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Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids

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Microbial production of chemicals is now an attractive alternative to chemical synthesis. Current efforts focus mainly on constructing pathways to produce different types of molecules[1–3]. However, there are few strategies for engineering regulatory components to improve product titers and conversion yields of heterologous pathways[4]. Here we developed a dynamic sensor-regulator system (DSRS) to produce fatty acid–based products in *Escherichia coli*, and demonstrated its use for biodiesel production. The DSRS uses a transcription factor that senses a key intermediate and dynamically regulates the expression of genes involved in biodiesel production. This DSRS substantially improved the stability of biodiesel-producing strains and increased the titer to 1.5 g/l and the yield threefold to 28% of the theoretical maximum. Given the large number of natural sensors available, this DSRS strategy can be extended to many other biosynthetic pathways to balance metabolism, thereby increasing product titers and conversion yields and stabilizing production hosts.

High productivities, titers and yields are essential for the microbial production of chemicals to be economically viable, particularly for low-value bulk chemicals and biofuels. However, product titers and yields are often limited by metabolic imbalances[5]. Expression of pathway genes at too low a level creates bottlenecks in biosynthetic pathways, whereas expression at too high a level diverts cellular resources to the production of unnecessary RNAs, proteins or intermediate metabolites that might otherwise be devoted to the desired chemical. Furthermore, heterologous enzymes or pathway intermediates are sometimes toxic to the host. Overproduction of toxic enzymes or intermediates leads to growth retardation or adaptive responses that reduce yield and productivity, such as genetic modifications that remove or inactivate the pathway genes[6]. Several strategies have been developed to regulate gene expression, including engineering the strengths of promoters[7], intergenic regions[8] and ribosome binding sites[9]. These methods provide static control of the gene expression level. If a control system is tuned for a particular condition in the bioreactor and the conditions change, the control system will not be able to respond and suboptimal product synthesis will result.

Ideally, the desired metabolic pathway would be dynamically regulated in response to the physiological state of the cell. Dynamic regulation would allow an organism to adapt its metabolic flux to changes within the host or in its environment in real time[10]. In one of the first examples of dynamic regulation of a heterologous pathway, acetyl phosphate was used as an indirect indicator for excess glucolytic flux to regulate the biosynthesis of lycopene[11]. An even better regulation system for an engineered pathway would sense the concentration of critical pathway intermediates and dynamically regulate the production and consumption of the intermediates, which would allow the delivery of intermediates at the appropriate levels and rates in order to optimize the pathway for its highest productivity as conditions change in the cell's environment.

The challenge in building such a regulatory system is having a sensor that can measure key intermediates in the synthesis cascade and having cognate regulators (in this study, regulatory DNA elements) that can control gene expression to improve production of the desired chemicals. Fortunately, nature has evolved sensors for a variety of intracellular molecules that could be used to sense the biosynthetic intermediate. However, the naturally occurring cognate regulators will rarely suffice to regulate an engineered pathway, as these regulators have evolved to regulate natural pathways that have lower flux, and they may not produce the desired chemical. The ability to engineer dynamic regulators for use with natural sensors would facilitate the creation of a DSRS for optimizing the engineered pathway and improving production of the desired chemical.

We sought to develop a DSRS that dynamically controls microbial production of biodiesels. Biodiesel, in the form of fatty acid ethyl ester (FAEE), is an excellent diesel fuel replacement due to its low water solubility and high energy density, and is suitable for microbial production because of its low toxicity to host cells[12]. An FAEE biosynthetic *E. coli* strain, A2A, has been recently developed; it is capable of converting glucose into FAEE with a yield of 9.4% of the theoretical maximum[3]. For practical replacement of petroleum-derived diesel fuel with biodiesel, further improvements in productivity and conversion yield are required. Additionally, A2A was unstable owing to the burden of producing FAEE and the toxicity of intermediates. Enhancing yields close to the theoretical maximum is extremely difficult and requires a nearly perfect balance in host metabolism, which relies on a

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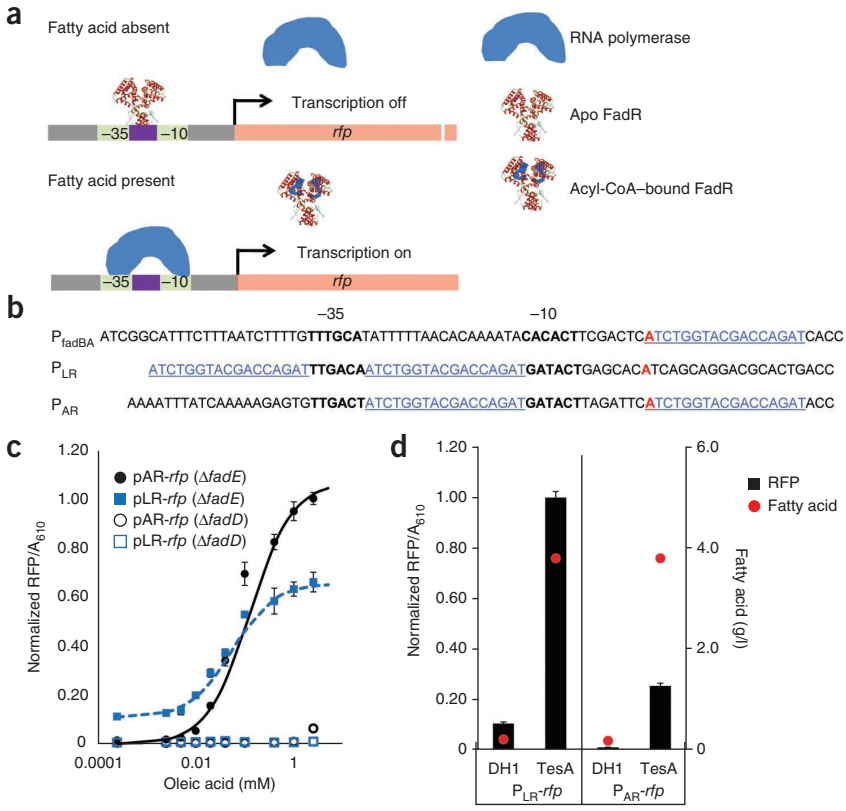

