ACS SyntheticBiology

Research Article

pubs.acs.org/synthbio

Flux Control at the Malonyl-CoA Node through Hierarchical Dynamic Pathway Regulation in *Saccharomyces cerevisiae*

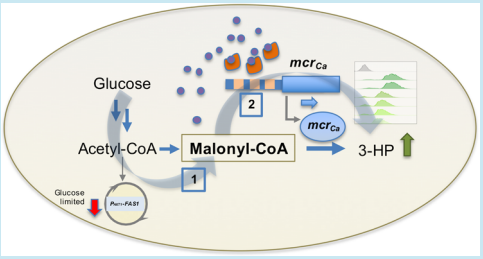
Florian David,[†,‡] Jens Nielsen,[†,‡,§] and Verena Siewers*[,†,‡]

[†]Department of Biology and Biological Engineering, [‡]Novo Nordisk Foundation Center for Biosustainability, Chalmers University of Technology, SE-41296 Gothenburg, Sweden

[§]Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2970 Hørsholm, Denmark

Supporting Information

ABSTRACT: The establishment of a heterologous pathway in a microbial host for the production of industrially relevant chemicals at high titers and yields requires efficient adjustment of the central carbon metabolism to ensure that flux is directed toward the product of interest. This can be achieved through regulation at key branch points in the metabolic networks, and here we present a novel approach for dynamic modulation of pathway flux and enzyme expression levels. The approach is based on a hierarchical dynamic control system around the key pathway intermediate malonyl-CoA. The upper level of the control system ensures downregulation of endogenous use of malonyl-CoA for fatty acid biosynthesis, which results in accumulation of this pathway intermediate. The lower level of the control system is based on use of a novel biosensor for malonyl-CoA to activate expression of a heterologous pathway using this metabolite for production of 3-hydroxypropionic acid (3-HP). The malonyl-CoA sensor was developed based on the FapR transcription factor of *Bacillus subtilis*, and it demonstrates one of the first applications of a bacterial metabolite sensor in yeast. Introduction of the dual pathway control increased the production of 3-HP by 10-fold and can also be applied for production of other malonyl-CoA-derived products.



KEYWORDS: *Saccharomyces cerevisiae, biosensor, dynamic pathway control, 3-hydroxypropionic acid*

Microbial cell factories are being engineered for the production of chemicals ranging from high-value pharmaceuticals, food and cosmetic ingredients to bulk products and fuels.[1−3] The first step in engineering these cell factories is usually the introduction of a heterologous biosynthetic pathway that leads to the formation of the desired product and employs a high expression level of all pathway genes. This can, however, lead to metabolic imbalances that impede the achievement of high yields, titers, and productivities. Such metabolic imbalances involve draining the precursor metabolites necessary for biomass synthesis or accumulating pathway intermediates that may be toxic to the cell. In order to ensure proper use of cellular resources, it is also important to control expression of genes encoding endogenous and heterologous pathway enzymes at specific time points to the appropriate level.

Therefore, expression modulation of certain pathway genes or modules is often applied using, e.g., promoters or ribosome binding sites of different strength or varying the gene copy number. This approach has been successfully applied to increase pathway flux in several cases, e.g., to increase the production of amorphadiene and taxadiene in *Escherichia coli*[4−6] or the formation of muconic acid in *Saccharomyces cerevisiae*.[7] Static pathway control, however, applies only to certain conditions and therefore often leads to reduced production when the fermentation conditions are changed.[8] It is, therefore, more desirable to incorporate dynamic control mechanisms when expressing heterologous pathways.[9,10]

Dynamic control can be implemented at different levels. At one level, it is possible to regulate pathway expression depending on extracellular triggers such as cell density using quorum sensing genetic circuits[10−12] or glucose concentration.[13] This allows for an autoregulated two-stage fermentation with a (primary) growth phase followed by a (secondary) production phase, which would be, e.g., beneficial in systems where biomass formation and product formation compete for the same precursor molecules. Another level of dynamic control includes feed-back and feed-forward loops, as they usually exist in natural systems to balance pathway fluxes, but implementation of such systems requires the development of intracellular metabolite sensors. An early example was the utilization of an acetyl phosphate sensor to control and thereby increase the formation of lycopene in *E. coli*.[14] In another study, Zhang et al. used an acyl-CoA responsive system to regulate the production of fatty acid ethyl esters in *E. coli*, leading to higher titers and increased strain stability,[15] and Dahl et al. identified *E. coli* promoters responsive to toxic isoprenoid pathway intermediates and employed these for the construction of feed-back and feed-forward loops for enhanced production of amorphadiene.[16] However, no examples have so far been presented in

Received: September 2, 2015
Published: January 10, 2016

DOI: 10.1021/acssynbio.5b00161
ACS Synth. Biol. 2016, 5, 224−233

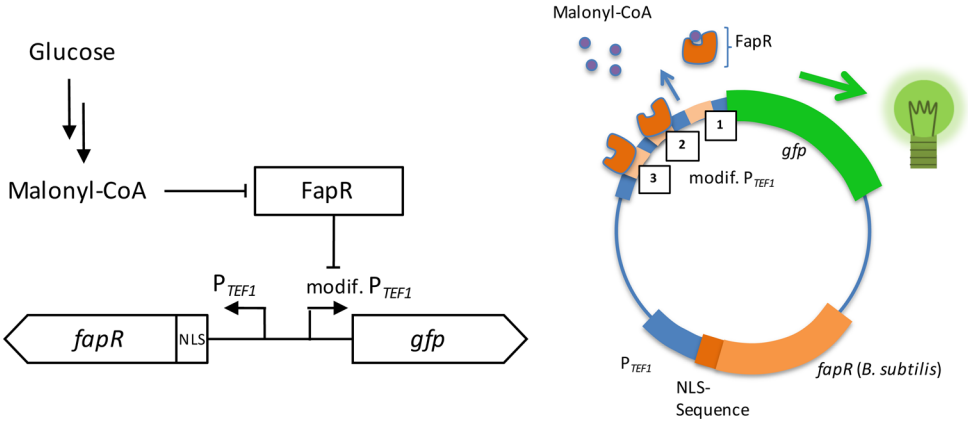


Figure 1. Malonyl-CoA sensor system. The system is based on the malonyl-CoA-sensitive transcription factor FapR derived from *B. subtilis* and FapR binding sites integrated into P_{TEF1} to control GFP expression as output signal. FapR functions as a repressor. The more malonyl-CoA that is present in the cell, the more repressor is released and GFP is expressed. The system was combined in one plasmid, pFDA9.

