

## RESEARCH ARTICLE

# Transcriptomic response of *Saccharomyces cerevisiae* to octanoic acid production

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**One sentence summary:** RNA-Seq analysis of a *Saccharomyces cerevisiae* strain producing octanoic acid identified RPL40B as an important gene for improved production of medium-chain fatty acids.

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## ABSTRACT

The medium-chain fatty acid octanoic acid is an important platform compound widely used in industry. The microbial production from sugars in *Saccharomyces cerevisiae* is a promising alternative to current non-sustainable production methods, however, titers need to be further increased. To achieve this, it is essential to have in-depth knowledge about the cell physiology during octanoic acid production. To this end, we collected the first RNA-Seq data of an octanoic acid producer strain at three time points during fermentation. The strain produced higher levels of octanoic acid and increased levels of fatty acids of other chain lengths (C6–C18) but showed decreased growth compared to the reference. Furthermore, we show that the here analyzed transcriptomic response to internally produced octanoic acid is notably distinct from a wild type's response to externally supplied octanoic acid as reported in previous publications. By comparing the transcriptomic response of different sampling times, we identified several genes that we subsequently overexpressed and knocked out, respectively. Hereby we identified RPL40B, to date unknown to play a role in fatty acid biosynthesis or medium-chain fatty acid tolerance. Overexpression of RPL40B led to an increase in octanoic acid titers by 40%.

**Keywords:** octanoic acid; transcriptome response to medium-chain fatty acids; RNA-Seq; RPL40B

## INTRODUCTION

Medium-chain fatty acids like octanoic acid (C8 fatty acid) have a wide range of applications in antimicrobials, surfactants and cosmetics, and can also serve as precursors for biofuels (Huang

et al. 2011; Rosenblatt et al. 2017; Sarria, Kruyer and Peralta-Yahya 2017; Henritzi et al. 2018). At present, octanoic acid and its derivatives are produced from oil seed crops or crude oil (Yan and Pfleger 2020). In comparison to petrochemical industry, the

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extraction from oily plants, such as oil palm, might appear environmentally less harmful but there are rising concerns about its extensive cultivation. Oil palm cultivation is often associated with deforestation of rainforest and the ecological consequences thereof, and in addition competes with food production (Schmidt 2015). The engineering of microbes for octanoic acid production from renewable biomass is therefore a promising alternative (Hu et al. 2019; Yan and Pflieger 2020).

Yeast produces fatty acids in the fatty acid synthesis cycle through action of the cytosolic fatty acid synthase (FAS), a large enzymatic complex encoded by the two genes *FAS1* and *FAS2*. Naturally, *Saccharomyces cerevisiae* produces mostly saturated and monounsaturated long-chain fatty acids with chain lengths of C16 and C18 (Klug and Daum 2014). There are different ways to redirect production from long-chain to short- and medium-chain fatty acids. Here, we use a yeast strain that carries a modified variant of *FAS1*, namely *FAS<sup>RK</sup>* (Gajewski et al. 2017). This enzyme contains an amino acid exchange from arginine to lysine ('R1834K') in the malonyl-palmitoyl transferase (MPT) domain of *Fas1p*. Thereby, the loading of the precursor malonyl-CoA is reduced, leading to premature release of short acyl chains, such as octanoyl-CoA. Octanoyl-CoA is then hydrolysed by thioesterases and free octanoic acid is released (Gajewski et al. 2017). For an economically feasible production, further strain improvements and process optimizations are necessary (Baumann et al. 2020).

To increase titers, it is essential to advance the understanding of the producer strain's physiology in the course of octanoic acid production (Jarboe, Liu and Royce 2011). To our knowledge, such an analysis has not been undertaken yet. So far, two transcriptome-wide studies have been performed with extracellular addition of octanoic acid to wild type strains (Legras et al. 2010; Liu et al. 2013). In the first one, a microarray analysis showed that incubation with 0.05 mM (7 mg/L) octanoic acid for 20 min caused oxidative stress and a similar response to decanoic acid was observed. Overall, 75 genes were found to be differentially regulated in comparison to the non-exposed control. *Pdr12* was identified as the main involved transporter and the transporter *Tpo1* was shown to play a minor role in octanoic acid expulsion (Legras et al. 2010). In the second study, the exposure to 0.3 mM (43 mg/L) octanoic acid until mid-log growth phase revealed differential expression of at least 2-fold of 136 genes in comparison to the non-exposed control. Here, membrane leakiness was identified as a possible mechanism of cell disruption and increasing the oleic acid content was shown to enhance yeast tolerance to octanoic acid (Liu et al. 2013).

To get a comprehensive insight into expression changes during production of octanoic acid, we performed RNA-Seq in three different growth phases of an *S. cerevisiae* octanoic acid producer strain and a reference strain. We used expression profiles from the different growth phases to guide strain engineering efforts and achieved a 40% increase in octanoic acid production.