Figure 1 Design of FA/acyl-CoA biosensors. (**a**) In the absence of fatty acid, FadR binds to the FadR-recognition site in a promoter, prevents RNA polymerase from binding to the promoter and represses *rfp* transcription. When fatty acid is present, fatty acid is activated to acyl-CoA, which antagonizes the DNA-binding activity of FadR, and RNA polymerase is able to bind to the promoter to turn on *rfp* transcription. (**b**) DNA sequence of promoters used to construct FA/acyl-CoA biosensors and compared with the native *fadBA* promoter (P_{fadBA}). The bold sequences represent the –10 and –35 regions. The FadR recognition site is colored blue. Transcript start sites are colored red. (**c**) Response of FA/acyl-CoA biosensors to exogenous oleic acid. Biosensor plasmids pAR-*rfp* (black circles) or pLR-*rfp* (blue squares) were used to transform *E. coli* DH1 Δ*fadE* (filled dots) or Δ*fadD* (empty dots). Various amounts of oleic acid were added to the medium, and fluorescence was measured and normalized after incubation at 37 °C for 24 h. (**d**) Response of FA/acyl-CoA biosensors to internally produced fatty acids. Biosensor plasmids were used to transform either wild-type *E. coli* DH1 or a fatty acid–producing strain (TesA overexpressed). After incubation for 3 d, both fatty acid production (red dots) and cell culture fluorescence (black columns) were measured. Note that it is difficult to compare the fluorescence output from the fatty acid supplementation experiments (**c**) with the fatty acid production experiments (**d**) because of the need for a solubilizing reagent to increase solubility of exogenously added fatty acids, fatty acid cellular localization in fatty acid–producing cells and the fatty acid composition.

well-designed regulatory system. We sought to develop a control system that would make the process of improving yields easier by dynamically balancing the enzymes responsible for producing the product.

The first step in engineering a DSRS is to develop biosensors for a key intermediate, which, in the FAEE biosynthetic pathway, is fatty acyl-CoA and, to a lesser extent, free fatty acids. We engineered fatty acid/acyl-CoA (FA/acyl-CoA) biosensors based on the naturally occurring fatty acid–sensing protein FadR and its cognate regulator (**Fig. 1a**). FadR is a ligand-responsive transcription factor that binds to specific DNA sequences and controls the expression of several genes involved in fatty acid biosynthesis, degradation and transport through the membrane[13]. The DNA-binding activity of FadR is specifically antagonized by acyl-CoAs[14]. Although previous results from an electrophoretic mobility shift assay showed that free fatty acids can also affect the DNA-binding activity of FadR, fatty acids were only effective in the micromolar concentration range as compared to nanomolar concentrations for acyl-CoAs[15]. An additional complication is that native FadR-regulated promoters have limited dynamic ranges (that is, induced expression level relative to uninduced expression level). The *E. coli fadBA* promoter (P_{fadBA}) has a fivefold dynamic range upon the addition of 5 mM oleic acid[16], and the *fabA* promoter (P_{fabA}) has a two- to tenfold dynamic range depending on the acyl chain length[17]. To increase the dynamic range, we designed two synthetic FA/acyl-CoA–regulated promoters, P_{LR} and P_{AR}, based on a phage lambda promoter (P_L) and a phage T7 promoter (P_{A1}), respectively[18]. In detail, the 17-bp FadR-binding DNA sequence from P_{fadBA} (the strongest known binding site for FadR, K_d = 0.2 nM[19]) was integrated into two locations in the phage promoters, flanking the –35 region in P_{LR} and the –10 region in P_{AR} (**Fig. 1b**). The engineered promoters were placed 5′ of the gene encoding red fluorescence protein (*rfp*) in plasmids pLR-*rfp* and pAR-*rfp*, respectively. In the absence of fatty acid, FadR is expected to bind to the 17-bp DNA sequences, which interferes with RNA polymerase binding to the phage promoter and inhibits *rfp* transcription. When fatty acid is present, the fatty acid is activated to acyl-CoA by acyl-CoA synthase (encoded by *fadD*). Acyl-CoA in turn binds to FadR and releases FadR from the synthetic promoter, initiating *rfp* transcription (**Fig. 1a**).