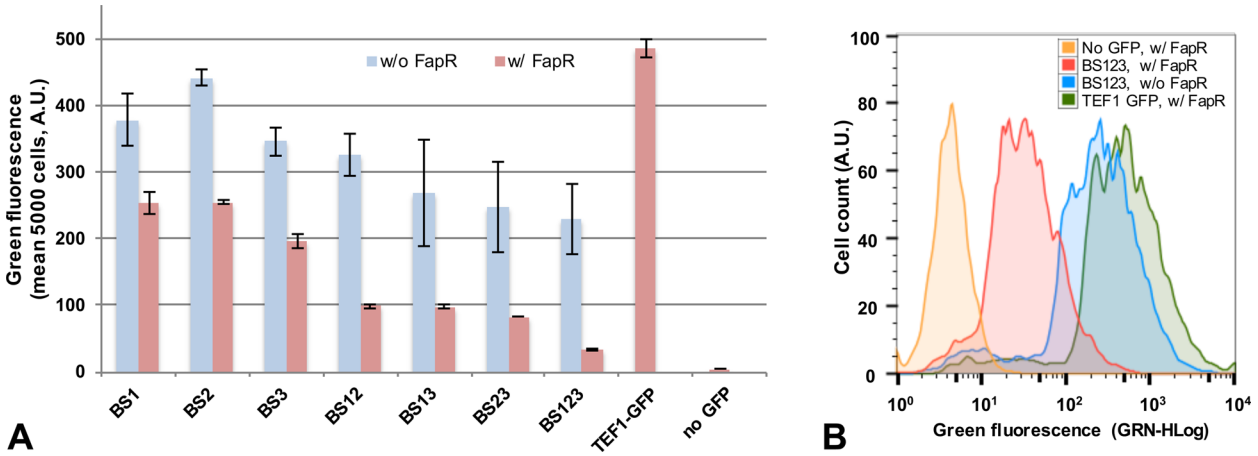


Figure 2. Qualitative malonyl-CoA sensor validation. (A) Different sensor constructs with various sensitivities were developed by modifying number and position of FapR binding sites (BS1/2/3) in the P_{TEF1} sequence. Yeast strain CEN.PK113-11C carrying particular constructs pFDA1−pFDA7 with (pFDA8) and without (p413TEF) expression of the FapR sensor were compared. Samples were measured in the exponential phase at an OD_{600} of 0.4, and the mean fluorescence of 5000 cells was determined by flow cytometry. The experiment was carried out in triplicates of three independent clones. (B) Validation of the malonyl-CoA sensor by flow cytometry measurements of CEN.PK113-11C strains carrying the following plasmids: no GFP, with FapR (p416TEF, pFDA8); BS123, with FapR (pFDA7, pFDA8); BS123, without FapR (pFDA7, p413TEF); or TEF1-GFP, with FapR (p416TEF-GFP, pFDA8).

yeast systems, which is partially due to the high complexity of their transcriptional regulation networks.[17]

Besides implementation of dynamic control in yeast, it would also be desirable to integrate the two different levels of dynamic control, i.e., regulation by growth stage of the culture combined with control by intracellular metabolite concentration, which we here refer to as hierarchical dynamic control. To demonstrate this, we first developed a sensor for intracellular malonyl-CoA in *S. cerevisiae* and used this for regulation of a pathway leading from malonyl-CoA to 3-hydroxypropionic acid (3-HP). Thereafter, we combined this system with an additional level of dynamic control by subjecting fatty acid biosynthesis, an essential pathway competing for malonyl-CoA, to regulation by the extracellular glucose concentration.[13] Thus, the metabolic flux can be gradually directed from biomass production to product formation during a fed-batch fermentation process, while expression levels of pathway genes are fine-tuned depending on precursor availability.

Malonyl-CoA is a crucial intermediate in fatty acid biosynthesis and also a precursor molecule for a number of industrially interesting products such as polyketides[18] or the platform chemical 3-HP.[19,20] In Gram-positive bacteria, gene regulation in response to malonyl-CoA concentration is mediated by the transcriptional repressor FapR, first described in *Bacillus subtilis*.[21] In the absence of malonyl-CoA, a FapR homodimer binds to its 34 bp operator (*fapO*) region. Binding of malonyl-CoA to the repressor leads to a conformational change of FapR, thus releasing it from the operator.[22] The FapR−*fapO* system was transferred to *E. coli* to build a malonyl-CoA sensor,[23] and the sensor was then employed to construct feed-back and feed-forward circuits for dynamic control of the native fatty acid synthesis pathway, which increased fatty acid titers.[24,25] In mammalian cells, coupling with the VP16 activation domain turned FapR into a transcriptional activator that was used to monitor intracellular malonyl-CoA concentrations.[26] Here, we applied the prokaryotic FapR−*fapO* system to dynamically control the heterologous 3-HP biosynthetic pathway in *S. cerevisiae*.

DOI: 10.1021/acssynbio.5b00161
ACS Synth. Biol. 2016, 5, 224−233

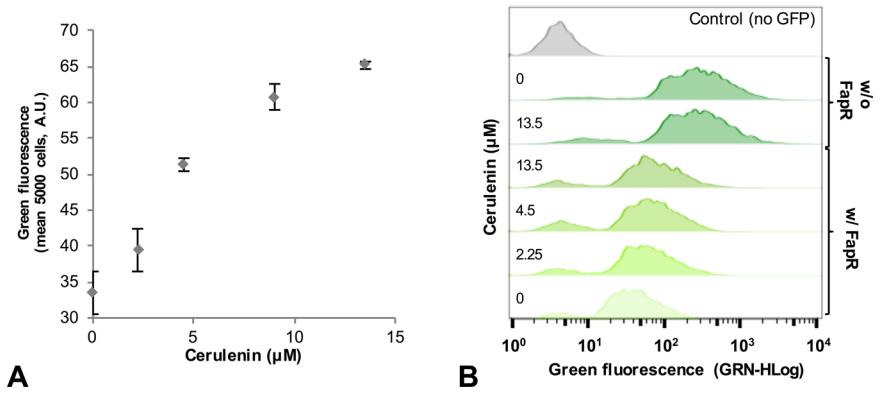


Figure 3. Quantitative validation of malonyl-CoA sensor. (A) Fatty acid synthase inhibitor cerulenin was used to increase intracellular malonyl-CoA pools to quantitatively validate the malonyl-CoA sensor system. The inhibitor was added to exponentially growing cells at an OD_{600} of 0.1, and fluorescence was measured 2 h after exposure. The strain carrying the malonyl-CoA sensor system with three FapR binding sites (BS123) was validated (CEN.PK113-11C carrying pFDA8, pFDA7) in triplicates (three different clones). (B) Flow cytometry measurements of exponentially growing cells 2 h after exposure to various cerulenin concentrations. Control samples comprise strains without FapR expression and without GFP expression (CEN.PK113-11C strains: BS123, with FapR (pFDA7, pFDA8); BS123, without FapR (pFDA7, p413TEF); no GFP, with FapR (p416TEF, pFDA8)).

RESULTS

Establishing a Malonyl-CoA Sensor in *S. cerevisiae*. Establishing an accurate metabolite sensor system needs to take several important factors into account. The aim is to create not only a qualitative on/off scheme but also a sensitive system that specifically and gradually responds to an input signal with a controlled output in a relevant range. Also, a modular setup of the system so that the sensitivity can be adjusted is of great importance. Here, we focused on the development of a malonyl-CoA sensor in *S. cerevisiae* by heterologously expressing the malonyl-CoA-responsive transcription factor FapR derived from *B. subtilis*.[21] To functionally transfer this bacterial system to a eukaryote, the SV40 nuclear localization signal (NLS) was added to the N-terminus of FapR to ensure transport into the nucleus. As a next step, we introduced FapR-specific binding sites, the 34 bp *fapO* operator, in the constitutively active strong yeast promoter *TEF1*.[27] The positions should preferably have as little influence as possible on the natural promoter activity. They were chosen based on a prediction for known binding sites of yeast transcription factors ±50 bp before and after the *TEF1* transcription start site.[28,29] The FapR binding sites were positioned so that they did not delete or interrupt any of the predicted recognition sites. To generate a feasible output signal, we coupled this engineered promoter to the expression of a green fluorescent protein (GFP). This system should hereby respond to a larger malonyl-CoA pool with increased GFP expression through FapR repressor-mediated control (Figure 1). We deliberately put FapR expression also under control of the *TEF1* promoter to ensure less deviation of actual sensor expression and GFP expression due to modulation of intrinsic growth phase-dependent *TEF1* promoter control, thereby keeping the actual response sensitivity at the same level.

Increasing the Malonyl-CoA Sensor Range. For establishing a relevant sensor range, we evaluated the number and position of FapR binding sites in the *TEF1* promoter (Figure 2). Binding sites were located in a ±50 bp region either downstream (BS1) or upstream (BS2 and BS3) of the transcriptional start site. GFP expression was tested either in the presence or absence of the *fapR* gene. Thereby, it was possible to study both the effect of FapR binding site insertion into the *TEF1* promoter and the actual effect of promoter repression when FapR was present. From this, it was found that the more binding sites that were integrated into the *TEF1* promoter, the more of the basal promoter activity was lost (Figure 2).

