E2.8	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					
		F8-E2.10	*[YML023C]**	*APT1*	*UNG1*	*YML020W*	*OST6*	*YML018C*	*[PSP2]**		
	E3 (top 5 %)	F8-E3.1	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					
	E3 (top 1 %)	F8-E3.2	*[YKL183C-A]**	*[LOT5]*	*FAS1*	*[PRS1]**					

[ ] intact ORF, possibly missing up- or downstream regions
*[ ]** 3' end of gene is missing
[ ]& 5' end of gene is missing

[a]Single colonies were analyzed from flask 7 and 8 (F7, F8) after different enrichment rounds (E1−E3) from highest fluorescent sorted events (top 7.5%, 5%, 1%). Plasmids from these cells were extracted and the contained genes identified via BLAST search.

Thereafter, we conducted another enrichment round, collecting the top 5% and top 1%, respectively, of fluorescent events (E3 sorting, Figure S5). LBY39 containing the empty vector was grown and measured each time in the flow cytometer as a control. The populations from flasks 7 and 8 from E2 and E3 showed a lower mean fluorescence than the control strain LBY39 containing the empty vector (Figures S4 and S5). The control strain, however, was always inoculated freshly to an OD = 0.1 and not sorted like the populations from flasks 7 and 8. Therefore, the fluorescence signal might have varied because of the growth-dependent octanoic acid amounts or because of a saturation of biosensor signal after long cultivation periods.

Identification and verification of candidate plasmids. Next, we analyzed the colonies of the enriched libraries. From the sorted events of E2 and E3, about 80% resulted in colony growth (approximately 200 colonies per sorted plate). To extract library plasmids from these colonies, we randomly picked about 10 colonies per plate, inoculated them, extracted plasmids and retransformed them into *E. coli* for plasmid amplification. Not all transformations led to *E. coli* colony growth. Nevertheless, we were able to successfully extract six plasmids from flask 7 of E2 and one plasmid of E3. From flask 8, we extracted eight plasmids of E2 and two plasmids of E3. To identify genes, we sequenced the ORFs on the plasmids and ran BLAST searches. Thereby, we successfully assigned all plasmids to the library as shown in Table 1.

Of the 17 plasmids we identified, 12 corresponded to a library plasmid containing two complete ORFs (*LOT5*, *FAS1*), as well as two truncated ORFs (*YKL183C-A*, *PRS1*). It was striking that the library plasmid containing the wild type variant of *FAS1* was enriched in the screening after E2 and E3. We assumed that this might be the result of improved growth conferred through wild type *FAS1*. To evaluate this hypothesis, we transformed producer strain LBY38 with this library plasmid containing wild type *FAS1*, the library plasmid containing wild type *FAS2*, or the control vector pGP564, respectively. 48 h after inoculation, we extracted fatty acids and quantified them via GC measurement. Indeed, we found that

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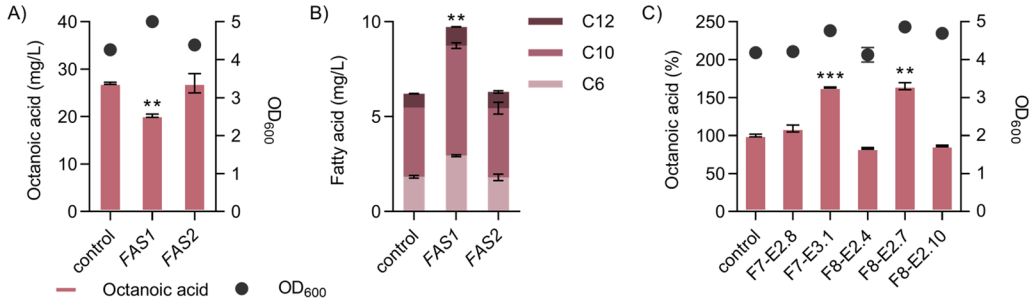

Figure 5. Effect of identified library plasmids on fatty acid titers and growth. OD_{600} (dots) and octanoic acid titers (bars) (A, C) and C6, C10 and C12 fatty acid titers (B) of octanoic acid producer strain LBY38 containing either the empty vector pGP564 (control) or library plasmids with wild type *FAS1* or *FAS2*, respectively, or other library plasmids as indicated. Fatty acids were extracted, methylated and quantified via GC 48 h after inoculation in buffered SCD^{-leu} medium. LBY38: $FAS1^{RK}$, Δ*faa2*. $n = 2$, error bars = ± SD. Statistical analysis (indicated columns vs control) was performed using two-tailed unpaired *t* test (**$P < 0.01$, ***$P < 0.001$). *P* values in graph B are ** for C6, C10, and C12.