We first evaluated the responses of biosensors to exogenous fatty acid. We used biosensor plasmids, either pLR-*rfp* or pAR-*rfp*, to transform a *fadE* knockout of *E. coli* DH1, and added oleic acid to the medium. The enzyme product of *fadE* catalyzes the second step in fatty acid degradation, and deletion of *fadE* is expected to slow down the degradation of exogenous oleic acid and maintain the oleic acid concentration in the culture medium. *E. coli* DH1 Δ*fadE* transformed with either plasmid showed oleic acid–dependent activation of fluorescence over a broad concentration range from 0.1 μM to the solubility limit of oleic acid under our experimental conditions, 5 mM (**Fig. 1c**). In the case of pAR-*rfp*, a 60-fold fluorescence change was observed upon the addition of oleic acid, greater than that reported for any of the native fatty acid–regulated promoters. The apparent half-maximal effective concentration of oleic acid was 35–60 μM, much higher than the K_d of FadR binding to either oleoyl-CoA or oleic acid[15], indicating that only a small proportion of oleic acid had diffused into the cell. In fact, when *fadD* was deleted, no induction of RFP expression was detected with the addition of up to 1 mM oleic acid (**Fig. 1c**), suggesting that with 1 mM oleic acid in the medium, the intracellular concentration was <5 μM, the K_d of FadR binding to oleic acid[15]. The inability to activate RFP expression in the Δ*fadD*

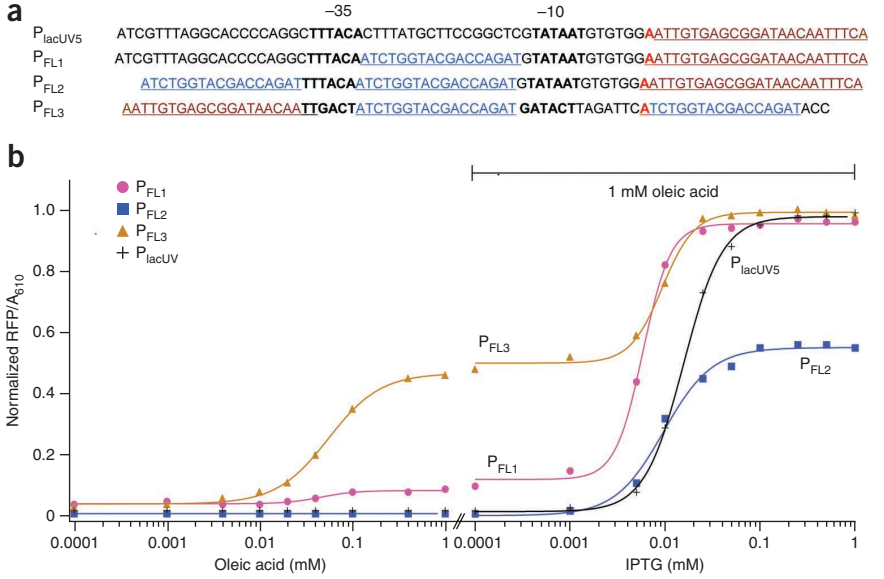

Figure 2 Hybrid FA/acyl-CoA–regulated promoters. (**a**) Hybrid promoters created by the combination of P_{lacUV5} with P_{AR} and P_{LR}. The bold sequences represent the –10 and –35 regions. Blue, FadR-recognition sites. Brown, LacI-binding sequences. Red, transcript start sites. (**b**) Response of hybrid promoters to exogenously added oleic acid and IPTG. Hybrid promoters were used to transform *E. coli* DH1 Δ*fadE* cells. Various amounts of inducers were added to the medium and cell culture fluorescence was measured after 24 h. Oleic acid concentrations were increased from 0.1 μM to 1 mM, followed by increasing IPTG concentration in the presence of 1 mM oleic acid.

strain also indicates that our biosensors responded primarily to acyl-CoA under the experimental conditions, and served as fatty acid sensors only indirectly (**Fig. 1c**).

We next tested the response of FA/acyl-CoA biosensors to internally produced fatty acids. To do so, either pLR-*rfp* or pAR-*rfp* was transformed into a fatty acid–producing *E. coli* strain. This strain harbors *tesA* under the control of P_{lacUV5} and produced 3.8 g/l fatty acid after cultivation for 3 d. As compared to wild-type *E. coli* DH1 harboring pLR-*rfp* and pAR-*rfp*, the fatty acid–producing strain harboring the same plasmids was tenfold and 25-fold more fluorescent, respectively (**Fig. 1d**), and turned the cell culture visibly red (**Supplementary Fig. 1**). The time course of fluorescence correlated well with the time course of fatty acid production, confirming that the *rfp* expression was induced by acyl-CoAs activated from the produced fatty acids (**Supplementary Fig. 2**). Overall, our results indicated that the designed biosensors detected both exogenously added and endogenously produced fatty acids. These sensors can be also used for high-throughput screening of fatty acid–producing strains, and more importantly, to regulate metabolic pathways (**Supplementary Fig. 2**).

