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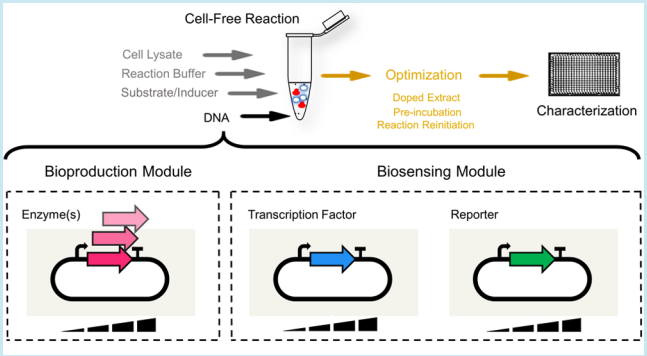
Optimizing Cell-Free Biosensors to Monitor Enzymatic Production

Amir Pandi,[†] Ioana Grigoras,[‡] Olivier Borkowski,[*,‡] and Jean-Loup Faulon[*,†,‡,§]

[†]Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas 78352, France
[‡]iSSB Laboratory, Génomique Métabolique, Genoscope, Institut François Jacob, CEA, CNRS, Université Evry, Université Paris-Saclay, 91057 Evry, France
[§]SYNBIOCHEM Center, School of Chemistry, University of Manchester, Manchester M13 9PL, U.K.

Supporting Information

ABSTRACT: Cell-free systems are promising platforms for rapid and high-throughput prototyping of biological parts in metabolic engineering and synthetic biology. One main limitation of cell-free system applications is the low fold repression of transcriptional repressors. Hence, prokaryotic biosensor development, which mostly relies on repressors, is limited. In this study, we demonstrate how to improve these biosensors in cell-free systems by applying a transcription factor (TF)-doped extract, a preincubation strategy with the TF plasmid, or reinitiation of the cell-free reaction (two-step cell-free reaction). We use the optimized biosensor to sense the enzymatic production of a rare sugar, D-psicose. This work provides a methodology to optimize repressor-based systems in cell-free to further increase the potential of cell-free systems for bioproduction.



KEYWORDS: *biosensor, E. coli cell-free system, transcriptional repressor, D-psicose, bioproduction, cell-free optimization*

Cell-free systems are emerging platforms for quick characterization of biological parts and circuits in synthetic biology and metabolic engineering.[1,2] These low-cost abiotic tools provide high-throughput characterization and decrease whole-cell growth-dependent limitations such as toxicity, noise, and resource competition. Recent advances have enabled various applications of cell-free systems from part characterization to biosensor and pathway prototyping as well as to study biological phenomena.[3−6]

Transcription-translation (TX-TL) cell-free systems have brought new facilities to metabolic engineers to bioproduce fine chemicals.[7,8] They also bring the possibility of leveraging synthetic biology tools such as biosensors to monitor and dynamically engineer metabolic pathways.[7] The TX-TL crude extract, used to express the genes of metabolic pathways, is a bacterial lysate which can be prepared from wild-type, engineered strains or cells harboring overexpressed enzymes.[9] As in whole-cell systems, biosensors can provide monitoring capability in cell-free systems for diagnosis and pathway engineering applications. Cell-free biosensors for quorum molecules, amino acids, nucleic acids, vanillin, and benzoic acid have been implemented and characterized.[4,10,11] In a recent study, the fold repression has been improved using promoter and TF engineering.[6] CRISPR has been implemented in the *Escherichia coli* TX-TL cell-free system, and a preincubation step has been applied to improve its behavior.[12]

Here, we study and improve cell-free biosensing of a valuable compound, D-psicose, to monitor its bioproduction. D-Psicose is a rare sugar with properties to fight against obesity and diabetes.[13] In this study, we first seek to characterize and improve the D-psicose biosensor in the cell-free system. The improvement methods used (i) a TF-doped extract based on cells harboring TF plasmid to prepare the cell lysate, (ii) a preincubation strategy based on the production of the TF in the extract prior to adding the reporter DNA, or (iii) a reinitiation strategy (two-step cell-free reaction) based on an 8 h reaction expressing the TF gene followed by the addition of the reporter DNA plus a fresh cell-free mix. In the next step, we show that the optimized biosensor can be used to monitor D-psicose production by D-psicose 3-epimerase (DPEase) from fructose. The strategies that we introduce here bring cell-free metabolic/enzyme engineering and biosensor development together for pathway monitoring or dynamic regulation.

