

Tuning genetic control through promoter engineering

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Gene function is typically evaluated by sampling the continuum of gene expression at only a few discrete points corresponding to gene knockout or overexpression. We argue that this characterization is incomplete and present a library of engineered promoters of varying strengths obtained through mutagenesis of a constitutive promoter. A multifaceted characterization of the library, especially at the single-cell level to ensure homogeneity, permitted quantitative assessment correlating the effect of gene expression levels to improved growth and product formation phenotypes in *Escherichia coli*. Integration of these promoters into the chromosome can allow for a quantitative accurate assessment of genetic control. To this end, we used the characterized library of promoters to assess the impact of phosphoenolpyruvate carboxylase levels on growth yield and deoxy-xylulose-P synthase levels on lycopene production. The multifaceted characterization of promoter strength enabled identification of optimal expression levels for *ppc* and *dxs*, which maximized the desired phenotype. Additionally, in a strain preengineered to produce lycopene, the response to deoxy-xylulose-P synthase levels was linear at all levels tested, indicative of a rate-limiting step, unlike the parental strain, which exhibited an optimum expression level, illustrating that optimal gene expression levels are variable and dependent on the genetic background of the strain. This promoter library concept is illustrated as being generalizable to eukaryotic organisms (*Saccharomyces cerevisiae*) and thus constitutes an integral platform for functional genomics, synthetic biology, and metabolic engineering endeavors.

functional genomics | metabolic engineering

Protein engineering via directed evolution and gene shuffling (1, 2) has been extensively applied for the systematic improvement of protein properties such as antibody-binding affinity (3), enzyme regulation (4), and increased or diverse substrate specificity (5). A similar approach whereby continuously improved mutants are generated along a selection-defined trajectory in the sequence space can also be applied for the systematic improvement or modification of other types of biological sequences, e.g., ribozymes (6, 7). We show here that promoters can also be engineered via directed evolution to achieve precise strengths and regulation and, by extension, can constitute libraries exhibiting broad ranges of genetic control.

Typically, the deletion (8) and strong overexpression (9) of genes have been the principal strategies for elucidation of gene function. These two methods sample the continuum of gene expression at only a few discrete points, determined by experimental feasibility (10) and not necessarily biological significance. Thus, the full dependency of phenotype on gene expression may not be accessible due to the limitations inherent in these methods. Gene expression is controlled by a number of factors in the cell, including promoter strength, cis- and transacting factors, cell growth stage, the expression level of various RNA polymerase-associated factors, and other gene-level regulation. Of course, gene expression may not always correspond with enzymatic activity given protein level regulation, which may also be present. Nevertheless, several groups have attempted to control gene expression through the creation of promoter libraries (11–13). In this work, we present the development of a fully characterized, homogeneous, broad-range, functional promoter library and demonstrate its applicability to the

analysis of such a genetic control. By characterizing the strength of these promoters in a quantitative manner with various metrics and subsequently integrating these constructs into the genome, it is possible to deduce the precise impact of the gene dosage on the desired phenotype.

An alternative method for controlling gene expression is through the use of a single inducible promoter tested at various levels of inducer. Although inducible promoters allow for a continuous control of expression at the macroscopic level, practical applications of these systems are limited by prohibitive inducer costs, hypersensitivity to inducer concentration, and transcriptional heterogeneity at the single-cell level (14, 15). The latter factor, in particular, can limit the effect of inducers in a culture to a simple increase of the number of cells expressing the gene of interest instead of the overexpression of the gene in all cells. Inducible systems are suitable in certain applications (e.g., recombinant protein overproduction) (16); however, the elucidation of gene function and genetic control on phenotype requires well characterized promoter libraries, which behave in a similar manner at the single-cell level. As a result, the creation of a promoter library based on a constitutive promoter would eliminate the need to regulate inducer concentrations and avoid heterogeneities in cellular response.

Methods

Strains and Media. *Escherichia coli* DH5 α (Invitrogen) was used for routine transformations, as described in the protocol. *E. coli* K12 (MG1655) and *E. coli* K12 PT5-*dxs*, PT5-*idi*, and PT5-*ispFD* (provided by DuPont) were used for promoter engineering examples. In specified strains, lycopene expression was performed by using the pAC-LYC plasmid (17) and assayed as described (18). Assay strains were grown at 37°C with 225 rpm orbital shaking in M9-minimal media (19) containing 5 g/liter D-glucose. When necessary, the M9 media were supplemented with 0.1% casamino acids. All other strains and propagations were cultured at 37°C in LB media. Media were supplemented with 68 μ g/ml chloramphenicol/20 μ g/ml kanamycin/100 μ g/ml ampicillin, as necessary. Glucose monitoring was conducted by using the r-Biopharm (Swansea, U.K.) kit. Cell density was monitored spectrophotometrically at 600 nm. All PCR products and restriction enzymes were purchased from New England Biolabs and used Taq polymerase. M9 minimal salts were purchased from USBiological (Swampscott, MA), and all remaining chemicals were from Sigma-Aldrich. Primers were purchased from Invitrogen, and sequence information is listed in *Supporting List*, which is published as supporting information on the PNAS web site.