The second step for engineering a DSRS is to use the measurement by the biosensor to regulate an output. Although the FA/acyl-CoA–regulated promoters can be directly used to regulate the FAEE biosynthetic pathway, it is essential to eliminate leaky expression from these promoters if a tightly regulated pathway is desired to maximize product yields. To do so, we integrated regulatory DNA elements responsive to two sensors, FadR and LacI, into one promoter, giving rise to three hybrid promoters, P_{FL1}, P_{FL2} and P_{FL3} (**Fig. 2a**). These promoters were designed to respond to changes of both fatty acid and an exogenous inducer, IPTG. When they were analyzed at various inducer concentrations, all hybrid promoters were fully activated only when both the fatty acids and IPTG were present. In P_{FL1} and P_{FL2} the LacI-binding site was inserted 3′ of the transcription start site, and *rfp* expression was tightly repressed in the absence of IPTG. In contrast,

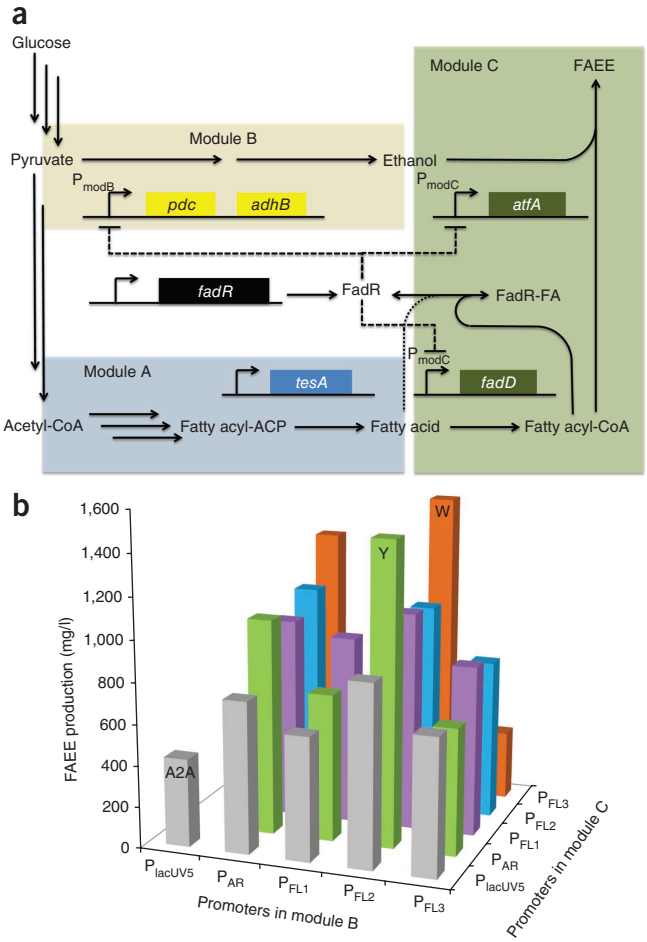

Figure 3 Regulation of FAEE production by the DSRS. (**a**) The FAEE biosynthetic pathway was divided into three modules. Module A contains the *E. coli* native fatty acid synthase and a cytoplasmic thioesterase gene (*tesA*) and produces fatty acids. Module B contains a pyruvate decarboxylase gene (*pdc*) and an alcohol dehydrogenase gene (*adhB*) and produces ethanol. Module C contains an acyl-CoA synthase gene (*fadD*) and a wax-ester synthase gene (*atfA*) and produces FAEE as the end product. The DSRS contains the repressor gene *fadR* and FadR-regulated promoters (represented by P_{modB} and P_{modC}). When there is no fatty acid accumulation, the expressed FadR represses P_{modB} and P_{modC}, and inhibits the synthesis of ethanol and acyl-CoA. When fatty acids accumulate, they are first activated to acyl-CoAs by the gene product of chromosomal *fadD*. Acyl-CoAs then bind to FadR (FadR-FA) and release FadR from its DNA-binding sites, simultaneously activating the biosynthesis of ethanol and more acyl-CoA and the expression of wax-ester synthase, which converts ethanol and acyl-CoA to FAEE. (**b**) FAEE production yields measured by GC-FID. FAEE-producing strains were induced with 1 mM IPTG and incubated at 37 °C for 3 d. A2A is a reference strain from a previous publication[3]. Strains using the same promoter in module C are presented with the same bar color.