Integration of a single binding site decreased the promoter activity by 5−30%, whereas the modified promoter containing all three binding sites retained about 50% of its original activity. Looking at the promoter activity under FapR expression, it becomes clear that the bacterial transcription factor FapR functionally repressed GFP expression in this eukaryotic system. The repression effect increased with more binding sites integrated into the *TEF1* promoter. Neither the integration of FapR binding sites nor the expression of FapR had an effect on the constitutive nature of the promoter, and GFP expression levels remained at a constant level throughout cultivation (Supporting Information Figure S1). Combining results of reduction in promoter activity due to insertion of binding sites and repression of GFP expression through FapR binding resulted in a specific sensor range for each construct. This spans from absence of repressor binding (without FapR) to FapR binding (with FapR), here observed under exponential growth conditions. This is most likely not the full sensor range, as there may be a certain level of basal derepression through malonyl-CoA present in the cell. Since each additional binding site introduced has a higher impact on promoter repression in the presence of FapR than on reducing the basal promoter activity, there is a correlation between the apparent sensor range and the number of binding sites integrated into the *TEF1* promoter. The sensor range is here defined as the fold increase in GFP fluorescence when comparing cells not expressing FapR to those expressing FapR: 2-fold when integrating one FapR binding site, 3-fold when integrating two binding sites, and 7-fold when integrating three binding sites. The most tightly regulated system with three binding sites and a 7-fold sensor range was used for further validation studies.

Figure 2B gives a representative overview of the analyzed flow cytometry data. The two controls comprising the nonfluorescent cells and cells carrying the construct with GFP expression under native *TEF1* promoter control and at the same time expressing FapR clearly separate from each other. Also, only a small overlap and a good separation is seen when

DOI: 10.1021/acssynbio.5b00161
ACS Synth. Biol. 2016, 5, 224−233

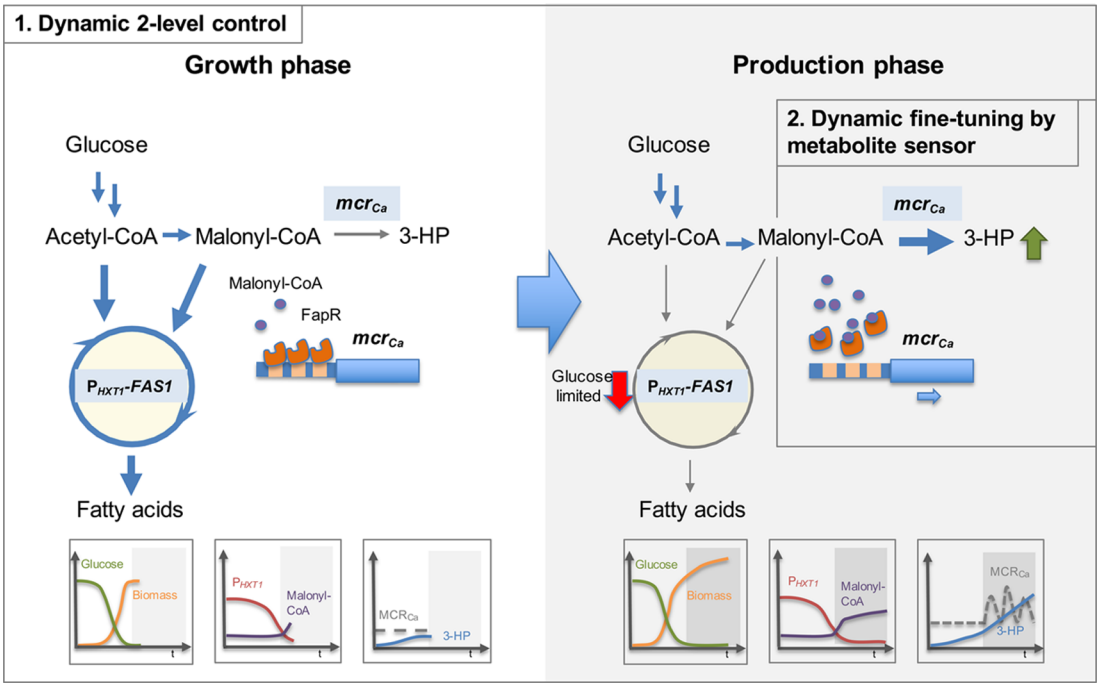


Figure 4. Hierarchical dynamic pathway control for 3-HP production. The system is based on a first growth phase where fluxes are similar to those under wild-type conditions and biomass can be quickly generated, which is followed by a production stage where the flux is channeled to the 3-HP production pathway. On a primary level of control, the fatty acid synthase gene *FAS1* is expressed under the control of a glucose concentration-sensitive P_{HXT1} promoter, enabling an increase in the concentration of malonyl-CoA under glucose-limited conditions. On a second level, a metabolite sensor for malonyl-CoA is used to continuously fine-tune gene expression of mcr_{Ca} for 3-HP production in the production phase. Anticipated changes in metabolite concentrations, P_{HXT1} promoter activity, and mcr_{Ca} expression are schematically illustrated.

comparing cells with and without FapR expression when GFP was under control of the modified *TEF1* promoter (BS123). Thus, through screening different positions and numbers of FapR binding sites inserted in the *TEF1* promoter, a suitable dynamic range of the biosensor was established.

Sensitivity Assessment of the Malonyl-CoA Sensor. Besides establishing a relevant sensor range, the system also has to have a certain sensitivity and gradual response to the metabolite of interest at concentrations that are physiologically relevant. From previous studies, it is known that there is a direct quantitative correlation between the fatty acid synthase (FAS) inhibitor cerulenin and intracellular malonyl-CoA concentrations.[24] Cerulenin binds the active site of the ketoacyl synthase domain of FAS2, thereby inhibiting the overall activity of Fas and leading to an increased concentration of malonyl-CoA, the direct substrate for the FAS system.[30,31] Cerulenin was added to exponentially growing cells, and fluorescence was measured by flow cytometry 2 h after exposure (Figure 3). The sensor system shows a gradual response corresponding to the applied cerulenin concentration (0−13.5 μM) and reached saturation at concentrations above 13.5 μM (data >13.5 μM not shown). Cerulenin blocks malonyl-CoA consumption, thereby increasing its intracellular concentration and leading to a release of FapR and increased GFP expression. This response increased with cerulenin concentration and exposure time (Supporting Information Figure S2). At the same time, the addition of cerulenin had a crucial effect on growth rate due to inhibition of the FAS system. As shown in Figure 3B, the addition of cerulenin had no effect on GFP expression in the control without FapR expression. These results therefore clearly show that the FapR sensor system successfully senses malonyl-CoA in a sensitive and gradual manner.

Metabolic Pathway Control for 3-HP Production. We next applied the malonyl-CoA sensor system for metabolic pathway control and fine-tuning gene expression, aiming at improving 3-HP production in *S. cerevisiae*. 3-HP production has recently been established in *S. cerevisiae* using different production pathways.[20,32,33] It can be produced from malonyl-CoA by one enzymatic step through a malonyl-CoA reductase derived from *Chloroflexus aurantiacus* (MCR_{Ca}).[33] Here, we aimed for continuous pathway control by self-adjusting target gene expression of mcr_{Ca} to the intracellular malonyl-CoA concentration. To implement metabolic pathway control, we put the expression of this enzyme under control of the modified *TEF1* promoter comprising three binding sites for FapR (BS123). Thus, we created a self-regulating system, which gradually expresses the mcr_{Ca} gene directly related to the malonyl-CoA available (Figure 4). First, we compared strains with mcr_{Ca} expression under control of the modified *TEF1* promoter (BS123) with and without FapR expression. In this comparison, the constitutive *TEF1* promoter without FapR regulation showed higher 3-HP production, around 0.1 g/L, compared to that with expression of the FapR repressor, where the final titer was decreased to 0.05 g/L (Figure 5). This is consistent with the initial evaluation of the engineered *TEF1* promoter that has 7-fold lower expression activity under FapR repression during exponential growth. Considering the 7-fold decrease in GFP, but only 2-fold decrease in 3-HP production, this may indicate that malonyl-CoA availability is a limiting factor to reach higher 3-HP titers.