## RESULTS

### Fermentation profiles of an octanoic acid producer compared to reference

To get the first insight into transcriptional changes in different growth phases associated with octanoic acid production, we performed RNA-Seq transcriptome profiling. As a background strain we used SHY34, (Wernig et al. 2020) a triple knockout strain ( $\Delta fas1 \Delta fas2 \Delta faa2$ ). In this strain the two *FAS* genes are knocked

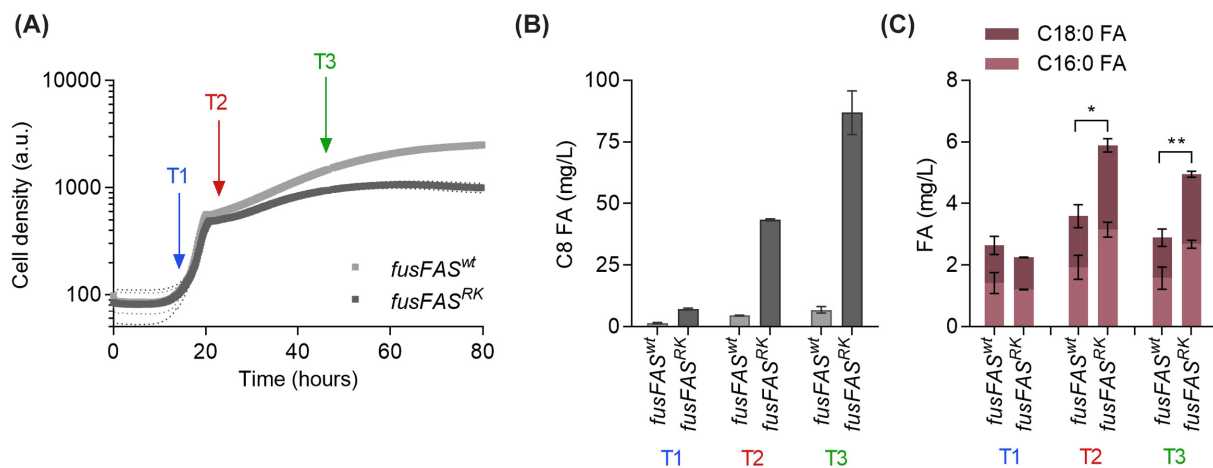
out as well as *FAA2*, encoding a fatty acyl-CoA synthetase, deletion of which prevents degradation of short- and medium-chain fatty acids (Leber et al. 2016; Henritzi et al. 2018). We transformed this strain either with a reference plasmid containing *FAS1* and *FAS2* in their wild type form fused into one sequence, i. e. *fusFAS<sup>wt</sup>*, or with a plasmid carrying *FAS1<sup>RK</sup>* and *FAS2* fused into one sequence, namely *fusFAS<sup>RK</sup>*. The *fusFAS<sup>RK</sup>* sequence contains an amino acid exchange from arginine to lysine in the MPT domain (R1834K), generating an octanoic acid producer (Wernig et al. 2020).

The two strains grew with a similar growth rate in exponential phase and only after the diauxic shift, growth was slower in the octanoic acid producer strain compared to the reference strain leading to a lower final OD in the octanoic acid producer (Fig. 1A). Such impairment of growth has similarly been observed for non-producers spiked with octanoic acid (Henritzi et al. 2018). Samples were taken in early exponential growth phase (14 h, T1), shortly after the diauxic shift (22 h, T2) and in ethanol consumption phase (46 h, T3), respectively. To confirm octanoic acid production, samples were taken from the culture supernatants at each sampling time for fatty acid extraction and quantification via GC measurement (Fig. 1B). In the reference strain, octanoic acid was produced in negligible amounts (< 7 mg/L) at all three sampling times whereas in the octanoic acid producer, the amount increased gradually from 7 mg/L (T1) to 43 mg/L (T2) to 87 mg/L (T3). We also observed an enhanced production of other short- and medium-chain fatty acids, namely C6, C10 and C12 fatty acids, in the octanoic acid producer strain, with the most pronounced difference to the reference strain at T3 (Figure S1, Supporting Information). Interestingly, even secreted amounts of saturated long-chain fatty acids, i.e. C16:0 and C18:0 fatty acids, were higher in the octanoic acid producer compared to the reference strain at T2 and T3 (Fig. 1C).

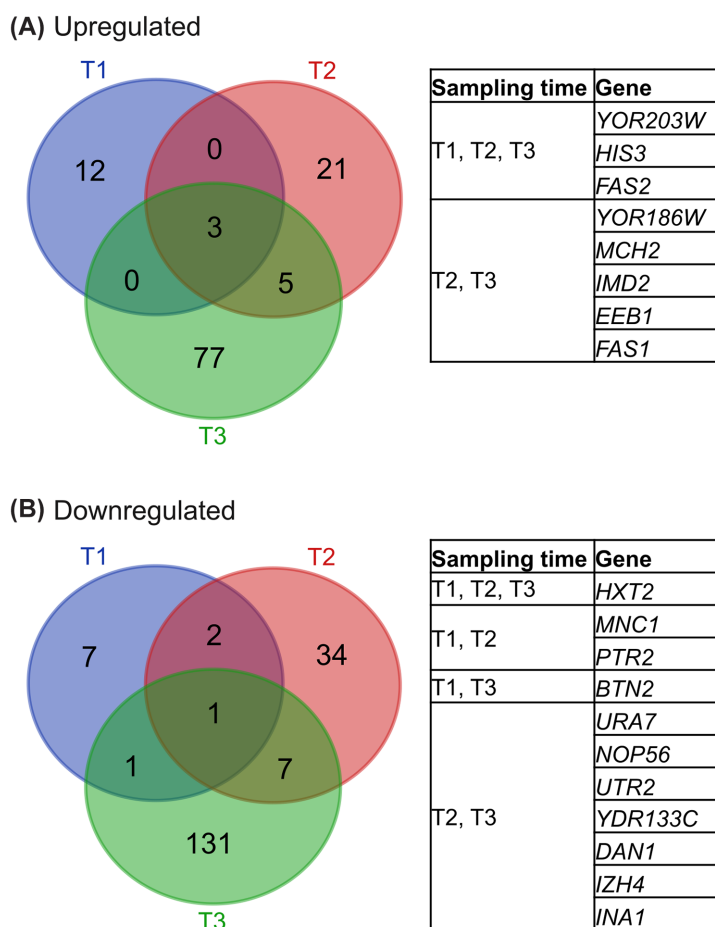
### Analysis of transcriptomic response in different phases of octanoic acid production