RESULTS AND DISCUSSION

First, we aimed to study and optimize the efficiency of the D-psicose biosensor in the cell-free system. *E. coli* BL21 lysate along with reaction buffers and DNA vectors are essentials to run a cell-free reaction.[14] In the cell-free system, different genes can be cloned individually, mini/maxiprepped, and pipetted at any concentration to fine-tune biological circuits. We inserted the transcription factor (*psiR*) and reporter (*ppsiA-sfgfp*) in

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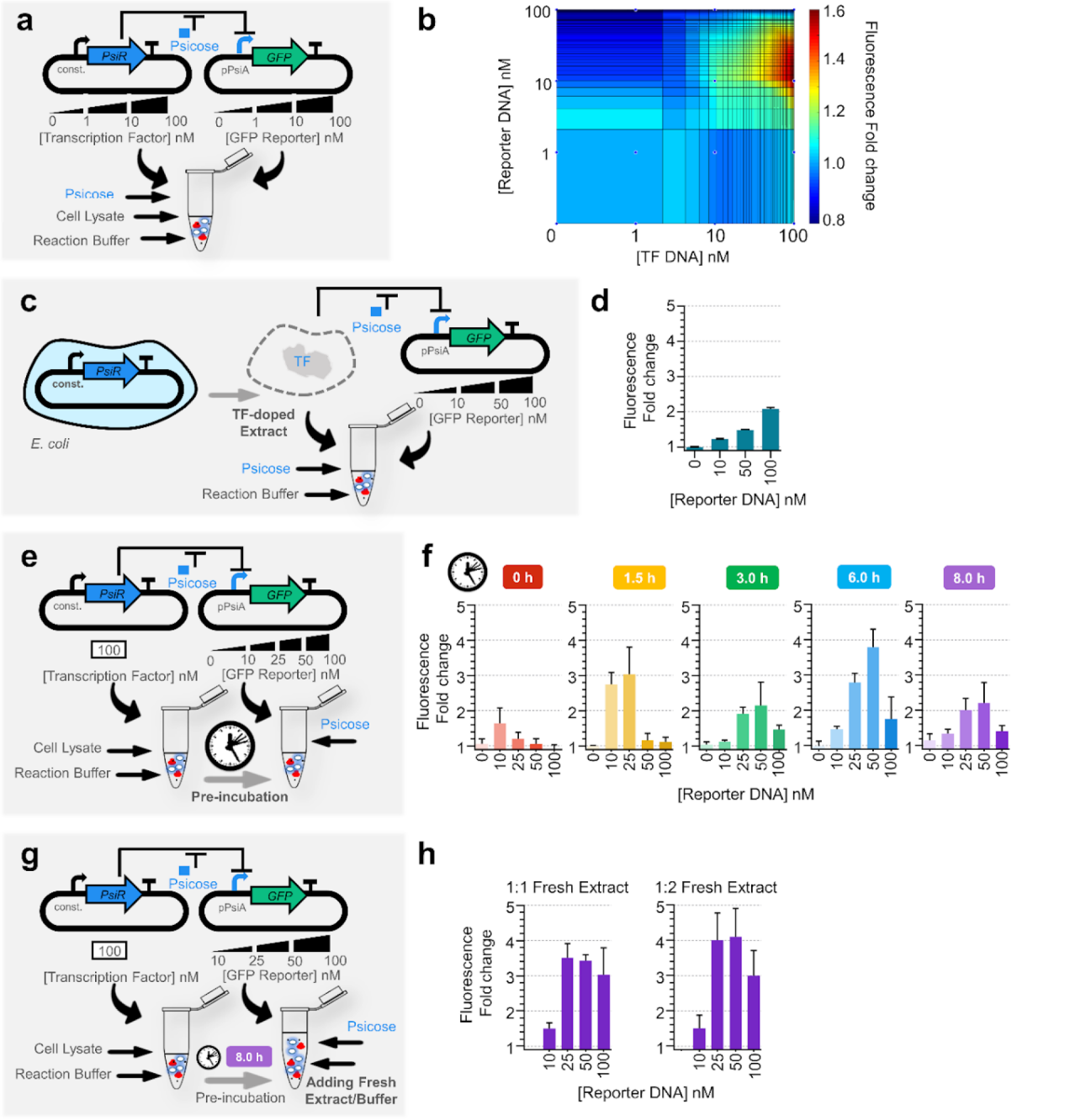