To increase malonyl-CoA supply while at the same time testing the FapR-mediated regulation of mcr_{Ca} expression, FAS inhibitor cerulenin was added to the cultures. As described before, due to inhibition of the FAS system the malonyl-CoA

DOI: 10.1021/acssynbio.5b00161
ACS Synth. Biol. 2016, 5, 224−233

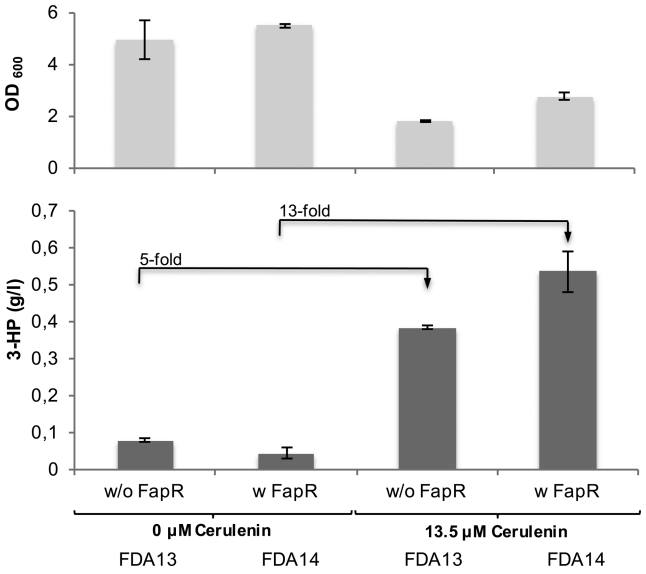


Figure 5. Application of malonyl-CoA sensor for 3-HP pathway regulation under cerulenin exposure. Fatty acid synthase inhibitor cerulenin was used to increase intracellular malonyl-CoA pools to improve precursor supply for 3-HP production and at the same time trigger mcr_{Ca} expression when under FapR control. CEN.PK113-11C was used, which carried plasmids with FapR (pFDA8, pYC1-BS123) and without FapR (p413TEF, pYC1-BS123) as control. The inhibitor was added to exponentially growing cells at an OD_{600} of 0.2 in 20 g/L glucose minimal medium. OD_{600} and 3-HP concentration were measured 24 h after exposure. The experiment was carried out in triplicates of three independent clones.

concentration will increase and, in the case of FapR control, trigger the expression of the mcr_{Ca} gene. Cerulenin was added at a concentration of 13.5 μM to exponentially growing cells at an OD of 0.2. The 3-HP titers and cell densities were determined 24 h after exposure. Inhibiting the FAS system through cerulenin addition clearly increased 3-HP production up to 5-fold when FapR was not expressed and up to 13-fold when FapR was expressed (Figure 5). Similar trends were observed when using a lower concentration of cerulenin (2.25 μM) (Supporting Information Figure S3). Here, there was no effect on the generation of biomass, but there was still a pronounced effect on 3-HP production. With this static control through cerulenin addition, the FapR-controlled gene expression of mcr_{Ca} was shown to be functional while at the same time leading to increased 3-HP titers.

From an industrial bioprocess perspective, the addition of cerulenin would not be practical in a large-scale fermentation due to high cost. Therefore, we implemented a hierarchical dynamic control system on the genetic level through modifying the *FAS1* gene expression characteristics. This gene codes for the β-subunit of the fatty acid synthase complex in *S. cerevisiae*, whereas the α-subunit is encoded by *FAS2*. When regulating expression of the *FAS1* gene, the *FAS2* gene is coregulated through a negatively acting downstream repression site, thereby leading to a coordinated up- or downregulation of the entire FAS system.[34] We used the P_{HXT1} promoter to render this regulation glucose concentration-dependent. A similar approach was previously used to successfully redirect the carbon flux from sterol synthesis toward α-santalene production.[13] As soon as the external glucose concentration is low, this promoter is repressed and *FAS1* gene expression is downregulated, leading to an increased intracellular malonyl-CoA concen-

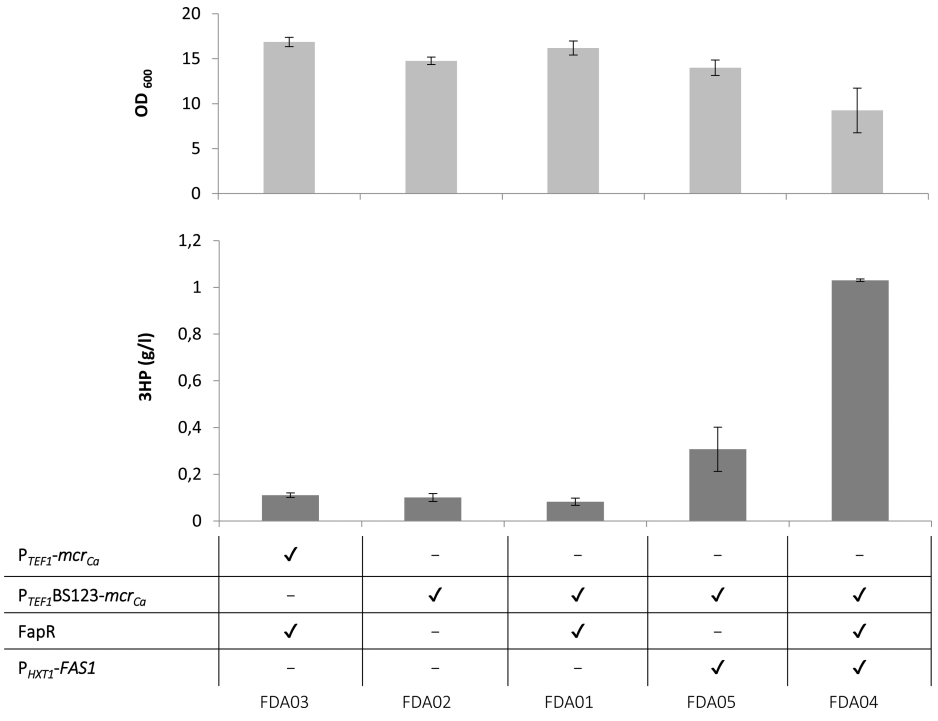


Figure 6. Application of malonyl-CoA sensor for dual dynamic 3-HP pathway regulation. The initial CEN.PK113-11C strain was compared to the modified strain CEN.PK113-11C:P_{HXT1}-*FAS1* under glucose-limiting conditions, with and without FapR control of mcr_{Ca} expression. Strains FD01−FDA05 were compared. Cells were cultivated in 5 mL of minimal medium in 50 mL tubes, with 10 g/L initial glucose concentration. Feed beads (release of 0.25 g/L/h) were added to the medium after an initial batch phase of 9 h. Final samples were taken for OD_{600} and 3-HP measurements after 96 h. Samples were measured in triplicates of three independent clones.