From the samples described above, we extracted RNA and performed RNA-Seq analysis. A principal component analysis (PCA) of the RNA-Seq data set revealed a close clustering of the three replicates at each sampling time in both strains (Figure S2, Supporting Information). The data of the reference strain and the octanoic acid producer strain also clustered closely together, suggesting a limited number of differentially expressed genes (Figure S3, Supporting Information). At T1, only 15 genes were upregulated ( $\log_2FC > 1$ ,  $FDR < 0.01$ ) in the octanoic acid producer in comparison to the reference strain—at T2 this was the case for 29 genes and at T3 for 85 genes. The number of downregulated genes was in a similar range, with 11 genes downregulated at T1, 44 genes at T2 and 144 genes at T3. The number of differentially expressed genes between the two strains therefore increased with fermentation time. A list of all differentially expressed genes can be found in Supplementary File 1. GO term enrichment analyses for all three sampling times are attached as Supplementary File 2.

To get a comprehensive view of the transcriptomic landscape of the octanoic acid producer in different growth phases, we compared the RNA-Seq data of the three sampling times (Fig. 2). We observed only eight genes upregulated and 11 genes downregulated at more than one sampling time compared to the reference. For upregulation, these included *FAS1<sup>RK</sup>*, *FAS2* as well as *HIS3*—the latter being the selection marker of the



**Figure 1.** Characterization of growth and octanoic acid production. **(A)** Growth was monitored on a cell growth quantifier in triplicates for strains SHY34 + *fusFAS<sup>wt</sup>* (reference strain) and SHY34 + *fusFAS<sup>RK</sup>* (octanoic acid producer). Sampling times are indicated with arrows (T1 = 14 h; T2 = 22 h and T3 = 46 h). **(B, C)** C8, C16:0 and C18:0 fatty acids (FA) were extracted and quantified via GC.  $n = 3$ , error bars =  $\pm$  standard deviation. Statistical analysis was performed using two-tailed t test (\* $P < 0.05$ , \*\* $P < 0.01$ ).



**Figure 2.** Differential gene expression for the octanoic acid producer compared to reference. Number of genes that are upregulated **(A)** or downregulated **(B)** in the octanoic acid producer strain in comparison to the reference strain ( $\log_2FC > 1$ ,  $FDR < 0.01$ ) between sampling times (T1 = 14 h; T2 = 22 h and T3 = 46 h). Gene lists include genes that are differentially regulated at more than one sampling time as indicated.

*fusFAS<sup>RR</sup>* plasmid used in the experiment. A higher copy number or expression of *fusFAS<sup>RR</sup>* was recently shown to increase octanoic acid titers (Wernig et al. 2020). It is likely that the expression was upregulated in the octanoic acid producer to ensure that enough long-chain fatty acids are produced for cell growth since chain length control in *fusFAS<sup>RR</sup>* is leaky (Gajewski et al. 2017). YOR203W was found to be upregulated at all sampling times, however, according to the SGD database, it is unlikely to encode a functional protein, and its assigned sequence partially overlaps with *HIS3*, suggesting an incorrect assignment in the RNA-Seq read mapping. *HXT2*, a glucose transporter, was the only gene found to be downregulated at all three sampling times.

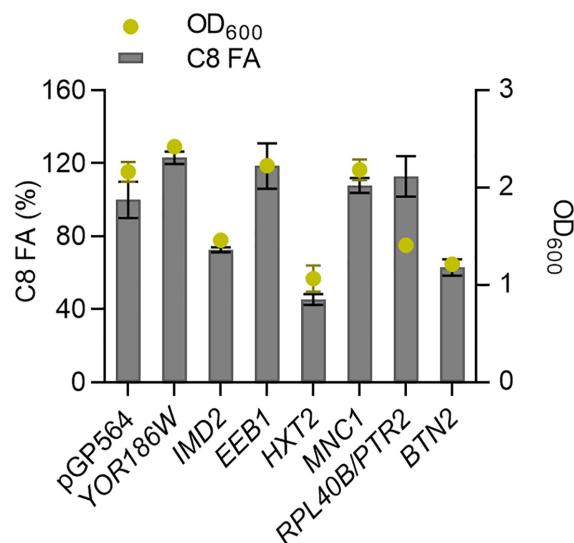
### Comparison to published transcriptome data

A total of two other transcriptome-wide studies have been performed so far, however, both of them with extracellular addition of octanoic acid to wild type *S. cerevisiae* strains (Legras et al. 2010; Liu et al. 2013). In the first study, a microarray analysis by Legras and colleagues, (Legras et al. 2010) 75 genes were found to be differentially regulated ( $\log_2FC \geq 0.3$ ,  $FDR < 0.05$ ) in comparison to the non-exposed control after incubation with only 0.05 mM (7 mg/L) octanoic acid for the short period of 20 min (Legras et al. 2010). In the second study by Liu et al. (2013) the exposure to six times the amount, i. e. 0.3 mM (43 mg/L) octanoic acid, led to differential expression ( $\log_2FC > 1$ ,  $P < 0.01$ ) in 136 genes in comparison to the non-exposed control (Liu et al. 2013). Interestingly, the overlap between these two published studies and our analysis was limited to a very small number of genes (Figures S4 and S5, Supporting Information). In all three studies the transporter *Pdr12* was found to be upregulated (RNA-Seq: T3), which has previously been associated with octanoic acid expulsion (Legras et al. 2010). However, additional plasmid-based expression of *Pdr12* has not led to an increase in octanoic acid titers in our hands, suggesting that endogenous expression is sufficient (Figure S6, Supporting Information).