Figure 1. Characterization and optimization of cell-free psicose biosensor. (a) Schematic representation of D-psicose cell-free biosensor with different concentrations of TF DNA and reporter DNA. (b) The surface response of the fold change of the biosensor in a combinatorial space of the TF and reporter DNA with 100/0 mM D-psicose. (c) Schematic representation of cell-free biosensor with expressed TF gene in the cells which were used to prepare the lysate for cell-free reactions (TF-doped extract). (d) Fold change between 100 and 0 mM D-psicose of the TF-doped biosensor at distinct concentrations of the reporter DNA. (e) Schematic representation of cell-free biosensor with preincubation of 100 nM TF DNA, the optimal concentration of TF based on Figure 1b. (f) Fold change between 100 and 0 mM D-psicose of the TF-preincubated DNA after 1.5, 3, 6, and 8 h, followed by the addition of the reporter DNA at different concentrations. (g) Schematic representation of cell-free biosensor with reinitiation of the cell-free reaction (or two-step cell-free reaction) after 8 h with fresh extract, D-psicose, and the reporter DNA. (h) Fold change of the fluorescence in reactions re-supplied with the same (1:1 fresh extract) or twice (1:2 fresh extract) the volume of the cell-free reaction. The data and error bars are the mean and standard deviation of three measurements from three independent reactions done on the same day using the same lysate and maxiprepped plasmids. Bar plots of raw fluorescence data are presented in Supplementary Figures S3−S5.

separate vectors to fine-tune their concentrations independently (Figure 1a). We altered the concentration of TF DNA and reporter DNA mixed with 100 or 0 mM D-psicose (inducer). A high concentration of D-psicose is needed to activate the transcription factor, which is not surprising behavior for a primary metabolite.[15] The surface plot in Figure 1b presents the fluorescence fold change, the ratio of the fluorescence values when 100 or 0 mM of D-psicose is added to the mix (Supplementary Figure S1 shows that D-psicose does not affect the production of the GFP reporter in the absence of the TF). This sensor showed a low fold change with a maximum of 1.6 (with 100 nM TF DNA and 10 nM

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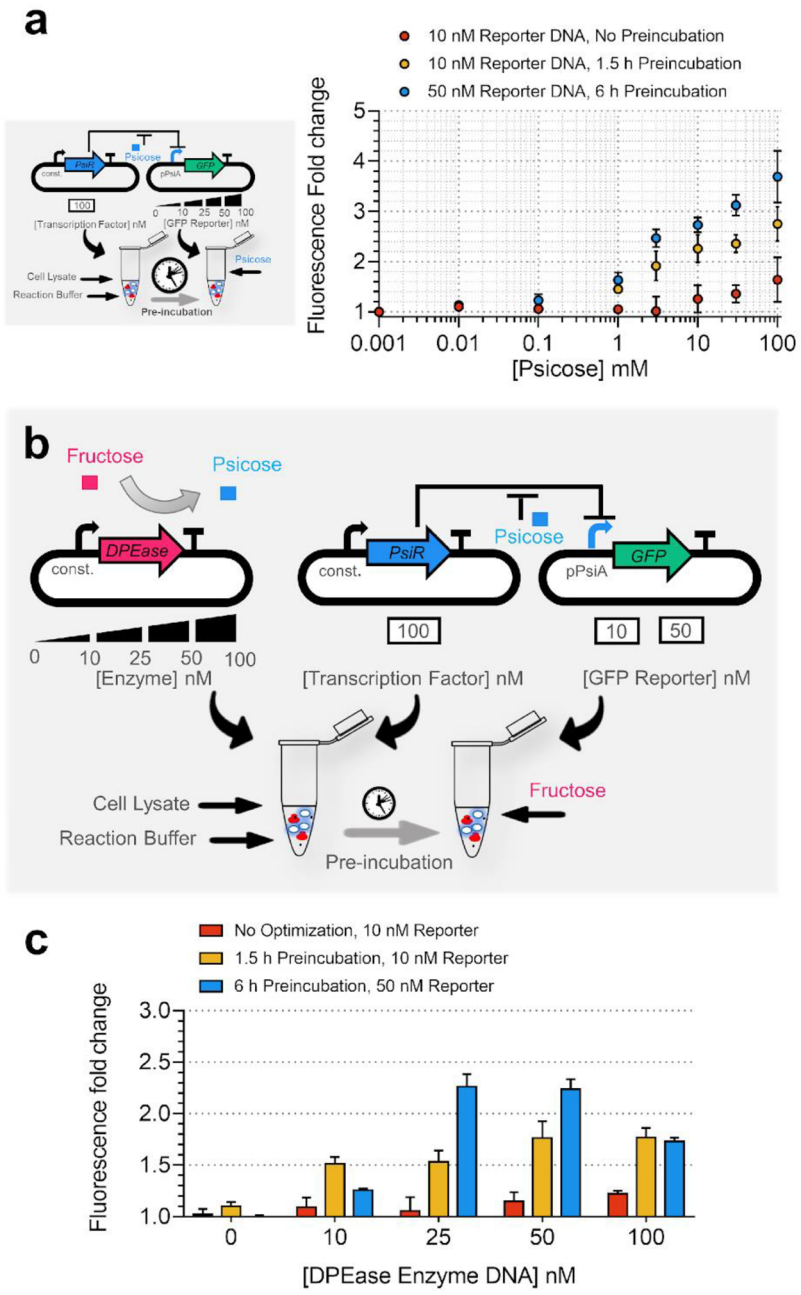