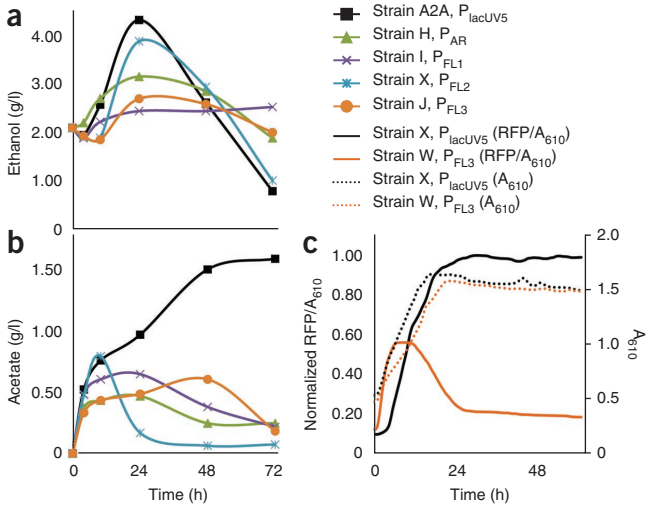

Figure 4 Metabolite analysis and dynamic behavior of FAEE-producing strains. Five strains using either P_{lacUV5} or the FA/acyl-CoA–regulated promoters (strain A2A, H, I, X and J using P_{lacUV5}, P_{AR}, P_{FL1}, P_{FL2} and P_{FL3}, respectively; see **Supplementary Table 1**) to control the expression of genes in the ethanol pathway were cultivated for FAEE production. (**a**,**b**) Cell cultures were collected and the amounts of ethanol (**a**) and acetate (**b**) were analyzed by high-performance liquid chromatography (HPLC). (**c**) Comparison of time-dependent behavior of a dynamic promoter, P_{FL3} (orange curves) in strain W, and an inducible promoter, P_{lacUV5} (black curves) in strain X. In these strains, *rfp* was cloned in front of the *fadD* gene under the control of P_{FL3} or P_{lacUV5}. During FAEE production, cell culture fluorescence was measured and normalized to cell density (solid lines). Growth of the two strains is shown in dotted lines.

P_{FL3} (**Fig. 2b**) was created by the insertion of the LacI-binding site 5′ of the −35 region. In the absence of IPTG, P_{FL3} behaved similarly to P_{AR}, exhibiting oleic acid–dependent activation. This observation is consistent with previous studies that repression of a promoter 5′ of the −35 region is less effective than between the −10 and −35 or 3′ of the −10 region[20]. Titration of P_{FL3} with IPTG in the presence of 1 mM oleic acid continued to activate P_{FL3}. In general, all hybrid promoters were repressed until induced by IPTG, and the induction levels could be tuned automatically by the endogenous FA/acyl-CoA level. As such, they can be readily used to regulate production of fatty acid–based chemicals, such as the biodiesel FAEE.

The previously developed FAEE biosynthetic pathway contains three modules[3] (**Fig. 3a**). Module A uses the native *E. coli* fatty acid biosynthetic pathway and a heterologous thioesterase, TesA (encoded by *tesA*), to produce free fatty acids. Module B is an ethanol biosynthetic pathway that converts pyruvate into ethanol. And module C contains an acyl-CoA synthase (encoded by *fadD*) and a wax-ester synthase (encoded by *atfA*), whose enzyme products condense the products from the previous two modules to FAEEs. Tight regulation of the modules in this pathway is needed because (i) ethanol is toxic, such that accumulation to undesirable levels reduces cell growth and productivity, and consumes carbon sources that could otherwise be used for FAEE biosynthesis; (ii) activation of fatty acids to fatty acyl-CoAs is a reversible process because TesA is able to hydrolyze fatty acyl-CoAs to free fatty acids just as readily as it can hydrolyze fatty acyl–acyl carrier proteins[21]; (iii) acyl-CoA can be readily degraded through β-oxidation[13]; and (iv) accumulation of acyl-CoA robs the cell of fatty acids needed for cell membrane biosynthesis and CoA needed for many metabolic reactions.

Ideally, modules B and C would be controlled by the availability of fatty acid, and ethanol and acyl-CoA would be produced in no greater than sufficient quantities needed for FAEE biosynthesis. To achieve this goal, we cloned the biosensor-derived promoters upstream of modules B and C to control the expression of *fadD*, the ethanol biosynthetic pathway (*adhB* and *pdc*) and *atfA* (**Fig. 3a**). At low intracellular fatty acid concentration, FadR represses production of ethanol and unnecessary acyl-CoA. When there is sufficient fatty acid available, fatty acid would be first activated to acyl-CoA by the enzyme product of chromosomal *fadD*, acyl-CoA would then antagonize the DNA-binding activity of FadR, resulting in expression of genes that encode enzymes to produce ethanol, activate more free fatty acid to fatty acyl-CoA, and convert ethanol and acyl-CoA to FAEE. To search for the proper regulatory system with desired strength and dynamic range, we created 20 FAEE-producing strains by changing the combination of biosensor-derived promoters in each module (**Supplementary Table 1**).

The FAEE production yields were analyzed by gas chromatography–flame ionization detector (GC-FID). Most strains harboring the DSRS had enhanced production titers and yields (**Fig. 3b**). Among them, two strains, Y and W, which contain P_{FL2} controlling the expression of genes in module B (ethanol pathway) and P_{AR} or P_{FL3} controlling the expression of genes in module C (*fadD-atfA*), had the highest yields. These strains produced threefold higher titers of FAEE compared to that produced by the previously engineered A2A strain, reaching 1.5 g/l after 3 d incubation, corresponding to 28% of the maximum theoretical yield.

To confirm that the yields were enhanced because of the dynamic regulation created by the DSRS rather than simply a change of promoter strength, we used a series of constitutive promoters to control the same pathway and compared their effects in FAEE production. Six constitutive promoters (P_{C1} to P_{C6}, from the Registry of Standard Biological Parts, http://partsregistry.org/; **Supplementary Fig. 3a**) were chosen to replace the biosensor-derived promoters in the DSRS. P_{C1} to P_{C6} have varied sequences in the −10 and −35 regions and cover a wide range of promoter strengths from weak to strong, including the dynamic range of the biosensor-derived promoters (**Supplementary Table 2**). In total, 30 control strains were created and tested. All the strains having constitutive promoters produced considerably less FAEE compared to strain W or Y (**Supplementary Fig. 3b**). Instead, large amounts of free fatty acids accumulated in these cultures (**Supplementary Table 1**), suggesting imbalanced metabolism.