To understand expression changes in the octanoic acid producer, we wanted to further analyse the effect of differentially expressed genes. To limit the number of genes for further analysis, we decided to focus on those genes that were differentially expressed at more than one sampling time in our data. We reasoned that such genes might have a more profound effect on octanoic acid production and constitute more promising targets for engineering than genes that are differentially regulated at only one sampling time.

### Overexpression of identified genes with a multi-copy plasmid library

We decided to test the effect of plasmid-based overexpression of all genes that were found to be up- or down-regulated, respectively, in at least two sampling times in our data (see gene tables in Fig. 2). In addition, we also tested the effect of overexpression of some genes overlapping with data from Liu and colleagues (Liu et al. 2013). For overexpressing the respective genes, we used a plasmid-library (Jones et al. 2008) that contains the majority of the yeast genome cloned as approximately 10 kb fragments into multi-copy plasmid backbones. Two genes (*DAN1*, *NOP56*) were not represented in the library and were therefore cloned separately on a multi-copy plasmid backbone. To avoid plasmid burden effects, we tested the plasmids in a strain that contains *FAS1<sup>RR</sup>* and *FAS2* in their wild type loci (LBY38). Towards the end of the exponential growth phase (18 h) we evaluated the octanoic



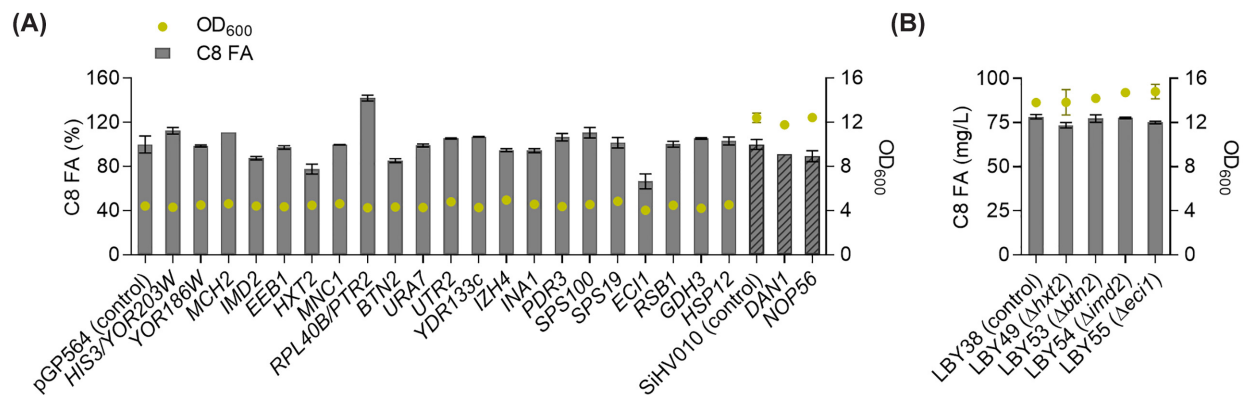
**Figure 3.** Fermentation of octanoic acid producer with overexpressed genes. The octanoic acid producer strain LBY38 was transformed with plasmids of a multi-copy library or a control plasmid (pGP564) and cultured for 18 h in buffered SCD<sup>-leu</sup> medium. Tested library plasmids contained the genes indicated on the x-axis. Octanoic acid was extracted and quantified by GC measurement, and is shown in % of the control (pGP564).  $n = 2$ , error bars =  $\pm$  standard deviation.

acid titers for some of the strains and observed a variation of titers in accordance with differing growth (Fig. 3). LBY38 containing plasmids with *IMD2*, *HXT2* or *BTN2*, respectively, had  $OD_{600}$  values and titers of only about 50% of the control, indicating delayed growth and therefore lower octanoic acid production.

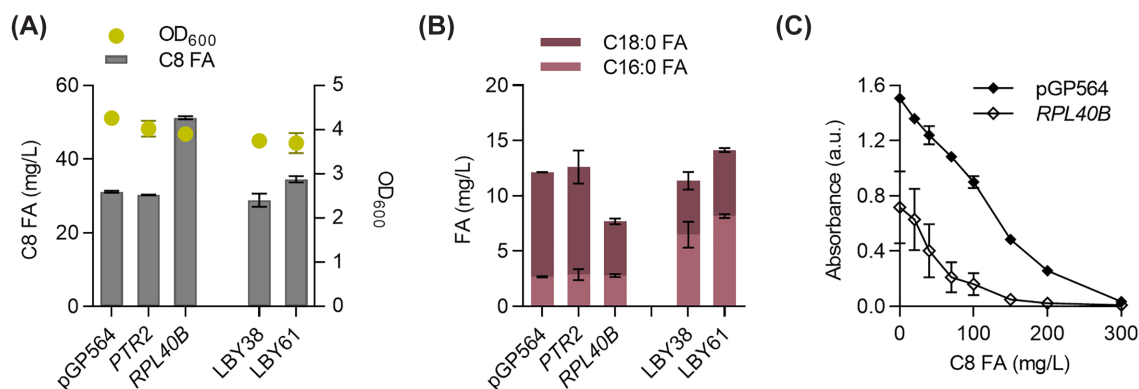
In ethanol consumption phase (46 h), all strains had reached similar  $OD_{600}$  values, which facilitated comparison between octanoic acid titers (Fig. 4A). After 46 h of fermentation, the expression of the library plasmid containing two neighboring genes of interest, *RPL40B* and *PTR2*, yielded a 40% higher octanoic acid titer; whereas *HXT2*, *BTN2* and *IMD2* expression led to a 25%, 15% and 13% decrease, respectively. The expression of the library plasmid containing *ECI1* led to a decrease in titer to about 60% of the reference. All other plasmids did not (< 10% difference) influence octanoic acid titers. To further characterize the effects on octanoic acid production, we generated individual knockout strains for *HXT2*, *BTN2*, *IMD2* and *ECI1* in the LBY38 background. We analysed octanoic acid titers after 46 h of growth in buffered SCD medium. All four knockout strains,  $\Delta hxt2$ ,  $\Delta btn2$ ,  $\Delta imd2$  and  $\Delta eci1$ , showed similar growth as well as octanoic acid titers as the control LBY38 (Fig. 4B).