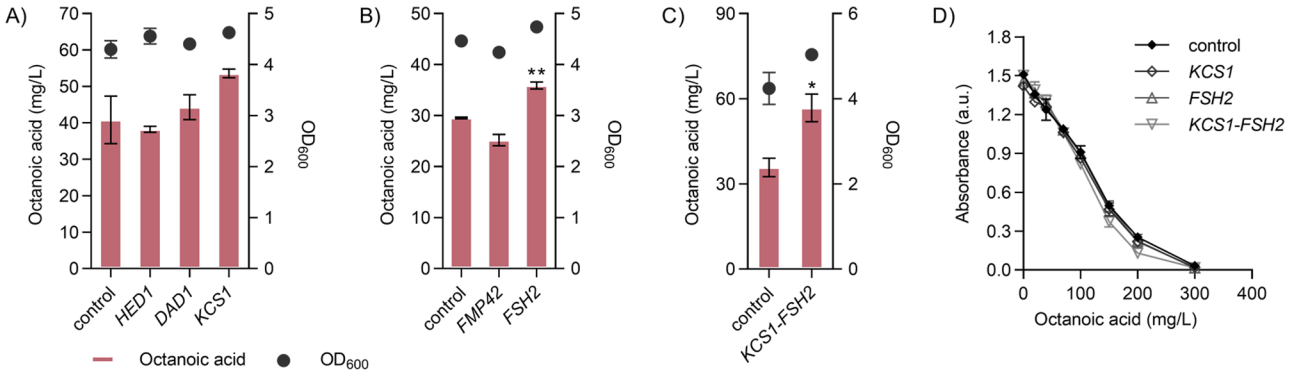

Figure 6. Identification of genes increasing octanoic acid titers but not resistance. (A–C) OD_{600} (dots) and octanoic acid titers (bars) of octanoic acid producer strain LBY38 containing either the empty vector pGP564 (control) or plasmids with indicated genes. Fatty acids were extracted, methylated and quantified via GC 48 h after inoculation in buffered SCD^{-leu} medium. LBY38: $FAS1^{RK}$, Δ*faa2*. $n = 2$, error bars = ± SD. Statistical analysis (indicated columns vs control) was performed using two-tailed unpaired *t* test (*$P < 0.05$, **$P < 0.01$). (D) Octanoic acid nonproducer CEN.PK2–1C containing either the empty vector pGP564 (control) or plasmids with the indicated genes was inoculated to OD = 0.05 and supplied with 0–300 mg/L octanoic acid. Growth was measured by absorbance after 20 h. $n = 3$, error bars = ± SD.

the *FAS1*-containing library plasmid led to slightly decreased octanoic acid titers but improved growth and, interestingly, higher C6, C10, and C12 fatty acid levels, whereas the library plasmid containing *FAS2* did not have an effect on either of these factors (Figure 5A and B). To confirm that this is the result of the *FAS* genes and not any of the other genes encoded on the library plasmids, we transformed LBY38 with plasmids solely containing *FAS1*, *FAS2*, or $FAS1^{RK}$ or different combinations thereof. We were able to confirm that expression of wild type *FAS1* in LBY38 decreases octanoic acid titers (Figure S6), as previously observed in another strain background.[26] Thereby, toxic effects on the cell are probably reduced. This suggests, that *FAS1* expression gives the cells an advantage over other cells and therefore appears enriched after iterative screening rounds. The observation that strains containing the *FAS1* library plasmid still have fluorescence in the range of the top fluorescing cells of the population might result from increased production of C6 and C10 fatty acids. C6 fatty acid also strongly activates the biosensor but is less toxic than C8 fatty acid and for C10 fatty acid an activation of *pPDR12* could not be excluded in previous experiments.[18]