DOI: 10.1021/acssynbio.5b00161
ACS Synth. Biol. 2016, 5, 224−233

tration (Figure 4). We hereby implemented a two-stage dynamic system that enables an initial growth phase similar to that of a wild-type strain followed by a 3-HP production phase under glucose-limiting conditions. Here, a fed-batch approach is an ideal setup to couple both a batch phase where biomass is generated with a production phase under external feeding control. To simulate fed-batch like conditions, we used glucose feed beads with a constant release of 0.25 g/L/h. After an initial batch phase with glucose concentrations of 5 and 10 g/L, feed beads were added and samples were taken after 96 h.

Under these conditions, different strains were compared: the background strain CEN.PK113-11C with mcr_{Ca} expression under control of the native *TEF1* promoter, the modified P_{TEF1}BS123 promoter with and without FapR expression, and the engineered strain FDA00 (CEN.PK113-11c P_{HXT1}-*FAS1*) with and without FapR expression (Figure 6).

As described before, the background CEN.PK113-11C strain shows less 3-HP production when expressing the FapR regulator, probably because the intracellular malonyl-CoA concentration is not sufficiently high to trigger sufficient mcr_{Ca} expression. When using the engineered strain with *FAS1* expression under P_{HXT1} promoter control, much more 3-HP, up to 0.4 g/L, was produced. It could be proven that this hierarchical two-stage control was successful in redirecting malonyl-CoA flux into 3-HP production instead of fatty acid production. When combining both the dynamic two-stage control with the malonyl-CoA sensor approach for continuous fine-tuning of gene expression in the production phase, a further increase in 3-HP production, up to 1.0 g/L, could be achieved (Figure 6). These results, therefore, clearly show that under these more stringent conditions of glucose limitation fine-tuning the gene expression of mcr_{Ca} becomes important and beneficial. The same trend was confirmed during fed-batch cultivations in a parallel bioreactor setup (Supporting Information Figure S4). Clearly, higher 3-HP titers, yields, and rates were examined under P_{HXT1} and FapR-mediated metabolic pathway control for 3-HP production. Under these conditions, high titers of about 0.8 g/L 3-HP were reached after 25 h.

DISCUSSION

Here, we established, for the first time, a hierarchical dynamic pathway control system in *S. cerevisiae*. We highlighted the advantages of this system by substantially increasing malonyl-CoA-derived 3-HP production. As a first step, an internal biosensor based on a malonyl-CoA-responsive transcription factor was established. The chosen heterologous repressor-based system has the advantage of creating robust and modular setup, which does not rely on complex interactions with other intrinsic transcription factors or require the active recruitment of the eukaryotic RNA polymerase. By integrating specific FapR binding sites into the constitutively active yeast promoter *TEF1*, we created an orthologous system that could, in principle, be used for any other metabolite-responsive repressor-type transcription factor that either binds or is released from its recognition site under increased metabolite concentrations of interest. The number and positions of binding sites were chosen based on available data related to potential binding sites of yeast transcriptions factors within the *TEF1* promoter region 50 bp up- and downstream the transcriptional start. Here, more predictive approaches could be used to increase the sensor range even further and allow for a tighter regulation.[35] For example, based on an extensive yeast promoter screening, a distinct correlation between expression and binding site multiplicity, dependencies on the distance between the binding site location and gene start, and a clear 10 bp periodic relationship of transcription factor binding sites were shown.[36] For creating a feasible intracellular biosensor system, one faces several challenges, like balancing sensitivity and dynamic response range adapted to relevant intracellular metabolite concentrations. There is a steady equilibrium between bound/unbound sensor and the particular intracellular metabolite concentration. In our case, the actual change in the promoter sequence also plays a major role in changing the responsiveness of the system. By inserting multiple FapR binding sites, the basal promoter activity is decreased, making it at the same time more susceptible to the repressor. This strategy of multiplying binding sites was shown previously to be the most feasible for setting up a repressor-based biosensor approach for controlled fatty acid production in *E. coli*.[15] Another strategy to generally increase the stringency of biosensor function is to use hybrid promoters, thereby introducing another level of coregulation, e.g., through addition of coinducers like IPTG[15] or copper.[37]

Taking the stringency and sensor range of the here developed malonyl-CoA sensor into account, this system is also an ideal tool for screening cell libraries for efficient malonyl-CoA producers. From the flow cytometry data, a clear separation was seen between cells expressing and not expressing FapR, and a distinct fluorescence shift was observed for cells having a cerulenin-induced increased malonyl-CoA pool (Figures 2B and 3B). This allows for specific gating, subsequent fluorescence activated cell sorting (FACS), and targeted screening for variants with increased malonyl-CoA production capabilities. During the preparation of this manuscript, a similar approach was published where a malonyl-CoA sensor was used to screen for specific target genes increasing the malonyl-CoA pool.[38] Such strains would be well-suited as platform strains for the production of fatty acids, polyketides, and flavonoids, all compounds of interest for production of biofuels, commodity/fine chemicals, and pharmaceuticals.

We further combined our novel malonyl-CoA biosensor with dual-level dynamic control. In this system, the process environment dynamically controls the malonyl-CoA availability, more specifically by the extracellular glucose concentration. This was realized by subjecting fatty acid synthase expression to control by the glucose concentration-sensitive P_{HXT1} promoter. Hereby, fatty acid biosynthesis is downregulated at the later stages of a batch fermentation and during the (glucose-limited) feed phase of a fed-batch fermentation. This is likely to result in an increase in the internal malonyl-CoA concentration, which is a precursor metabolite for 3-HP production. With this system, sufficient biomass can be generated in the initial growth phase followed by a production phase where the metabolism is directed toward 3-HP generation. The approach has the great advantage of not relying on external inducers; it decouples growth from the 3-HP production phase in an autoregulated manner. This two-stage fermentation strategy was shown to be beneficial in reaching higher final product titers, yields, and rates and can be directly translated for larger scale production as it does not rely on external inducers.[10]

To validate this system, we used feed beads to establish a constant feeding rate, keeping the glucose concentration low. In the next step, a controlled bioreactor fed-batch setup with a constant feed was used for further validation. Under such conditions, the specific growth rate decreases exponentially

DOI: 10.1021/acssynbio.5b00161
ACS Synth. Biol. 2016, 5, 224−233

with increased biomass concentration. With this hierarchical control system, expression of the mcr_{Ca} gene is dynamically fine-tuned using the FapR biosensor system, which is beneficial for the cellular system for two different reasons. First, the metabolic burden resulting from constant production of the heterologous MCR_{Ca} enzyme is reduced. The gene expression is adapted to the actual precursor availability, which makes the system more efficient and results in fewer cellular resources being wasted in futile cycles. Second, not all malonyl-CoA is immediately channeled toward 3-HP production, thus making it available for cell proliferation and maintenance, especially in the initial growth phase.

As shown, the hierarchical two-level control and fine-tuning of mcr_{Ca} gene expression lead to a 10-fold increase in 3-HP production, resulting in a titer of 1 g/L. So far, when using yeast as a cell factory, only extensive metabolic engineering made it possible to reach titers in this range. Previous multistep engineering of metabolic pathways upstream of malonyl-CoA, like increasing the NADPH supply and abolishing Snf1-dependent regulation of Acc1, resulted in titers only up to 0.5 g/L.[33] We imagine that a combination of our approach of dynamic control with metabolic engineering of upstream pathways may be used to increase 3-HP production even further.

The approach of dynamic metabolic control integrating signals from the cellular environment with intracellular metabolism and regulatory circuits also has a great potential to be used for further application in dynamic expression of heterologous pathways and fine-tuning of cellular phenotypes. Especially in the nonuniform environment of industrial fermenters, these regulation systems might be essential tools to increase yield, titers, and especially process robustness.

The developed approach can be seen as a prototype of a general strategy to engineer and dynamically control metabolic pathways and could, in principle, be applied easily to any malonyl-CoA-derived product in *S. cerevisiae*; when it is extended to other orthologous transcription factors and pathways, it could be used even further for redirecting metabolic fluxes and balancing precursor supply.