### Overexpression of RPL40B leads to increase in octanoic acid production

To determine which of the two genes - *RPL40B* or *PTR2* - is responsible for the observed positive effect, we cloned each with its native promoter and terminator on a separate plasmid backbone. The fermentation showed that the plasmid-based expression of *RPL40B* caused an increase in octanoic acid titers of 40%, whereas *PTR2* expression did not influence octanoic acid titers (Fig. 5A). The amount of secreted saturated C18 fatty acid was lower in the strain with plasmid-based *RPL40B* expression compared to the reference (Fig. 5B). When introducing a second copy of *RPL40B* into the genome under the control of the strong *pPGK1* promoter, we could not observe this increase in titers (Fig. 5A), suggesting that the native *RPL40B* regulatory elements



**Figure 4.** Test of overexpression and deletion of targets in octanoic acid producer. **(A)** The octanoic acid producer strain LBY38 was transformed with plasmids of a multi-copy library and cultured in buffered SCD<sup>-leu</sup> medium. Tested library plasmids contained the genes indicated on the x-axis (dark grey bars). The genes DAN1 and NOP56 were not part of the library and therefore were cloned individually into SiHV010 multi-copy backbone, transformed into LBY38 and cultured in buffered YPD<sup>hye</sup> (striped bars). Titters are shown in % of the respective control strains. **(B)** A total of four genes were knocked out individually in LBY38 and the strains were fermented in buffered YPD. For all samples, octanoic acid was extracted after 46 h of growth and quantified by GC measurement.  $n = 2$ , error bars =  $\pm$  standard deviation.



**Figure 5.** Effect of PTR2 and RPL40B overexpression. **(A, B)** The octanoic acid producer strain LBY38 was transformed with plasmids containing PTR2, RPL40B or the control vector (pGP564). A second copy of RPL40B was integrated into the genome under control of pPGK1 in the *PYK2* locus in LBY38 resulting in strain LBY61. Strains were cultured in buffered SCD medium and fatty acids (FA) were extracted after 46 h and quantified by GC measurement.  $n = 2$ , error bars =  $\pm$  standard deviation. **(C)** Non-producer CEN.PK2-1C containing either the vector pGP564 (control) or RPL40B were inoculated in triplicates to OD<sub>600</sub> = 0.05 and supplied with 0–300 mg/L octanoic acid. Growth was measured by absorbance after 20 h.  $n = 3$ , error bars =  $\pm$  standard deviation.

are required for the positive effect on octanoic acid production. To investigate the effect of RPL40B on robustness towards octanoic acid, we transformed the non-producer CEN.PK2-1C with either a control plasmid or the multi-copy plasmid containing RPL40B. After incubation for 20 h in a 96-well plate (without shaking) with 0–300 mg/L octanoic acid, the strain with plasmid-based expression of RPL40B showed strongly decreased growth under all octanoic acid concentrations and even without addition of octanoic acid (Fig. 5C).

## DISCUSSION

To date, no transcriptomics data of an *S. cerevisiae* octanoic acid producer strain was available. To fill this gap, we generated a high-quality RNA-Seq data set for three sampling times during fermentation. By comparing the growth, fatty acid and expression profile of the producer to a reference strain, we were able to get a profound insight into transcriptomic changes during octanoic acid production.

In the course of fermentation, octanoic acid biosynthesis increased in the producer strain from 7 mg/L after 14 h to 87 mg/L after 46 h. This increase correlated with the increase in differential expression in comparison to the reference strain,

suggesting that increasing octanoic acid titers influence overall strain physiology. In terms of growth, the two strains behaved similarly until the diauxic shift when growth of the octanoic acid producer slowed down more quickly and reached lower final OD values. The main production of octanoic acid started shortly before or during the diauxic shift, which is consistent with the production profile observed in our previous studies (Gajewski et al. 2017; Wernig et al. 2020). Although we did not measure the concentrations of glucose and ethanol in the course of fermentations shown in Fig. 1, the sampling points can be clearly assigned to the glucose (T1) and ethanol consumption phases (T2 and T3), respectively, based on the previously published correlation of precisely measured growth curves and carbon source profiles (Bruder et al. 2016). This implies that octanoic acid production mainly occurs during the consumption of ethanol, presumably due to an increased supply of the precursor molecule acetyl-CoA on this carbon source. The onset of octanoic acid production parallels the slowdown in growth; this can probably be attributed to toxic effects of octanoic acid on the cell physiology (Viegas et al. 1989; Legras et al. 2010; Liu et al. 2013; Henritzi et al. 2018), which is further potentiated in the presence of ethanol (Viegas, Sá-Correia and Novais 1985; Viegas et al. 1989). Octanoic acid disrupts the composition of the yeast plasma membrane