Apart from the enriched *FAS1* library plasmid, we identified five more plasmids. Even though none of these plasmids was enriched, we assumed that they could improve growth and/or octanoic acid titers being present after E2 or E3. We transformed the producer LBY38 with each of the five plasmids and quantified produced octanoic acid 48 h after inoculation. Two of the plasmids, namely, F7-E3.1 (flask 7, enrichment 3) and F8-E2.7 (flask 8, enrichment 2), led to an increase in octanoic acid titers (Figure 5C).

Identification and Verification of Candidate Genes. As both plasmids, F7-E3.1 and F8-E2.7, contain several genes (Table 1), we were seeking to find the ones responsible for the positive effect. For this purpose, we recloned the genes and repeated fermentations (Figures S7 and 6). Thereby, we identified *FSH2*, putatively encoding a serine hydrolase,[27] as the gene from plasmid F7-E3.1 responsible for the increase in octanoic acid titer (Figure 6B). From plasmid F8-E2.7, *KCS1*, encoding an inositol hexa-/heptakisphosphate kinase,[28,29] seemed to be the responsible gene, even though the results were less clear (Figure 6A). When overexpressing both *FSH2* and *KCS1* from one multicopy plasmid, we observed the strongest increase in octanoic acid titer, with 55% higher amounts than the control strain, indicating a synergistic effect of both genes (Figure 6C). Suspecting that the detected genes might influence robustness toward octanoic acid, we transformed octanoic acid nonproducer CEN.PK2–1C with plasmids containing *FSH2*, *KCS1* and both, respectively, and supplied octanoic acid in concentrations from 0 to 300 mg/L. However, both *FSH2* and *KCS1* did not increase robustness toward octanoic acid (Figure 6D).

To study the physiology of cells overexpressing *KCS1* and *FSH2* in more detail, we conducted two more experiments. First, we fermented the octanoic acid nonproducer CEN.PK2–

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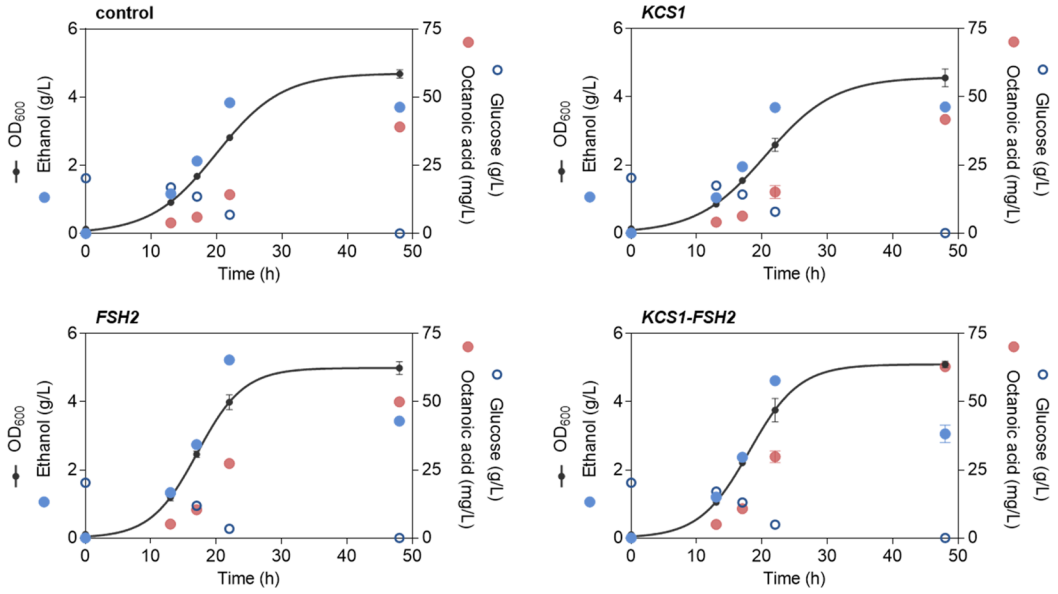

Figure 7. Growth and metabolic performance of selected strains. OD_{600} and glucose, ethanol, and octanoic acid titers of strain LBY38 containing either the vector control or plasmids with the indicated genes. Strains were grown in buffered SCD^{-leu} medium in shake flasks and samples were taken for HPLC or GC measurement, respectively, over the course of fermentation. n = 3, error bars = ± SD. LBY38: *FAS1*^{RK}, Δ*faa2*.