METHODS

Media and Culture Conditions. *S. cerevisiae* strain CEN.PK113-11C (*MAT*a *SUC2 MAL2−8*^{c} *his3Δ1 ura3−52*; P. Kötter, University of Frankfurt, Germany)[39] was used as the background strain. Synthetic dextrose (SD) medium was applied to select for recombinant yeast strains, containing 6.7 g/L of yeast nitrogen base without amino acids (Difco Laboratories, Sparks, MD, USA), 0.77 g/L of complete supplement mixture (CSM, without uracil and histidine) (MP Biomedicals, Solon, OH, USA), and 2% glucose. YPD plates containing 10 g/L yeast extract, 20 g/L casein peptone, 20 g/L glucose, and 20 g/L agar were supplemented with 200 mg/L G418 (Formedium) to select for strains carrying the kanMX cassette. For marker loop out, cells were cultivated in YPG medium, which is identical to YPD medium but has a galactose (2%) supplement instead of glucose.

For sensor evaluation and 3-HP production, yeast strains were grown in defined minimal medium[40] with 7.5 g/L of $(NH_4)_2SO_4$, 14.4 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, and 20 g/L glucose, adjusted to pH 6.0 with 5 N NaOH. For routine cloning procedures, *E. coli* DH5*α* was used grown in Luria−Bertani (LB) broth with 80 mg/L ampicillin supplement. 3-HP was purchased from TCI Europe N.V. (Zwijndrecht, Belgium).

Plasmid Construction. All oligonucleotide primers, primer pairs, and strains used in this study are listed in Supporting Information Tables S1−S3. For PCR reactions, Phusion high-fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA) was used.

For constructing p416TEF-GFP, the GFP gene was amplified from pFA6a-GFPS$_{65}$T-HIS3MX6[41] by the primer pair GFP-fw and GFP-rv, and the product was cloned via CPEC[42] into p416TEF[43] (amplified via primer pair p416TEF-fv, p416TEF-rv).

In vector p416TEF-GFP, modified parts of the *TEF1* promoter (BS1/2/3) carrying different FapR binding site combinations (see the Supporting Information) were introduced. These were ordered from Genscript (Piscataway, NJ, USA) and were amplified using the primer pair TEF1-insert-fw and TEF1-insert-rv. The vector backbone of p416-GFP was amplified using primers TEF1-vector-fw and TEF1-vector-rv, creating overlapping regions with the modified *TEF1* promoter fragments. Using the CPEC cloning method, the vectors pFDA1, pFDA2, pFDA3, pFDA4, pFDA5, pFDA6, and pFDA7 were constructed.

Primer pair NLS-FapR-fw and FapR-rv was used for *fapR* amplification from *B. subtilis* genomic DNA and at the same time for introducing a SV40 NLS at the N-terminus of FapR. The vector backbone of p413TEF[43] was amplified using primer pair p413TEF-NLSfapR-fw and p413TEF-NLSfapR-rv, thereby creating regions that overlapped with the amplified *fapR* fragment. Through CPEC, both fragments were cloned together, and plasmid pFDA8 (p413TEF-NLS-FapR) was created.

The BS123-GFP cassette from pFDA7 was integrated into pFDA8. It was amplified from pFDA7 using the primer pair NsiI-P$_{TEF1}$BS123-GFP_fw and NsiI-P$_{TEF1}$BS123-GFP_rv and cloned via the NsiI restriction site into pFDA8, thereby creating pFDA09 (p413TEF-NLS-FapR-P$_{TEF1}$BS123-GFP). The same approach was followed for cloning the BS123-GFP cassette into the plasmid p413TEF to create the control plasmid pFDA10 (p413TEF-P$_{TEF1}$BS123-GFP) without *fapR* expression. From restriction digestion analysis, the plasmid variant was chosen where both *TEF1* promoters are in opposite directions relative to each other.

For construction of the plasmid containing mcr_{Ca} under control of the modified *TEF1* promoter (P$_{TEF1}$BS123), plasmid pYC1[33] was used as the backbone plasmid. P$_{TEF1}$BS123 was amplified from pFDA10 (p416P$_{TEF1}$BS123-GFP) using the primer pair P1_MCR_TEFm_fv and P2_MCR_TEFm_rv. This fragment was integrated into NotI and BamHI predigested pYC1 by homologous recombination in yeast, creating construct pYC1-BS123.

All constructs were verified through restriction digestion and sequencing (Eurofins, Ebersberg, Germany).

Strain Construction. For testing different combinations of FapR binding sites inside the *TEF1* promoter, constructs pFDA1−pFDA7 were each combined with pFDA8 through transformation into CEN.PK113-11C, creating strains FDA06, FDA07, FDA08, FDA09, FDA10, FDA11, and FDA12, respectively. Strains were plated on SD-URA-HIS plates, and three colonies of each strain were confirmed via PCR.

Background strain CEN.PK113-11C was modified through replacing 1000 bp of the native promoter in front of the *FAS1* gene by 1000 bp of the *HXT1* promoter. Here, a deletion/

DOI: 10.1021/acssynbio.5b00161
ACS Synth. Biol. 2016, 5, 224−233

replacement cassette approach was used[44] to create the strain FDA00 (CEN.PK113-11C P_{HXT1}-*FAS1*). For the deletion cassette, two fragments were used (see sequences in the Supporting Information): P1.2(1+2) carrying *loxP*(71)-P_{TEF1}-*KanMX*-T_{TEF1}-P_{GAL1} and P1.2(2+3+4) carrying P_{GAL1}-*cre*-T_{CYC1}-*loxP*(66). Regions 500 bp downstream (H1) (−1000 until −1500 bp) and upstream (H2) (0 until +500 bp) of the *FAS1* promoter were amplified with corresponding primer pairs (pH1_fw, pH1_rv; pH2_fw; pH2_rv). 1000 bp of the *HXT1* promoter (−1000 bp) was amplified with primers pHXT1-fw and pHXT1-rv, which carry overlapping regions to H2 and P1.2(2+3+4). P1.2(1+2) was amplified via primers pJet_(1+2)_fw_ext and pJet_(1+ 2)_rv to create an overlapping region with H1. Using fusion PCR, two main cassettes were amplified: (1) H1-p1.2(1+2) and (2) p1.2(2+3+4)-pHXT1-H2. CEN.PK113-11C was transformed with the two cassettes using the frozen-EZ yeast transformation II kit (Zymo Research, Irvine, CA, USA), and strains were selected on YPD G418 plates. Marker loop out was induced through 48 h cultivation in YPG medium. Strains were confirmed by PCR and sequencing, creating new background strain FDA00.

For testing 3-HP production under dual dynamic pathway control, the following strains were created and tested: FDA01 (CEN.PK113-11C; pYC1-BS123, pFDA9), FDA02 (CEN.PK113-11C; pYC1-BS123, pFDA10), FDA03 (CEN.PK113-11C; pYC1, pFDA9), FDA04 (FDA00; pYC1-BS123, pFDA9), and FDA05 (FDA00; pYC1-BS123, pFDA10).

Strains were plated on SD-URA-HIS plates, and three colonies of each were confirmed via PCR.