Figure 2. Characterization of psicose production using the optimized biosensors. (a) Dose−response curve of two optimized sensors, 1.5 h preincubation with 10 nM reporter DNA and 6 h preincubation with 50 nM reporter DNA, plus the unoptimized D-psicose sensor from Figure 1b. (b) Schematic representation of cell-free biosensor with preincubation of 100 nM TF DNA applied to monitor the DPEase enzymatic production of D-psicose from 100 mM fructose. (c) Fold change between 100 and 0 mM fructose using different concentrations of DPEase enzyme DNA. We used two different optimized biosensors and the unoptimized D-psicose biosensor. The data and error bars are the mean and standard deviation of three measurements from three independent reactions done on the same day using the same lysate and maxiprepped plasmids.

reporter DNA). This low value is due to the biosensor design: PsiR poorly represses GFP production as both TF and GFP genes are expressed at the same time (Supplementary Figures S2 and S3a). While the repressor gene is being expressed, the GFP gene under the control of the responsive promoter is also expressed, especially at a high concentration of the reporter DNA. Therefore, the conditions with the maximum fold change are with a TF DNA at the maximum concentration (maximum repression in the absence of the inducer) and the reporter DNA at low concentration (minimum leakiness in the absence of the inducer). As a result, the fold change of a repressor-based cell-free biosensor is far from the scale of an activator-based cell-free biosensor.[16,17]

We applied three strategies to increase the fold change of the D-psicose biosensor. The first strategy was to dope the extract with the TF (Figure 1c). The living bacteria, used to prepare the cell lysate, contained a plasmid expressing the TF gene. With this approach, the TF is already present in the cell lysate and is ready to repress the promoter when the reporter DNA is added to the cell-free reaction. When the TF is already present in the lysate, the maximum fold change is obtained with the maximum concentration of the reporter DNA, 100 nM (Figure 1d and Supplementary Figure S3). However, the doping

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approach exhibited only a 30% improvement in the maximum fold change. As the TF is already present in the cell lysate, we moved to another strategy to tune the amount of TF plasmid and its expression to further improve the fold change.

The second strategy uses preincubation (Figure 1.e): we added 100 nM of TF DNA (that led to the maximum fold change observed in the initial experiment in Figure 1b) in the cell-free mix and incubated at 30 °C. After 1.5, 3, 6, or 8 h of incubation, we then added the inducer (D-psicose) and several concentrations of the reporter DNA: 10, 25, 50, and 100 nM. We looked for the best balance between reaching a sufficient amount of TF and GFP production by the reporter DNA as protein production diminishes over time.[14] As expected, increasing the preincubation time led to an increase of promoter repression (less GFP produced in the absence of D-psicose) but a decrease in GFP production (in both the presence or absence of D-psicose) (Supplementary Figure S4). Figure 1f shows that for different preincubation time periods, there are conditions that improved the fold change with regard to no preincubation as in the initial experiment (Figure 1b and red bar plot in Figure 1f). However, 1.5 and 6 h incubation time demonstrated fold changes higher than those at 3 and 8 h (Figure 1f). After 3 h of preincubation, the repression increases but is not sufficient to compensate for the decrease of GFP production (Supplementary Figure S4c), and after 8 h, the production of GFP is too low as cell-free protein production diminishes[14] (Supplementary Figure S4e). Therefore, the fold change is a result of the balance between repression of the promoter (increases by longer preincubation time) and resource availability for the GFP production (decreases by longer preincubation time).