1C containing plasmids with *FSH2*, *KCS1*, or both, respectively, and measured growth as well as glucose and ethanol titers over the course of fermentation in comparison to the empty vector control (Figure S8). Second, we performed the same experiment with the octanoic acid producer LBY38, in which we additionally measured octanoic acid titers (Figure 7). When expressed in CEN.PK2−1C, we did not observe an effect of *KCS1* or *KCS1-FSH2* on either of the parameters measured. Interestingly, CEN.PK2−1C carrying only *FSH2* exhibited a slightly but significantly (*P < 0.05) increased glucose consumption by 8−12% as measured from aliquots after 12 and 15 h of growth, respectively. The analysis of LBY38 cultures (Figure 7) led to similar results concerning *KCS1* overexpression as no significant effects on any of the measured parameters was observable. Overexpression of *FSH2* led to higher OD_{600} values after 13, 17, and 22 h of growth, as well as faster glucose consumption (17 and 22 h) and ethanol production (13, 17, and 22 h) and consumption (48 h; all values significant with at least *P < 0.05). Furthermore, higher octanoic acid titers (at least *P < 0.05) were measured at all sampling times in the strain overexpressing *FSH2* in comparison to the control. The yields (measured as milligrams octanoic acid per gram of consumed glucose at 22 h) were 54% higher when *FSH2* was overexpressed. Although the effects of *KCS1* overexpression were not significantly different from the control, the cumulative positive effects on octanoic acid titers of overexpressing both *KCS1* and *FSH2* in LBY38 were confirmed (Figure 7). Overexpression of only *FSH2* increased octanoic acid titers by 29% after 48 h of growth in comparison to the parental strain, whereas combined *KCS1-FSH2* overexpression increased titers by 60% and yields by 84%. The double overexpression strain also showed faster glucose consumption and ethanol metabolization similarly to the *FSH2* overexpression results. The fact that not only the titers, but also the yields of octanoic acid were increased suggests that the improvement is not solely due to a faster metabolism, but also to a redistribution of the carbon flux. The underlying mechanisms will certainly need to be addressed in future studies.

The biological role of *FSH2* is unknown. A large-scale study showed that Fsh1p localized to the cytosol[30] and in a study combining computational and experimental proteomics, it was assigned to a new group of serine hydrolases together with *FSH1* and *FSH3*.[27] Overexpression of *FSH1* was reported to decrease the abundance of phospholipids and increase that of triacylglycerols, lipid droplets, and free fatty acids.[31] As *FSH1* and *FSH2* share 21−26% homology,[31] it seems likely that *FSH2* also plays a role in lipid metabolism and, thereby, leads to increased octanoic acid titers when overexpressed—but due to a lack of knowledge about *FSH2*, this is speculative. The molecular and biological function of *KCS1*, is well characterized. It encodes an inositol pyrophosphate synthase, which phosphorylates a variety of inositol phosphate substrates.[28,29,32] Inositol pyrophosphates serve as signaling molecules in many cellular processes. *KCS1* was shown to play a role in vesicular trafficking, cell wall integrity and stress response.[29,33] Deletion of *KCS1* resulted in strong phenotypes with increased cell size and impaired proliferation.[33] A recent publication showed that *KCS1* knockout increased S-adenosyl-L-methionine levels in *S. cerevisiae* cells and caused enhanced transcription levels of glycolytic genes as well as ATP levels.[34] Possibly, *KCS1* overexpression affects glycolysis, thereby leading to enhanced octanoic acid titers; however, the exact link remains to be investigated. Further analyses, such as proteomics or RNA-Seq analyses with *FSH2* and *KCS1* overexpression strains would be interesting to study the exact mechanisms in detail.