Characterization of Malonyl-CoA Sensor in *S. cerevisiae*. For cultivation, minimal medium was used with 20 g/L glucose. Precultures derived from single colonies were incubated for 18 h in 5 mL of medium. Main cultures were inoculated at an OD_{600} of 0.1 and grown in 10 mL of medium in 100 mL shake flasks. Samples were taken during the exponential growth phase (glucose) at an OD_{600} of 0.4 about 6 h after inoculation. Cells were diluted to an OD_{600} of 0.02 to keep the concentration <500 cells/μL. Fluorescence was measured via flow cytometry using a Guava easyCyte 8HT (Merck Millipore, Billerica, MA, USA). Cells were excited by a blue laser at 488 nm (75 mW), and green fluorescence signals were measured through a 525/30 band pass filter. Signals were triggered against the FSC, and specific gain values were set (FSC: 19.03, SSC: 418, GRN: 5.42). Fluorescence mean values were determined based on 5000 cell counts. FlowJoX software (Ashland, OR, USA) was used to create images.

For monitoring the influence of cerulenin, exponential growing cells at an OD_{600} of 0.2 were exposed to different concentrations of cerulenin (0, 2.25, 4.5, 9, 13.5, 45 μM). Cerulenin stock solutions (in 98% ethanol) were used at a 1:1000 dilution.

Characterization of Malonyl-CoA Sensor for 3-HP Production. Strains [CEN.PK 113-11C; FDA00 (CENPK11C P_{HXT1}-*FAS1*)] with various combinations of plasmids were compared for 3-HP production under different conditions. FDA01 and FDA02 were tested under cerulenin exposure (2.25 and 13.5 μM) for 3-HP production after 24 h. The inhibitor was added to exponentially growing cells at an OD_{600} of 0.2 in 20 g/L glucose minimal medium. The experiment was carried out in triplicates of three independent clones.

FDA01 and FDA02 were also compared to strains with a P_{HXT1}-*FAS1* background with FapR sensor expression and MCR expression under P_{TEF1}BS123 and P_{TEF1} promoter control (FDA03−FDA05). Experiments were carried out in tubes or shake flasks with 5 or 10 mL of minimal medium, respectively (10 g/L initial glucose). Cultivation conditions were 30 °C at 200 rpm orbital shaking. Cells were inoculated at on OD_{600} of 0.1. Feed beads (SMFB63319, Kuhner Shaker, Basel, Switzerland)[45] with a release rate of 0.25 g/L/h were added to the medium after an initial batch phase of 9 h after inoculation. Final samples were taken for final 3-HP measurements after 96 h. For the bioreactor experiments, a parallel cultivation system (Dasgip/Eppendorf, Germany) was used. One liter of 10 g/L glucose containing minimal medium was inoculated at an OD_{600} of 0.1 from an overnight preculture. DO and pH values were set to >20% and pH 5, respectively. For the bioreactor fed-batch experiments, a first batch phase (21 h) was followed by a feed phase of constant glucose feed at 0.5 g/L/h of a 100 g/L minimal medium-based glucose feed solution.

3-HP was analyzed by a Summit HPLC (Dionex, Sunnyvale, CA, USA) as described in Chen et al.[33]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00161.

Time course for GFP expression under control of the modified *TEF1* promoter, quantitative validation of malonyl-CoA sensor, application of malonyl-CoA sensor for 3-HP pathway regulation under cerulenin exposure, and application of malonyl-CoA sensor for dual dynamic 3-HP pathway regulation (Figures S1−S4); oligonucleotide primers, strains, and plasmids used in this study (Tables S1−S3); ordered sequences (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: siewers@chalmers.se.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support by Carl Tryggers Stiftelse för Vetenskaplig Forskning, and the Novo Nordisk Foundation is gratefully acknowledged. We thank Oliver Englund Örn, Märta Jansson, Wictor Olsson, Yvette Palm, and Alexandra Imre for skillful assistance with experiments and Yun Chen and Alexandra Bergman for providing plasmid pYC1 and the *kanMX-cre* cassette, respectively.

■ REFERENCES

(1) Hong, K. K., and Nielsen, J. (2012) Metabolic engineering of *Saccharomyces cerevisiae*: A key cell factory platform for future biorefineries. *Cell. Mol. Life Sci. 69*, 2671−2690.

(2) Chen, X., Zhou, L., Tian, K., Kumar, A., Singh, S., Prior, B. A., and Wang, Z. (2013) Metabolic engineering of *Escherichia coli*: A sustainable industrial platform for bio-based chemical production. *Biotechnol. Adv. 31*, 1200−1223.

(3) Jullesson, D., David, F., Pfleger, B., and Nielsen, J. (2015) Impact of synthetic biology and metabolic engineering on industrial production of fine chemicals. *Biotechnol. Adv. 33*, 1395−1402.

(4) Anthony, J. R., Anthony, L. C., Nowroozi, F., Kwon, G., Newman, J. D., and Keasling, J. D. (2009) Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *Escherichia coli* for production of