leading to increased leakage and at high concentrations cell death (Legras et al. 2010; Liu et al. 2013). This effect was shown to be mitigated by an increase in the average chain length of membrane fatty acids, i.e. more C18 than C16 fatty acids, as well as an increase in unsaturated fatty acid, i.e. oleic acid (C18:1; Liu et al. 2013; Besada-Lombana et al. 2017). In the culture supernatant, unsaturated long-chain fatty acids were not detectable. Interestingly, at T2 and T3, we observed that the octanoic acid producer secreted more saturated long-chain fatty acids of chain lengths C16 and C18 than the reference strain. Since the strain used in the experiment is a  $\Delta fas1\Delta fas2\Delta faa2$  deletion strain, its only possibility to produce long-chain fatty acids is the leaky synthesis through plasmid-encoded *fusFAS<sup>RK</sup>*. Even though the 'R1834K' amino acid exchange in the MPT domain of FAS1 leads to premature release of short- and medium-chain fatty acids from the FAS cycle, this regulation is not completely tight (Gajewski et al. 2017). As the pressure to synthesize enough long-chain fatty acids, which are essential for growth and survival, is probably high, the copy number of the plasmid containing *fusFAS<sup>RK</sup>* seems to have been increased. The evidence for this is twofold: Firstly, we saw higher expression of the *fusFAS<sup>RK</sup>* construct with RNA-Seq for all time points in the octanoic acid producer strain and secondly, the expression of the marker gene on the plasmid, *HIS3*, was increased accordingly. In summary, this suggests that gene expression is not optimized for a maximum production of octanoic acid but rather ideal for producing sufficient long-chain fatty acids to survive and mitigate toxic effects of octanoic acid. This could also explain the limit in octanoic acid production that we seem to encounter, as the precursor malonyl-CoA is increasingly used for C16 and C18 rather than for C8 fatty acid biosynthesis.

To get a deeper understanding of transcriptional changes, we analyzed differential expression at the three sampling times in the producer compared to the reference. For this comparison, we chose a  $\log_2FC$  of at least 1 as a threshold ( $FDR < 0.01$ ) and chose a subset of the hits for further analysis. We evaluated the effect of additional, plasmid-based expression of all genes that were found to be up- or downregulated, respectively, at more than one RNA-Seq sampling time in the octanoic acid producer. For this purpose, we chose to use a multi-copy library which contains the entire yeast genome cloned onto 2 $\mu$  backbone plasmids and each plasmid contains roughly 2–10 genes (Jones et al. 2008). Even though the other genes on the plasmid might also affect octanoic acid production, it is unlikely that they so much influence titers as to mask the possible effect of the gene-of-interest. The expression of *HXT2*, *BTN2*, *IMD2* and *ECI1* from library plasmids led to a decrease in octanoic acid titers after 46 h of fermentation whereas individual knockouts did not have any effect on growth or octanoic acid titers (Fig. 4). Interestingly, growth and octanoic acid production were also delayed as a result of *HXT2*, *BTN2* and *IMD2* overexpressions (Fig. 3). In our RNA-Seq data, *HXT2* was found significantly downregulated at all sampling times. In addition, *HXT2* expression has also been found downregulated with externally supplied octanoic acid (Liu et al. 2013). As the downregulation was observable at all three sampling times, it seems to be rather independent of octanoic acid production. The expression of *BTN2*, similarly to *HXT2*, was downregulated at all sampling times in RNA-Seq ( $\log_2FC > 1$ ,  $FDR < 0.01$ ; except at T2:  $\log_2FC = 0.7$ ) and its additional plasmid-based expression led to lower octanoic acid titers. *BTN2* encodes a v-SNARE protein involved in intracellular protein transport and regulation of pH (Chattopadhyay et al. 2000; Chattopadhyay, Roberts and Pearce 2003). In another study,  $\Delta btn2$  strains showed an enhanced activity of vacuolar

H<sup>+</sup>-ATPase as well as diminished growth and decreased buffering capacity under sorbic acid supply, affecting intracellular pH homeostasis (Chattopadhyay et al. 2000). This contradicts the downregulation of *BTN2* expression at all sampling times in the octanoic acid producer, as the presence of octanoic acid might create more acidic conditions which the cell has to counteract. Also, our  $\Delta btn2$  strain did not show any difference in octanoic acid titers. The reason for the decrease in octanoic acid titers following plasmid-based *BTN2* expression is also unclear. These partly contradictory results could also arise from differences in protein turnover or possible post-translational modifications. The expression of *IMD2* was significantly upregulated in our RNA-Seq data at all three sampling times ( $\log_2FC > 1$ ,  $FDR < 0.01$ ; except at T1  $\log_2FC = 0.65$ ) and additional plasmid-based expression led to a 13% decrease in octanoic acid titers but did not influence growth. *IMD2* encodes an inosine monophosphate dehydrogenase, an enzyme involved in GTP biosynthesis. For *ECI1*, we observed a significant upregulation of expression at T3 ( $\log_2FC = 1.4$ ) in our data as well as under octanoic acid supply in a non-producer strain in previously published data (Liu et al. 2013). *ECI1* encodes a peroxisomal isomerase and is essential for  $\beta$ -oxidation of unsaturated fatty acids (Geisbrecht et al. 1998). We observed that *ECI1* plasmid-based expression resulted in less octanoic acid whereas its knockout (LBY55) showed similar titers as the reference strain. We suspect that upregulation of *ECI1* leads to increased  $\beta$ -oxidation of unsaturated fatty acids, thereby possibly covering the cell's need for acetyl-CoA. Why this also leads to lower octanoic acid titers, remains obscure, as *Eci1p* has not been described to be involved in medium-chain fatty acid degradation. The exact effect of all four enzymes on octanoic acid biosynthesis remains to be investigated.

We found that most genes identified to be differentially expressed at more than one sampling time, did not have a significant effect on octanoic acid titers when overexpressed. This suggests that the upregulation of these genes in the octanoic acid producer had effects not directly related to the octanoic acid titers. One possibility is that the observed gene expression changes help to mitigate effects on precursor availability or for instance toxic effects of the acid.