In summary, we demonstrated the utility of employing a biosensor in a high-throughput screening via FACS to detect new targets that increase octanoic acid titers and yields. This screening shows how useful the development of biosensors for industrially relevant compounds is. The developed method is very versatile and could also be employed with other gene libraries to detect even more new targets. This high-throughput screening approach enabled us to detect two entirely new

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genetic targets, *KCS1* and *FSH2*, which could not have been detected in a rational engineering approach. This is the first report that *KCS1* and *FSH2* affect fatty acid biosynthesis and which, in combined overexpression, increased octanoic acid titers by 55−60% and yields by 84% compared to the parental strain.

METHODS

Strains and Plasmid Construction. Yeast strains used throughout this study are listed in Table 2, and plasmids and oligonucleotides in Tables S1 and S2, respectively. For genomic integration of the biosensor (Δ*ura3*::*pPDR12-EnvyGFP-tCYC1*) into LBY31, CRISPR/Cas9 was used as described previously.[35] The CRISPR/Cas9 plasmid was amplified in two PCR fragments, assembled *in vitro* in an isothermal reaction using T5 exonuclease, polymerase and ligase[36] and transformed into *E. coli*. After verification via Sanger sequencing, it was transformed into the target strain together with the respective insertion fragment. The same approach was used for: integration of the positive feedback loop (Δ*pWAR1*::*pPDR12*) into LBY31 and Δ*faa2* knockout in LBY39. The insertion fragments were amplified with overhangs from the following template DNAs: biosensor from LBY27 genomic DNA; positive feedback loop from LBV14 plasmid DNA; and Δ*faa2* donor DNA from SHY34[26] genomic DNA.

Table 2. Yeast Strains

strain	genotype	reference
CEN.PK2−1C	*MATa; MAL2-8c; SUC2; ura3-52; his3Δ1; leu2−3_112; trp1−289*	Euroscarf, Frankfurt am Main, Germany
VGY2	CEN.PK2-1c Δ*pFAS1*-1-300-1-392 Δ*pFAS2*-1-200::*pHXT7*-1-392, $FAS1^{R1834K}$	Wernig et al., 2021 (submitted)
LBY27	CEN.PK113-11C Δ*pyk2*::*pPDR12-EnvyGFP-tCYC1*	18
LBY31	VGY2 Δ*ura3*::*pPDR12-EnvyGFP-tCYC1* Δ*pWAR1*::*pPDR12*	this study
LBY38	VGY2 Δ*faa2*	Wernig et al., 2021 (submitted)
LBY39	LBY31 Δ*faa2*	this study

For cloning plasmids with one or more genes (LBV91-113), the desired fragments were amplified by PCR from the original library plasmids with overhangs to the sequences flanking the *Bam*HI/*Pst*I cut sites of pGP564. pGP564 was digested with *Bam*HI/*Pst*I and transformed together with the insertion fragments into CEN.PK2−1C and plasmids were assembled via homologous recombination in yeast. Yeast transformations were performed according to Gietz and Schiestl.[37] Cells were streaked out on selective SCD medium[38] lacking leucine (SCD^{-leu}) to select for *LEU2*. The colonies from the yeast plates were collectively transformed into electrocompetent *E. coli* DH10*β* (Gibco BRL, Gaithersburg, MD) and transformants were selected on lysogeny broth (LB) agar plates[39] supplemented with kanamycin (50 $\mu g\ mL^{-1}$). Selected plasmids were extracted according to standard procedures and verified via Sanger sequencing.

Preparation of Library Plasmids for Screening. The library was present in 17 96-well *E. coli* glycerol stocks. To ensure that all plasmids were represented, we inoculated the *E. coli* strains in 1 mL of LB^{Kan} in 17 96-deepwell plates (Flat-bottom blocks, Qiagen) and grew them overnight under shaking (250 r.p.m.). The cultures of each deepwell plate were mixed and plasmids extracted via 17 midi-preps (GeneJET Plasmid-Midiprep-Kit, Thermo Fisher Scientific). For later use, a mixture of all plasmid extracts was prepared.