With our third strategy, we tried to overtake the decrease in protein production after 8 h with a two-step cell-free reaction (or reinitiation of the cell-free reaction). After 8 h preincubation of the first reaction mix with 100 nM TF plasmid, we added fresh cell-free mix (lysate and buffers) along with the reporter DNA (Figure 1g). After 8 h, 15 μL (1:1 fresh extract) or 30 μL (1:2 fresh extract) of the fresh cell-free mix, along with different concentrations of the reporter DNA, were added to the initial 15 μL reaction incubated with the TF plasmid. In both cases (1:1 fresh extract and 1:2 fresh extract), the fold change increased (Figure 1h) with regard to the purple bar plot in Figure 1f that had the same preincubation time. This improvement is because, by adding fresh reaction mix, we provided fresh resources for the production of GFP. A fold change of 4 was obtained when 30 μL of the fresh cell-free mix with 50 nM of reporter DNA was added (Figure 1h and Supplementary Figure S5).

Although the reinitiation approach improved the fold change, this is a more costly and time-consuming approach. Additionally, we achieved relatively high fold changes with only preincubation for 1.5 and 6 h. Finally, we chose 100 nM TF, preincubated during 1.5 and 6 h, followed by the addition of 10 nM (see Supplementary Figure S6) and 50 nM of the reporter DNA, respectively, as our optimized biosensors to quantify D-psicose in our cell-free system. To measure the quality of the optimized biosensor, we added different concentrations of D-psicose in the cell-free mix (Figure 2a). We observed that preincubation during 1.5 or 6 h, followed by the addition of 10 or 50 nM of reporter DNA, respectively, allowed a linear dose−response behavior of the biosensor (Figure 2a).

Next, we used the optimized biosensors to monitor the production of D-psicose from fructose using DPEase, an enzyme from *Clostridium cellulolyticum* (Figure 2b). In this experiment, first, 100 nM of TF DNA plus different concentrations of DPEase DNA were preincubated. Then, we added 10 or 50 nM of reporter DNA plus 100 mM fructose. Figure 2c shows the monitored D-psicose produced from fructose using the enzyme DPEase. The unoptimized biosensor (red bars in Figure 2c) produces only a limited level of fluorescence at its maximum. In Figure 2c, by increasing the concentration of the enzyme, first, the fluorescence level raises and then decreases (blue bars in Figure 2c) or reaches saturation (yellow bars in Figure 2c). The reduction in the fluorescence at high concentrations of the enzyme can be explained by the competition for a fixed amount of resources present in the lysate.[5,16]

Although our pathway is composed of only one enzyme, our study introduces a workflow which can be applied for multienzyme pathways. Note that the resource competition can increase when multiple enzymes are produced. Moreover, in this study, we demonstrated that a repressor-based biosensor that suffers from low fold change can be improved to quantify the production of a metabolite. Such improved biosensors can be used to monitor pathway activity for prototyping or to implement dynamic regulation. Cell-free biosensors enable faster screening of the enzymatic pathways where combinations of different enzymes at different concentrations can be explored, therefore speeding up the design−build−test cycle in metabolic/enzyme engineering.

METHODS

Molecular Biology. The source of the transcription factor (TF)−promoter pair is *Agrobacterium tumefaciens*, and the DPEase enzyme is from *C. cellulolyticum*. All sequences are available in Supplementary Table S1. The sequences were cloned in the pBEAST backbone,[16] a derived version of the pBEST[18] vector using Golden Gate assembly in *E. coli* Mach1 chemically competent cells. To build the reporter plasmid, the psicose responsive promoter (p*psiA*) was inserted upstream of *sfgfp* in pBEAST. *DPEase* and *psiR* genes were cloned under control of J23101 and B0032 RBS. Plasmids for cell-free reactions were prepared using Macherey-Nagel Maxiprep kit from overnight bacterial cultures.