The library plasmid containing two genes of interest, namely *RPL40B* and *PTR2*, led to a strong increase in octanoic acid titer after 46 h of fermentation. Further analysis showed that plasmid-based expression of *RPL40B* was responsible for the improvement. Interestingly, secreted C18:0 fatty acid titers were halved in this strain. As *RPL40B* expression was downregulated at T3 in the transcriptomics analysis ( $\log_2FC = 0.45$ ), we suspect that this decrease reduces toxicity by lowering octanoic acid synthesis. In addition, a decrease in *RPL40B* expression may help maintain long-chain fatty acid synthesis at sufficient levels. Consequently, this would increase the strain's fitness. The genomic integration of a second copy of *RPL40B* under the control of the strong *pPGK1* promoter did not increase octanoic acid titers significantly and had no effect on C18 fatty acid titers. It seems that genomic expression of a second gene copy was not strong enough to exhibit the effects observed with expression from a multi-copy plasmid. Intriguingly, *RPL40B* expression from a multi-copy plasmid in non-producer CEN.PK2-1C, resulted in reduced growth. It is possible that this negative effect on growth is only observable in CEN.PK2-1C because LBY38 is already growth inhibited by inherent octanoic acid production. The decreased growth resulting from *RPL40B* overexpression in CEN.PK2-1C could also be attributed to lowered C18 fatty acid production by the native FAS complex and thereby impaired

robustness. Rpl40b is a subunit of the ribosome (Fernández-Pevida et al. 2012) but to our knowledge has never been reported to play a direct role in fatty acid biosynthesis. Future work could investigate the global effect of increased Rpl40b expression via ribosome profiling ('RiboSeq') (Weinberg et al. 2016), proteome analyses and ribosome assembly studies. Another interesting approach could be to study knockout or knockdown mutants of RPL40B or create mutants of other ribosomal subunit-encoding genes such as RPL40A.

In the aforementioned published transcriptomic data sets (Legras et al. 2010; Liu et al. 2013), which were generated by adding octanoic acid externally to *S. cerevisiae* wild type strains, RPL40B had not been found to be differentially regulated. Overall, there was little overlap between our RNA-Seq data set and the two other data sets. On the one hand, the diverging results could be attributed to the difference in experimental conditions, such as strain background, media and pH. More importantly, differential expression seems to be highly growth phase-dependent and to differ for internally produced and externally supplied octanoic acid. This underlines the importance of collecting transcriptomic data not just for externally added compounds, but for actual producer strains. Our comprehensive transcriptomics data set facilitated the detection of an unreported genetic target by enabling an in-depth view into yeast physiology during octanoic acid production. We were able to identify the new target RPL40B, overexpression of which led to 40% more octanoic acid.

## MATERIALS AND METHODS

### Strains and plasmid construction

Yeast strains and plasmids used throughout this study are listed in Table S1 (Supporting Information). The genes *DAN1* and *NOP56* for overexpression were amplified from SHY34 (Wernig, Boles and Oreb 2019) genomic DNA with primers containing the respective overhangs for cloning via Golden Gate (Lee et al. 2015; oligonucleotides are listed in Table S2, Supporting Information). The PDR12-tPDR12 fragment as well as promoter *pTDH3* were amplified from CEN.PK113-11C genomic DNA with primers containing the respective overhangs for cloning via homologous recombination into pRS42H/EcoRV. The library plasmid containing RPL40B and PTR2 (A10-C10 from (Jones et al. 2008)) was split into two plasmids as follows: Each gene was amplified with its endogenous up- and downstream regions from the original plasmid with primers containing overhangs for the pGP564 backbone for homologous recombination in yeast (CEN.PK2-1C). pGP564 was digested with *Bam*HI/*Pst*I prior to use. Genomic knockout of *HXT2* was performed via CRISPR/Cas9 as described previously (Generoso et al. 2016). The CRISPR/Cas9 plasmid for *hxt2* knockout was assembled via Golden Gate—SiHV138 was used and the GFP-dropout region was replaced by pre-assembled, double stranded gRNA oligonucleotides. For *BTN2*, *IMD2* and *ECI1* knockout via CRISPR/Cas9 was not possible and ORFs were knocked out with the Cre-loxP recombinase system with the *hphNT1* marker as described in (Güldener et al. 1996). For this purpose, the resistance cassette was amplified from plasmid pUG6H with primers containing overhangs for the up- and downstream regions, respectively, of the targeted ORF. For genomic integration of a second copy of RPL40B, the promoter *pPGK1* was amplified via PCR from pYTK011 and RPL40B was amplified via PCR from the original plasmid including the downstream part. Used primers contained suitable overhangs for the

parts or *PYK2* up- or downstream regions, respectively. Integration was achieved via CRISPR/Cas9 plasmid Lbv103 (assembled via homologous recombination into pRCC-K backbone) into the *PYK2* locus, thereby removing the entire *PYK2* ORF. Yeast transformations were performed according to Gietz and Schiestl (Gietz and Schiestl 2007) or for SHY34 via an adapted procedure (Gajewski et al. 2017). Cells were streaked out on selective YPD (1% yeast extract, 2% peptone, both by BD, Franklin Lakes, USA and Difco Laboratories, Franklin Lakes, USA; 2% D-glucose, Roth, Germany) containing hygromycin (200 µg/mL) to select for *hphNT1*, G-418 (200 µg/mL) to select for *kanMX* or on selective SCD medium (Bruder et al. 2016) lacking leucine (SCD<sup>-leu</sup>) to select for *LEU2*. Electrocompetent *E. coli* DH10β (Gibco BRL, Gaithersburg, MD) was used for subcloning according to standard procedures, and transformants were selected on lysogeny broth (LB) agar plates (Sambrook, Fritsch and Maniatis 1989) supplemented with carbenicillin (100 µg/mL), chloramphenicol (20 µg/mL) or kanamycin (50 µg/mL).