Cultivation and Flow Cytometry Analysis. For precultures, several colonies of respective strains were inoculated in (selective) SCD with 100 mM potassium phosphate buffer adjusted to pH 6.5 and shaken (180 r.p.m.) at 30 °C overnight. The main cultures were inoculated (in replicates as indicated for each figure) to an OD_{600} of 0.1 in buffered (selective) SCD medium in shake flasks and grown for up to 72 h with shaking (180 r.p.m., 30 °C), or for the experiment depicted in Figure S8, grown in 300 mL flasks on a cell growth quantifier (aquila biolabs GmbH, Germany).[40] Aliquots were taken for flow cytometry measurements and analyses were performed using a Sony SH800SA with a 488 nm argon laser and a 100 μm sorting chip. Instrument gains were set as follows: Forward scatter values were set to 6, thresholds for backscatter and FL2 channel ("GFP signal" = 525 ± 50 nm) to 43% and 30%, respectively. Sample pressure was set to a maximum of 6 to keep the event per second rate below 10 000 events. For each analysis, 10^5 events were evaluated. For cell sorting, cell agglomerates (doublets) were excluded based on channels FSC-H/FSC-W, BSC-H/BSC-W, and FSC-A/BSC-A.

High-Throughput Screening via FACS. For transformation of LBY39 with the library, a mixture of the 17 plasmid extracts was used. About 22 000 colonies resulted from the transformation and we inoculated eight shake flasks with colonies from 3 to 4 transformation plates each. These were grown in buffered SCD^{-leu} medium (pH = 6.5) alongside control strains (LBY27, LBY39+pGP564) in shake flasks (180 r.p.m., 30 °C). Aliquots were taken after 48 h and measured via flow cytometry as described above. A gate was set to sort the 10% events with highest fluorescence (∼300 000 events, mode "purity") into 15 mL falcons containing fresh media (E1 sorting). For all 8 flasks, sorting was performed, and samples were grown again for 66 h in shake flasks (approximated starting $OD_{600} \sim 0.0025$). After 66 h, another round of sorting (E2 sorting) was performed for flask 7 and 8. The 7.5% highest fluorescent events were sorted into falcons as well as onto SCD^{-leu} solid medium. This procedure was repeated after another incubation period of 66 h in shake flasks, this time sorting only onto solid SCD^{-leu} of the top 5% and 1% fluorescent events, respectively (E3 sorting).

Identification and Verification of Plasmids after Screening. From the sorted plates resulting from the screening, we randomly picked about 10 colonies per sorted plate for growth in SCD^{-leu}. Plasmids were extracted by standard procedures with an additional step of disrupting the yeast cells with glass beads (Ø0.25−0.5 mm, Roth, Karlsruhe, Germany) by vigorous shaking in lysis buffer for 8 min. For amplification, the plasmids were transformed in electrocompetent *E. coli* DH10*β* (Gibco BRL, Gaithersburg, MD). Plasmids were extracted from *E. coli* by standard procedures and sequenced with Sanger sequencing with primers LBP85, LBP237, or LBP238. Identification of library plasmids was performed via BLAST searching the *Saccharomyces cerevisiae* Genome Database (http://www.yeastgenome.org/).

Cultures for Fatty Acid Production. *S. cerevisiae* strains were grown as previously described[4] with some adjustments. For precultures, several colonies of a strain were inoculated in (selective) SCD with 100 mM potassium phosphate buffer adjusted to pH 6.5 and grown overnight (180 r.p.m., 30 °C).

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Main cultures were inoculated to an OD_{600} of 0.1 in 50 mL of the respective medium and incubated in 300 mL shake flasks under the same conditions. For sampling, cultures were harvested by centrifugation and 10 mL of the supernatant was used for fatty acid extraction.