DOI: 10.1021/acssynbio.5b00161
ACS Synth. Biol. 2016, 5, 224−233

the anti-malarial drug precursor amorpha-4, 11-diene. *Metab. Eng. 11*, 13−19.
(5) Nowroozi, F. F., Baidoo, E. E. K., Ermakov, S., Redding-Johanson, A. M., Batth, T. S., Petzold, C. J., and Keasling, J. D. (2014) Metabolic pathway optimization using ribosome binding site variants and combinatorial gene assembly. *Appl. Microbiol. Biotechnol. 98*, 1567−1581.
(6) Ajikumar, P. K., Xiao, W.-H., Tyo, K. E. J., Wang, Y., Simeon, F., Leonard, E., Mucha, O., Phon, T. H., Pfeifer, B., and Stephanopoulos, G. (2010) Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. *Science 330*, 70−74.
(7) Curran, K. A., Leavitt, J. M., Karim, A. S., and Alper, H. S. (2013) Metabolic engineering of muconic acid production in *Saccharomyces cerevisiae*. *Metab. Eng. 15*, 55−66.
(8) Holtz, W. J., and Keasling, J. D. (2010) Engineering static and dynamic control of synthetic pathways. *Cell 140*, 19−23.
(9) Comba, S., Arabolaza, A., and Gramajo, H. (2012) Emerging engineering principles for yield improvement in microbial cell design. *Comput. Struct. Biotechnol. J. 3*, 1−6.
(10) Venayak, N., Anesiadis, N., Cluett, W. R., and Mahadevan, R. (2015) Engineering metabolism through dynamic control. *Curr. Opin. Biotechnol. 34*, 142−152.
(11) Kobayashi, H., Kaern, M., Araki, M., Chung, K., Gardner, T. S., Cantor, C. R., and Collins, J. J. (2004) Programmable cells: interfacing natural and engineered gene networks. *Proc. Natl. Acad. Sci. U. S. A. 101*, 8414−8419.
(12) Anesiadis, N., Cluett, W. R., and Mahadevan, R. (2008) Dynamic metabolic engineering for increasing bioprocess productivity. *Metab. Eng. 10*, 255−266.
(13) Scalcinati, G., Knuf, C., Partow, S., Chen, Y., Maury, J., Schalk, M., Daviet, L., Nielsen, J., and Siewers, V. (2012) Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquitepene α-santalene in a fed-batch mode. *Metab. Eng. 14*, 91−103.
(14) Farmer, W. R., and Liao, J. C. (2000) Improving lycopene production in *Escherichia coli* by engineering metabolic control. *Nat. Biotechnol. 18*, 533−537.
(15) Zhang, F., Carothers, J. M., and Keasling, J. D. (2012) Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nat. Biotechnol. 30*, 354−359.
(16) Dahl, R. H., Zhang, F., Alonso-Gutierrez, J., Baidoo, E., Batth, T. S., Redding-Johanson, A. M., Petzold, C. J., Mukhopadhyay, A., Lee, T. S., Adams, P. D., and Keasling, J. D. (2013) Engineering dynamic pathway regulation using stress-response promoters. *Nat. Biotechnol. 31*, 1039−1046.
(17) Liu, G., Marras, A., and Nielsen, J. (2014) The future of genome-scale modeling of yeast through integration of a transcriptional regulatory network. *Quant. Biol. 2*, 30−46.
(18) Lussier, F.-X., Colatriano, D., Wiltshire, Z., Page, J. E., and Martin, V. J. J. (2012) Engineering microbes for plant polyketide biosynthesis. *Comput. Struct. Biotechnol. J. 3*, 1−11.
(19) Valdehuesa, K. N. G., Liu, H., Nisola, G. M., Chung, W.-J., Lee, S. H., and Park, S. J. (2013) Recent advances in the metabolic engineering of microorganisms for the production of 3-hydroxypropionic acid as C3 platform chemical. *Appl. Microbiol. Biotechnol. 97*, 3309−3321.
(20) Jensen, N. B., Borodina, I., Chen, Y., Maury, J., Kildegaard, K. R., Förster, J., and Nielsen, J. (2014) Microbial production of 3-hydroxypropionic acid, Patent WO2014198831.
(21) Schujman, G. E., Paoletti, L., Grossman, A. D., and de Mendoza, D. (2003) FapR, a bacterial transcription factor involved in global regulation of membrane lipid biosynthesis. *Dev. Cell 4*, 663−672.
(22) Schujman, G. E., Guerin, M., Buschiazzo, A., Schaeffer, F., Llarrull, L. I., Reh, G., Vila, A. J., Alzari, P. M., and de Mendoza, D. (2006) Structural basis of lipid biosynthesis regulation in Gram-positive bacteria. *EMBO J. 25*, 4074−4083.
(23) Xu, P., Wang, W., Li, L., Bhan, N., Zhang, F., and Koffas, M. A. G. (2014) Design and kinetic analysis of a hybrid promoter-regulator system for malonyl-CoA sensing in *Escherichia coli*. *ACS Chem. Biol. 9*, 451−458.
(24) Liu, D., Xiao, Y., Evans, B., and Zhang, F. (2015) Negative feedback regulation of fatty acid production based on a malonyl-CoA sensor-actuator. *ACS Synth. Biol. 4*, 132−140.
(25) Xu, P., Li, L., Zhang, F., Stephanopoulos, G., and Koffas, M. (2014) Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. *Proc. Natl. Acad. Sci. U. S. A. 111*, 11299−11304.
(26) Ellis, J. M., and Wolfgang, M. J. (2012) A genetically encoded metabolite sensor for malonyl-CoA. *Chem. Biol. 19*, 1333−1339.
(27) Partow, S., Siewers, V., Bjørn, S., Nielsen, J., and Maury, J. (2010) Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*. *Yeast 27*, 955−964.
(28) Montgomery, S. B., Griffith, O. L., Sleumer, M. C., Bergman, C. M., Bilenky, M., Pleasance, E. D., Prychyna, Y., Zhang, X., and Jones, S. J. M. (2006) ORegAnno: an open access database and curation system for literature-derived promoters, transcription factor binding sites and regulatory variation. *Bioinformatics 22*, 637−640.
(29) Teixeira, M. C., Monteiro, P. T., Guerreiro, J. F., Gonçalves, J. P., Mira, N. P., dos Santos, S. C., Cabrito, T. R., Palma, M., Costa, C., Francisco, A. P., et al. (2014) The YEASTRACT database: an upgraded information system for the analysis of gene and genomic transcription regulation in *Saccharomyces cerevisiae*. *Nucleic Acids Res. 42*, D161−D166.
(30) Johansson, P., Wiltschi, B., Kumari, P., Kessler, B., Vonrhein, C., Vonck, J., Oesterhelt, D., and Grininger, M. (2008) Inhibition of the fungal fatty acid synthase type I multienzyme complex. *Proc. Natl. Acad. Sci. U. S. A. 105*, 12803−12808.
(31) Tehlivets, O., Scheuringer, K., and Kohlwein, S. D. (2007) Fatty acid synthesis and elongation in yeast. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids 1771*, 255−270.
(32) Borodina, I., Kildegaard, K. R., Jensen, N. B., Blicher, T. H., Maury, J., Sherstyk, S., Schneider, K., Lamosa, P., Herrgård, M. J., Rosenstand, I., et al. (2015) Establishing a synthetic pathway for high-level production of 3-hydroxypropionic acid in *Saccharomyces cerevisiae* via β-alanine. *Metab. Eng. 27*, 57−64.
(33) Chen, Y., Bao, J., Kim, I. K., Siewers, V., and Nielsen, J. (2014) Coupled incremental precursor and co-factor supply improves 3-hydroxypropionic acid production in *Saccharomyces cerevisiae*. *Metab. Eng. 22*, 104−109.
(34) Wenz, P., Schwank, S., Hoja, U., and Schüller, H.-J. (2001) A downstream regulatory element located within the coding sequence mediates autoregulated expression of the yeast fatty acid synthase gene *FAS2* by the *FAS1* gene product. *Nucleic Acids Res. 29*, 4625−4632.
(35) Lubliner, S., Keren, L., and Segal, E. (2013) Sequence features of yeast and human core promoters that are predictive of maximal promoter activity. *Nucleic Acids Res. 41*, 5569−5581.
(36) Sharon, E., Kalma, Y., Sharp, A., Raveh-Sadka, T., Levo, M., Zeevi, D., Keren, L., Yakhini, Z., Weinberger, A., and Segal, E. (2012) Inferring gene regulatory logic from high-throughput measurements of thousands of systematically designed promoters. *Nat. Biotechnol. 30*, 521−530.
(37) Teo, W. S., and Chang, M. W. (2014) Development and characterization of AND-gate dynamic controllers with a modular synthetic *GAL1* core promoter in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng. 111*, 144−151.
(38) Li, S., Si, T., Wang, M., and Zhao, H. (2015) Development of a synthetic malonyl-CoA sensor in *Saccharomyces cerevisiae* for intracellular metabolite monitoring and genetic screening. *ACS Synth. Biol. 4*, 1308−1315.
(39) Van Dijken, J. P., Bauer, J., Brambilla, L., Duboc, P., Francois, J. M., Gancedo, C., Giuseppin, M. L. F., Heijnen, J. J., Hoare, M., Lange, H. C., Madden, E. A., Niederberger, P., Nielsen, J., Parrou, J. L., Petit, T., Porro, D., Reuss, M., Van Riel, N., Rizzi, M., Steensma, H. Y., Verrips, C. T., Vindeløv, J., and Pronk, J. T. (2000) An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. *Enzyme Microb. Technol. 26*, 706−714.

DOI: 10.1021/acssynbio.5b00161
ACS Synth. Biol. 2016, 5, 224−233

(40) Verduyn, C., Postma, E., Scheffers, W. A., and Van Dijken, J. P. (1992) Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast 8*, 501−517.
(41) Wach, A., Brachat, A., Alberti-Segui, C., Rebischung, C., and Philippsen, P. (1997) Heterologous *HIS3* marker and GFP reporter modules for PCR-targeting in *Saccharomyces cerevisiae*. *Yeast 13*, 1065−1075.
(42) Quan, J., and Tian, J. (2014) Circular polymerase extension cloning. *Methods Mol. Biol. 1116*, 103−117.
(43) Mumberg, D., Müller, R., and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene 156*, 119−122.
(44) Pan, R., Zhang, J., Shen, W. L., Tao, Z. Q., Li, S. P., and Yan, X. (2011) Sequential deletion of *Pichia pastoris* genes by a self-excisable cassette. *FEMS Yeast Res. 11*, 292−298.
(45) Jeude, M., Dittrich, B., Niederschulte, H., Anderlei, T., Knocke, C., Klee, D., and Büchs, J. (2006) Fed-batch mode in shake flasks by slow-release technique. *Biotechnol. Bioeng. 95*, 433−445.

DOI: 10.1021/acssynbio.5b00161
ACS Synth. Biol. 2016, 5, 224−233