Extract Preparation. The cell lysate preparation is based on the protocol of Sun et al.[14] Briefly, this is a 5-day protocol in three phases; (i) harvesting the cells: *E. coli* BL21 colonies (for TF-doped extract: cells transformed with TF DNA) grow on a plate overnight at 37 °C, 50 mL preculture at 37 °C during 8 h, 4 L of cultures at 37 °C until $OD_{600\ nm}$ = 1.5−2.0, (ii) extract preparation: multiple pellet washing with S30A buffer followed by sonication (instead of beads-beating in the original protocol) to obtain the extract, and (iii) cell-free reaction optimization: optimization by varying the Mg-glutamate and K-glutamate concentrations. After the cells were washed based on the Sun et al. protocol (Day 3 step 18) with S30A buffer (14 mM Mg-glutamate, 60 mM K-glutamate, 50 mM Tris, 2 mM DTT, pH 7.7), the cells were centrifuged at 2000*g* for 8 min at 4 °C. The pellet was resuspended in S30A (pellet mass (g) × 0.9 mL). The solution was split into 1 mL aliquots in 1.5 mL Eppendorf tubes. Eppendorf tubes were placed in a cold block and sonicated using a vibracell 72408 (Fisher Bioblock Scientific) with the following procedure: 20 s

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on, 1 min off, 20 s on, 1 min off, 20 s on. Output frequency 20 kHz, amplitude 25%.

The remaining steps of the protocol followed the procedure of Sun et al. for day 3, step 37. The process of mRNA and protein synthesis is performed by the molecular machinery present in the extract with no addition of external enzymes. The amino acid solution and energy solution mixes are kept as in the original protocol and are added to the cell extract. Reactions take place in 15.75 μL volumes at 30 °C in a 384-well plate. The final cell lysate contains 6 mM Mg-glutamate, 140 mM K-glutamate, 1.5 mM of each amino acid (except leucine), 1.25 mM leucine, 50 mM HEPES, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/mL tRNA, 0.26 mM CoA, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA, and 2% PEG-8000.

Cell-Free Experiments. For all cell-free reactions, 33% extract, 40% buffer, DNA plasmids, D-psicose or fructose, and water were mixed in PCR tubes to the final volume of 15.75 μL per reaction. Fifteen microliters of each reaction was pipetted in a 384-well plate (Thermo Fisher Scientific) to measure GFP fluorescence in a Biotek Synergy HTX plate reader. All reactions were incubated at 30 °C in the plate reader, and fluorescence (gain: 50, ex: 458 nm, em: 528 nm) kinetic data was recorded. For all presented results, the fluorescence measurement was taken after 8 or 4 h for preincubation experiments. All fold change data represent the ratio of the GFP fluorescence at a specific concentration of psicose or fructose with regards to fluorescence at 0 mM psicose or fructose for each concentration of reporter and TF DNA.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.9b00160.

Supplementary Figure S1, the behavior of the cell-free reaction with 10 nM reporter DNA at different concentrations of psicose and absence of the TF; Supplementary Figure S2, kinetic data of the fluorescence level for the maximum fold change point at Figure 1b; Supplementary Figure S3, fluorescence level for TF-doped extract with and without psicose (matches with Figure 1d); Supplementary Figure S4, fluorescence level for preincubation experiments with and without psicose (matches with Figure 1f); Supplementary Figure S5, fluorescence level for 8 h preincubation followed by addition of extra fresh extract (matches with Figure 1h); Supplementary Figure S6, dose−response curves for 1.5 h preincubation experiments; Supplementary Table S1, list of sequences used in this study (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: jean-loup.faulon@inra.fr.
*E-mail: olivier.borkowski@univ-evry.fr.

ORCID

Amir Pandi: 0000-0002-5204-756X
Ioana Grigoras: 0000-0003-2547-8935
Jean-Loup Faulon: 0000-0003-4274-2953

Author Contributions

A.P. and O.B. designed and performed the experiments and generated the results. A.P., O.B., I.G., and J.L.F. participated in the preparation of the manuscript.

Notes

The authors declare no competing financial interest.

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