### Cultures for RNA-seq analysis

For pre-cultures, several colonies of SHY34 with plasmid *fusFAS<sup>wt</sup>* or *fusFAS<sup>RK</sup>*, respectively, were inoculated in 20 mL YPD 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 triplicates to an OD<sub>600</sub> of 0.1 in 50 mL buffered YPD medium in 300 mL flasks on a cell growth quantifier (Aquila Biolabs GmbH, Germany; Bruder et al. (2016)). Samples were taken for fatty acid quantification as described below. For RNA extraction, ~10 OD units of cells/sample were pelleted in a pre-cooled falcon at 4°C (3000 rcf) for 3 min. Falcons were then immediately dropped into liquid nitrogen for a few minutes and stored at -80°C until further processing.

### RNA extraction, processing and sequencing

RNA extractions were performed on samples that were mechanically lysed with 0.5 mm acid washed beads using an MP-Biomedicals FastPrep-24 for three 1-minute cycles. Further extraction was performed using an RNeasy® Kit from Qiagen, Hilden, Germany. Libraries were prepared using the TrueSeq mRNA Stranded HT kit. Sequencing was carried out using an Illumina NextSeq 500 High Output Kit v2 (75 cycles), with a minimum of 10 million single-end reads per replicate. The Novo Nordisk Foundation Centre for Biosustainability (Technical University of Denmark) performed the RNA sequencing and library preparation. RNA-Seq data sets can be found using SRA accession PRJNA575618. RNA-Seq read mapping was performed after analysis in FASTQC. RNA-Seq mapping for differential expression was mapped with STAR (Dobin et al. 2013) and reads were assigned with featureCounts (Liao, Smyth and Shi 2014). Differential expression results can be found in Supplementary File 1.

### Cultures for fatty acid production

*Saccharomyces cerevisiae* strains were grown as previously described (Gajewski et al. 2017) with minor adjustments. For pre-cultures, several colonies of a strain were inoculated in 20 mL YPD with 100 mM potassium phosphate buffer adjusted to pH 6.5 and antibiotic (200 µg/mL hygromycin), if applicable, or in similarly buffered SCD medium lacking leucine (SCD<sup>-leu</sup>). After shaking (180 r.p.m.) at 30°C overnight, the main culture was inoculated to an OD<sub>600</sub> of 0.1 in the respective medium and cultured in 300 mL shake flasks under the same conditions. For sampling,

the 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 (Henritzi *et al.* 2018). 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 fatty acids was performed as previously described (Ichiyama and Fukubayashi 2010). Samples were dissolved in 200  $\mu$ L toluene, mixed with 1.5 mL methanol and 300  $\mu$ L 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 on ice for 10 min, 1 mL H<sub>2</sub>O 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 Perkin Elmer Clarus 400 instrument (Perkin Elmer, Germany) equipped with an Elite FFAP capillary column (30 m  $\times$  0.25 mm, film thickness: 0.25  $\mu$ m; PerkinElmer, Germany) and a flame ionization detector (Perkin Elmer, Germany) as described previously (Henritzi *et al.* 2018).

### Toxicity test

CEN.PK2-1C was transformed with plasmid pGP564 or RPL40B, respectively, and plated on SCD<sup>-leu</sup>. Pre-cultures were inoculated in triplicates in buffered SCD<sup>-leu</sup> and grown overnight at 30°C (180 r.p.m.). For main cultures, strains were inoculated to an OD<sub>600</sub> of 0.2 and incubated for 4 h under the same condition (30°C, 180 r.p.m.). The cultures were then diluted in fresh media to an OD<sub>600</sub> of 0.05 and transferred into a 96-well plate (clear with flat bottom) with 50  $\mu$ L per well. A dilution series was made with octanoic acid diluted in the same media, to reach final concentrations in the wells of 0–300 mg/L. Of these dilutions, 200  $\mu$ L were added per well. The three biological replicates per 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 20 h at 30°C without shaking before absorbance was measured again. From final absorbance values, a blank value (media without strain) was subtracted.

### Software

RNA-Seq read mapping and data analyses were performed with R packages limma, edgeR and piano. Data tables were stored in Microsoft Excel 2016. Fermentation graphs were made using the software Prism 9 (GraphPad, USA). Geneious Prime 2020.2 software was used for assembly planning.

### Data availability

RNA-Seq data sets of data generated in this study can be found using SRA accession PRJNA575618.

### ABBREVIATIONS

FAS: fatty acid synthase; FDR: false discovery rate; GC: gas chromatography; log<sub>2</sub>FC: log<sub>2</sub> fold change; MPT: malonyl-palmitoyl transferase; OD<sub>600</sub>: optical density at  $\lambda = 600$  nm.

### AUTHOR CONTRIBUTIONS

LB performed experiments including RNA-Seq fermentations, cloning, engineered strain fermentations and analysis. TD performed RNA extraction. LB and TD analysed RNA-Seq data and wrote the paper. MO and EB co-supervised the project. VS and JN provided advice and resources. All authors read and approved the manuscript.

### SUPPLEMENTARY DATA

Supplementary data are available at [FEMSYR](https://www.femsyr.com) online.

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**Conflict of interest.** EB is inventor of EP patent application number 15162192.7 filed on April 1, 2015, and of EP patent application number 15174342.4 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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