To determine if the DSRS increased product yields because of the improved metabolic balance, we performed metabolite analysis on five strains using either P_{lacUV5} or the biosensor-derived promoters to control the expression of genes in the ethanol pathway (**Supplementary Table 1**). When P_{lacUV5} was used, ethanol accumulated to 4.3 g/l at 24 h but decreased dramatically afterwards (**Fig. 4a**). In contrast, the biosensor-derived promoters produced ethanol more consistently. Less ethanol accumulated at 24 h and the ethanol productivity remained nearly constant during 3 d of production. Furthermore, strains using biosensor-derived promoters produced less acetate than the strain that harbored P_{lacUV5}, suggesting that central metabolism was balanced better with the biosensor-derived promoters (**Fig. 4b**). In addition, we characterized the time-dependent behavior of a dynamic promoter, P_{FL3}, in the best FAEE-producing strain W and compared it to that of the inducible promoter P_{lacUV5} in strain X (**Fig. 4c**). Under our production conditions, P_{FL3} turned on more quickly and dynamically adjusted itself to remain induced at the desired level, resulting in higher FAEE titer.

It has been well documented that accumulation of some metabolic intermediates or proteins in genetically modified microorganisms

is toxic and leads to a rapid loss of gene function[22,23]. We hypothesized that because the designed DSRS alleviated the imbalances in the metabolic pathways, it would stabilize the genetic constructs. We characterized the genetic stability of the strains with various versions of the DSRS and compared it to that of the original A2A and control strains. Strains harboring the DSRS had greater plasmid integrity and the predicted copy number ratios (**Supplementary Fig. 4**). In particular, *fadD*, which was previously shown to be the least stable gene in the system, was maintained at higher copy number in the strains with the DSRS compared to the unregulated system (**Supplementary Fig. 5**), indicating that the DSRS was able to improve gene stability.

To further understand the DSRS-enhanced FAEE production, we formulated a kinetic model consisting of 19 molecular species and 26 effective rate constants to perform an in-depth analysis of the DSRS and to compare it to the same pathway controlled by constitutive promoters (**Supplementary Fig. 6**). Consistent with the experimental results, global sensitivity analysis showed that relative improvements in FAEE production levels ($\alpha_{rel} > 1$) can be obtained with the topology of the DSRS across a broad range of promoter strengths (**Supplementary Fig. 7** and **Supplementary Tables 3** and **4**). In this system, the largest effects on FAEE production, as measured by the partial correlation coefficients with α_{rel}, were observed for the FadR-P_{modB} and FadR-P_{modC} promoter binding affinities (partial correlation coefficient with apparent $K_d = -0.31$ and -0.23, respectively), indicating that, as expected, tight regulation of expression from modules B and C is important for increasing yields (**Supplementary Fig. 7a**). Across the parameter intervals under study, the simulation data showed that the mean improvement in DSRS-regulated FAEE production results from lower levels of accumulated ethanol and lower concentrations of intracellular acyl-CoA (**Supplementary Fig. 8**). Thus, the simulation results are in agreement with both the experimental data and the design principles outlined above, demonstrating that the DSRS regulated the expression of modules B and C according to the availability of key intermediates and improved FAEE production relative to a pathway controlled by constitutive promoters.

Taken together, our results show that the DSRS balanced the metabolism of host cells, improved the stability of the FAEE biosynthetic pathway and enhanced production yields. Our strategy to design the DSRS based on a ligand-responsive transcription factor can be extended to design biosensors and regulatory systems for other molecules and metabolic pathways using the large pool of natural ligand-responsive transcription factors[24]. A bioinformatics search identified at least 36 ligand-responsive transcription factors in *E. coli*, whose DNA-binding activities are regulated by various types of molecules, including amino acids, nucleic acids, carbohydrates, lipids, central metabolites and many secondary metabolites (**Supplementary Table 5**). Furthermore, heterologous ligand-responsive transcription factors from other organisms can be grafted into the production host. For example, the Oaf1p/Oaf2p heterodimer is the FadR homolog in yeast, and HNF-4α is an acyl-CoA-responsive transcription factor in mammalian cells (**Supplementary Table 5**). DSRSs based on these ligand-responsive transcription factors could be used in these hosts or in heterologous hosts in a manner similar to what we have shown here to regulate the production of fatty acid–based products.

To further demonstrate the utility of dynamic regulation, we used a similar strategy to engineer a DSRS responsive to a phenolic compound, salicylic acid, by assimilating the *E. coli* ligand-responsive transcription factor MarR and engineering regulators responsive to MarR. Depending on the regulator used with the sensor, the dynamic range of the DSRS varied from 5- to 16-fold in response to the exogenously added salicylic acid (**Supplementary Fig. 9**). These biosensors could be readily used to regulate the production of salicylic acid–derived compounds, such as aspirin.