Fatty Acid Extraction and Derivatization. Fatty acid extraction and derivatization were performed as described previously.[12] Cells were separated from the medium (3500 rcf, 10 min), an internal standard (0.2 mg heptanoic acid) was added to 10 mL supernatant and mixed with 1 mL 1 M HCl and 2.5 mL methanol/chloroform solution (1:1). After vigorous shaking for 3 min, the mixture was centrifuged at 3000 rcf for 10 min and the chloroform layer was recovered and evaporated overnight. The methylation of the fatty acids was performed as previously described.[41] Samples were dissolved in 200 μL toluene, mixed with 1.5 mL methanol and 300 μL 8.0% (w/v) HCl solution (diluted in methanol), vortexed, and incubated at 100 °C for 3 h to form fatty acid methyl esters (FAME). After cooling at 4 °C for 10 min, 1 mL H_2O and 1 mL hexane were added to the sample, followed by thorough shaking, and the organic phase was transferred to a GC vial.

GC-FID analysis of FAMEs. GC analyses were carried out on a PerkinElmer Clarus 400 instrument (PerkinElmer, Germany) equipped with an Elite FFAP capillary column (30 m × 0.25 mm, film thickness: 0.25 μm; PerkinElmer, Germany) and a flame ionization detector (PerkinElmer, Germany) as described previously.[12]

Toxicity test. CEN.PK2−1C was transformed with plasmids pGP564, LBV106, LBV112 and LBV113, respectively, and plated on SCD^{-leu}. Precultures were inoculated in triplicates in buffered SCD^{-leu} and grown overnight with shaking (30 °C, 180 r.p.m.). For main cultures, strains were inoculated to an OD_{600} of 0.2 and incubated for about 5 h. The cultures were then diluted in fresh media to an OD_{600} of 0.05 and transferred into a 96-well plate (clear with flat bottom, greiner bio-one) with 50 μL/well. A dilution series was made with octanoic acid (Sigma-Aldrich, GC grade) diluted in the same media, to reach final concentrations in the wells of 0−300 mg/L when 200 μL of the respective dilutions was added to the strains per well. All three replicates of each strain were inoculated in technical triplicates in the well plates. The starting absorbance was measured in a plate reader (CLARIOstar, BMG Labtech, Ortenberg, Germany), and plates were incubated for about 20 h at 30 °C without shaking before absorbance was measured again. From final absorbance values, a blank value (media without strain) was subtracted.

HPLC Analysis. To quantify glucose and ethanol concentrations, samples were centrifuged, and 450 μL of the supernatant was mixed with 50 μL 50% (w/v) 5-sulfosalicylic acid. Samples were analyzed in a UHPLC+ system by Thermo Scientific (Dionex UltiMate 3000) equipped with HyperREZ XP Carbohydrate H+ 8 μm column and a refractive index detector (Thermo Shodex RI-101). The HPLC was operated at 30 °C with 0.5 mM sulfuric acid and a flow rate of 0.5 mL/min. Glucose and ethanol were used as standards with concentrations of 0.5−20 g/L.

Software. Flow cytometry data evaluations and graphical presentations were performed with R v3.6.3 using packages readr v1.3.1, plyr v1.8.6, and tidyverse v1.3.0. Data tables were stored in Microsoft Excel 2016. Other graphs were made using the software Prism 9 (GraphPad, USA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00600.

Supporting Figures and Tables. (PDF)

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Author Contributions

L.B. and M.O. conceived the study. L.B. performed all experiments and wrote the paper. S.B. assisted with FACS experiments and data analysis. J.K. and E.B. provided advice and resources. All authors read, reviewed, and approved the manuscript.

Notes

The authors declare the following competing financial interest(s): E.B. is inventor of EP patent application No. 15 162 192.7 filed on April 1, 2015, and of EP patent application No. 15 174 342.4 filed on June 26, 2015, by Goethe-University Frankfurt, concerning short-chain acyl-CoA producing FAS variants. There are no other competing interests.

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■ ABBREVIATIONS

E1−E3, enrichment 1−3; FACS, fluorescence-activated cell sorting; FAS, fatty acid synthase; GC, gas chromatography; MPT, malonyl-palmitoyl transferase; OD_{600}, optical density at λ = 600 nm; ORF, open reading frame; SD, standard deviation

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