We have demonstrated that one can construct a DSRS by assimilating two different natural ligand-responsive transcription factors and engineering regulators with different responses. Additionally, we demonstrated how one system could be practically used to regulate production of a desired chemical. Given these two examples and the numerous other naturally occurring sensors available, it should be possible to regulate nearly any biosynthetic pathway for which a natural sensor for the product or an intermediate exists or for which a sensor can be easily evolved. Furthermore, quorum sensors or carbon source sensors could be used to initialize the upstream genes to create a fully internally controlled pathway. With the development of advanced techniques for biosensor design (such as computer-aided design of proteins[4] or model-driven RNA device engineering[25]), it should one day be possible to dynamically regulate any metabolic pathway, regardless of whether a natural sensor is available or not, and increase yields, titers and productivities to make microbial production of commodity chemicals and fuels economically viable.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturebiotechnology/.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

F.Z. and J.D.K. conceived the project and designed the experiments. F.Z. performed the experiments. J.M.C. designed and performed the DSRS modeling. F.Z., J.M.C. and J.D.K. analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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ONLINE METHODS

Construction of FA biosensors. FA/acyl-CoA biosensor plasmids (pAR-*rfp*, pLR-*rfp*, together with pfadBA-*rfp*) were derived from a BioBrick plasmid, pBbB8k-*rfp* (BBR1 origin, kanamycin resistant)[26], by replacing the P_{BAD} promoter and the AraC repressor gene (*araC*) with FA/acyl-CoA-regulated promoters. In detail, DNA sequences of FA/acyl-CoA-regulated promoters were embedded into primers to amplify the whole pBbB8k-*rfp* plasmid except for the P_{BAD} and *araC* gene. The PCR products were purified and ligated to create circular plasmids using a one-step Golden-Gate assembly method[27]. The *fadR* gene was amplified from *E. coli* genomic DNA and inserted into pBbE8a (ColE1 origin, ampicillin resistant)[26] between BglII and XhoI sites to generate pBbE8a-*fadR*. For biosensor evaluation, either *fadE* or *fadD* knockout strains of *E. coli* DH1 were transformed with biosensor plasmids and pBbE8a-*fadR*. For the biosensor evaluation in a fatty acid–producing strain, a *tesA* gene[3] was cloned into pBbA5c (p10A origin, chloramphenicol resistant)[26] between BglII and XhoI sites to create pBbA5c-*tesA*. Biosensor plasmids together with pBbE8a-*fadR* and pBbA5c-*tesA* were used to transform *E. coli* DH1 Δ*fadE* for characterization.

Cell culture fluorescence measurement. Fresh rich-broth (RB) medium, containing per liter 10 g tryptone, 5 g NaCl and 1 g yeast extract, was inoculated with overnight cultures and incubated at 37 °C for 24 h. Fatty acid supplementation experiments were adapted from a previous publication[16]. In detail, oleic acid was neutralized with NaOH and added to RB medium containing 0.5% Tergitol NP-40. Cell culture fluorescence was recorded on a SpectraMax M2 plate reader (Molecular Devices) or an Infinite F200 (TECAN) plate reader using 96-well plates. Each well was filled with 150 µl of cell culture. Excitation at 535 ± 5 nm and emission at 620 ± 10 nm were used. All fluorescence was normalized with cell density by measuring the absorbance at 610 nm.

For FA production, minimal medium (M9 medium supplemented with 75 mM MOPS, 2 mM $MgSO_4$, 1 mg/l thiamine, 10 nM $FeSO_4$, 0.1 mM $CaCl_2$ and micronutrients including 3 µM $(NH_4)_6Mo_7O_{24}$, 0.4 mM boric acid, 30 µM $CoCl_2$, 15 µM $CuSO_4$, 80 µM $MnCl_2$ and 10 µM $ZnSO_4$) with 2% glucose as carbon source was used. After inoculation with overnight culture, the cell culture was incubated at 37 °C until A_{600} reached 0.8. Cells were induced with 1 mM IPTG and kept growing at 37 °C for 3 d. To monitor the development of cell culture fluorescence during FA production, 150 µl of induced cell cultures were transferred into a 96-well plate. The plate was incubated in either an Infinite F200 plate reader or an Infinite F200PRO (TECAN) plate reader at 37 °C with shaking. Cell culture fluorescence and absorption were recorded every 15 min. The same induced cultures were shaking in a 37 °C incubator and a fraction of 0.5 ml culture was transferred at various time points for fatty acid production analysis.

Construction and characterization of hybrid fatty acid–regulated promoters. Plasmids containing the hybrid FA/acyl-CoA-regulated promoters were constructed similarly as the FA/acyl-CoA biosensor plasmids except a LacI repressor gene was cloned together with the promoters. Cell culture fluorescence was measured similarly as described above except varied amounts of IPTG were added to the media.

Construction of DSRS into FAEE biosynthetic pathway. The previous engineered FAEE-producing strain A2A contains three plasmids, pKS1, pKS17 and pKS104 (ref. 3), with each of them carrying genes in each module (**Fig. 3**). The P_{lacUV5} promoter used in modules B and C was replaced with biosensor derived-promoters using similar cloning method as described for the construction of FA/acyl-CoA biosensors. The *fadR* gene under the control of a P_{BAD} promoter together with its repressor gene (*araC*) was cloned into pKS1 using the Golden-Gate assembly method. All three plasmids were transformed into *fadE* knockout *E. coli* strain for FAEE production.

FAEE production and analysis. All cell cultures were incubated in test tubes at 37 °C. Overnight cultures were used to inoculate 5 ml of minimal medium containing 2% glucose and antibiotics (50 mg/l ampicillin, 30 mg/l chloramphenicol, and 10 mg/l tetracycline). Cells were induced at A_{600} = 1.2 by 1 mM IPTG and kept growing at 37 °C for 3 d. To analyze production yields, 0.5 ml of cell culture was transferred and acidified with 50 µl of 6 N HCl. The cell culture was extracted twice with 0.5 ml of ethyl acetate. Free fatty acids were derivatized to fatty acid methyl esters (FAME) by the addition of 90 µl methanol, 10 µl 6 N HCl and 100 µl TMS-diazomethane (Sigma-Aldrich) and incubation at room temperature (~25 °C) for 10 min. FAEE and FAME were then analyzed using a Thermo Trace Ultra gas chromatograph (GC) equipped with a Triplus AS auto-sampler and a TR-5 column (Thermo Scientific). For each sample, the column was equilibrated at 45 °C for 2.25 min followed by a linear thermo gradient from 45 °C to 300 °C within 6.5 min. Final FAEE concentration was analyzed using a Xcalibur software. FAEE or FAME with acyl chain length from C_{12} to C_{20} were quantified and added as total FAEEs or fatty acids.

DNA analysis and qPCR. After FAEE production, 2 ml of cell culture were transferred and subjected to plasmid DNA preparation using QIAprep Spin Miniprep Kit (QIAGEN). Plasmid DNAs were then digested with BamHI and loaded to a 1% agarose gel for gel-electrophoresis. All the plasmids used for FAEE production contain only one BamHI site.

Plasmid copy numbers were determined by qPCR using a method adapted from a previous publication[28]. In detail, 1 ml of FAEE production culture was centrifuged at 5,000*g* for 5 min. The cell pellet was resuspended in 400 µl of 50 mM Tris, 50 mM EDTA, pH 8.0 followed by the addition of 0.6 µg plasmid DNA as external control. Cell membrane was permeabilized by the addition of 8 µl of 50 mg/ml lysozyme (Sigma-Aldrich) in 10 mM Tris, pH 8.0 followed by incubation at 37 °C for 30 min. To completely lyse the cell, 4 µl of 10% SDS and 12 µl of 14 mg/ml protease K solution (Invitrogen) were added to the sample followed by incubation at 50 °C for 30 min. Protease K was heat deactivated by incubation at 70 °C for 10 min. After sample temperature was equilibrated to room temperature, 8 µl of 25 mg/ml RNase A solution (Qiagen) was added to digest RNAs at 37 °C for 30 min. Total DNA was then chloroform extracted and ethanol precipitated. Real-time PCR was performed on a StepOnePlus Real-Time PCR System (AB Applied Biosystems) using 96-well reaction plates. Each well contained 20 µl reaction mixture with a final concentration of 50 ng/ml of sample DNA, 500 nM of each primer and 1X Fast SYBER Green Master Mix (AB Applied Biosystems). Real-time PCR cycling was 95 °C for 20 s followed by 40 cycles of 10 s at 95 °C, 20 s at 50 °C, and 15 s at 68 °C. Data were processed with the StepOne Software v2.0 (AB Applied Biosystems). Gene copy numbers were normalized to the external control and compared between samples.

Metabolite analysis. During FAEE production, 1 ml of cell culture was transferred and centrifuged at 18,000*g* for 5 min. The supernatant was filtered and applied to an Agilent 1100 series HPLC equipped with an Agilent 1200 series auto-sampler, an Aminex HPX-87H ion exchange column (Biorad), and an Agilent 1200 series DAD and RID detectors. Metabolites were separated using 4 mM H_2SO_4 aqueous solution with a flow rate of 0.6 ml/min at 50 °C.

DSRS modeling. Forty-five chemical reactions (**Supplementary Fig. 7**) were coded as differential equations and solved deterministically using the Dizzy ODE-adaptive solver[29] with custom software for set-up and analysis[25]. The number of FAEE molecules (N_{FAEE}) at time t was determined by solving the system of equations; because FAEE is a long-lived molecule, N_{FAEE} is directly related to the production rate, integrated across the experiment. Monte Carlo filtering was employed to determine how variations in the parameter inputs, $\mathbf{x} = [x_1, x_2, \ldots, x_n]$, affect system outputs, $\mathbf{y}(x) = [y_1(\mathbf{x}), y_2(\mathbf{x}), \ldots, y_n(\mathbf{x})]$, where $\mathbf{y}(x)$ corresponds to N_{FAEE}. Ten thousand sets of Monte Carlo distributions of parameter values taken from uniform distributions over the intervals shown in **Supplementary Tables 3** and **4** were used to solve the set of differential equations and determine $N_{FAEE,\ DSRS}$. For a system with constitutive (const) promoter expression, paired sets of equations were solved with FadR-promoter K_ds = 0, yielding $N_{FAEE,\ const.}$. Global sensitivity analysis was performed to quantify the contribution of individual parameters to relative FAEE production, taken as $\alpha_{rel} = N_{FAEE,\ DSRS}/N_{FAEE,\ const.}$, by computing partial rank correlation coefficients using R (R Foundation for Statistical Computing, Vienna). 95% confidence intervals were determined by bootstrapping from ten replicates with sample size $N = 2 \times 10^3$. The ranges of parameter values consistent with a given level of FAEE production were identified by filtering the parameter values by α_{rel}.

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