Saccharomyces cerevisiae strain BY4741 (*MATa*; *his3 Δ 1*; *leu2 Δ 0*; *met15 Δ 0*; *ura3 Δ 0*) used in this study was obtained from EURO-SCARF (Frankfurt). It was cultivated in yeast extract/peptone/dextrose medium (10 g of yeast extract per liter/20 g of Bacto Peptone (Becton Dickinson) per liter/20 g of glucose per liter). For yeast transformation, Frozen-EZ Yeast Transformation II (Zymo Research, Orange, CA) was used. To select and grow yeast trans-

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Abbreviations: *dxs*, deoxy-xylulose-P synthase; *ppc*, phosphoenolpyruvate carboxylase.

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formants bearing plasmids with *URA3* as selectable marker, a yeast synthetic complete (YSC) medium was used containing 6.7 g of yeast nitrogen base (Difco) per liter, 20 g of glucose/liter, and a mixture of appropriate nucleotides and amino acids (CSM-URA, Qbiogene, Irvine, CA), referred here as to YSC Ura⁻. Medium was supplemented with 1.5% agar for solid media. Yeast cells were routinely cultivated at 30°C in Erlenmeyer flasks shaken at 200 rpm. For sorting single cells (*TEF* promoter mutations) by FACS into microtiter plates, each well contained 200 μ l of YSC Ura⁻ supplemented with 10 mg/liter ergosterol and 420 mg/liter Tween 80 (20).

Library Construction. Nucleotide analogue mutagenesis was carried out in the presence of 20 μ M 8-oxo-2'-deoxyguanosine (8-oxo-dGTP) and 6-(2-deoxy- β -D-ribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-c][1,2]oxazin-7-one (dPTP) (TriLink BioTechnologies) (21). By using plasmid pZE-gfp(ASV) kindly provided by M. Elowitz (California Institute of Technology, Pasadena) as template (22) along with the primers PL_{sense}-AatII and PL_{anti}-EcoRI, 10 and 30 amplification cycles with the primers mentioned above were performed. The 151-bp PCR products were purified by using the GeneClean Spin Kit (Qbiogene). After digestion, the product was ligated at 16°C overnight and transformed into library efficiency *E. coli* DH5 α (Invitrogen). Approximately 30,000 colonies were screened by eye from minimal media-casamino acid agar plates, and 200 colonies, spanning a wide range in fluorescent intensity, were picked from the plates.

To create the *TEF* promoter mutation library for *S. cerevisiae*, the plasmid p416-TEF-yECitrine was used as a template for the error-prone PCR of the *TEF1* promoter by using the primers TEF.Sense and TEF.Anti. The mix of purified mutagenized PCR products was transformed into yeast together with p416-TEF, which was cut with SacI/XbaI before (*in vivo* cloning). The CEN/ARS plasmid P416-TEF (23), containing the native *TEF1* promoter from *S. cerevisiae*, the *CYC1* terminator, and the *URA3* gene as a selectable marker, was obtained from American Type Culture Collection. The plasmid pKT140 was obtained from EUROSCARF. This plasmid contains the coding sequence of yECitrine, a yeast codon-optimized version of the yellow fluorescent protein (24), which was used as a reporter protein in this study. To clone the yECitrine gene downstream of the *TEF* promoter, the coding sequence of yECitrine was amplified via PCR from the plasmid pKT140 by using the primer yEC.Sense and yEC.Anti. The PCR product was cut with ClaI and XbaI and ligated to ClaI/XbaI restricted vector p416-TEF. The resulting plasmid is referred to as p416-TEF-yECitrine.

Library Characterization

Initial Characterization. Approximately 20 μ l of overnight cultures of library clones growing LB broth were used to inoculate 5 ml of M9G medium supplemented with 0.1% wt/vol casamino acid (M9G/CAA), and the cultures were grown at 37°C with orbital shaking. After 14 h, a sample of the culture was centrifuged at 18,000 \times g for 2 min, and the cells were resuspended in ice-cold water. Flow cytometry was performed on a Becton Dickinson FACScan, and the geometric mean of the fluorescence distribution of each clonal population was calculated. To ensure that bulk population-averaged measurements could reflect the underlying single-cell behavior, only clones with clean monovariate distributions of fluorescence were retained for further analysis. Twenty-seven clones were isolated in this way. Sequencing revealed that these 27 clones represented 22 unique promoter sequences.

Promoter Strength Metric. Shake flasks containing 50 ml of M9G/CAA medium were inoculated with 1% vol/vol of an overnight LB culture of a library clone. The culture turbidity ($A_{600\text{ nm}}$) and fluorescence (Packard Fusion microplate fluorescence reader, PerkinElmer) were monitored as a function of time. Fluorescence readings taken during the exponential growth phase were plotted as a function of turbidity. The best-fit slope to this line represents

the exponential-phase steady-state concentration of GFP, f_{ss} . Because f_{ss} is affected by the cell growth rate oxygen-dependent maturation constant of GFP and the protease-mediated degradation of GFP as well as the promoter-driven synthesis of new GFP, it is not a suitable metric for promoter strength. Instead, we used a previously published dynamic model (25) that accounts for all of these factors. Under this model and under the assumption that the rate constant of protease-mediated degradation is the same for mature GFP as its precursor polypeptide, P , the rate of promoter-driven production of GFP can be expressed as in Eq. 1.

$$P = f_{ss} \left(\mu \left(1 + \frac{\mu}{m} \right) + D \left(2 + \frac{\mu}{m} \right) \right) \quad [1]$$

In Eq. 1, μ is the growth rate, m is the maturation constant for the oxygen-dependent fluorophore activation of GFP, and D is the first-order rate constant for protease-mediated degradation. Estimates of m and D of 1.5 h⁻¹ and 0.23 h⁻¹, respectively (26, 27), were obtained from the literature. The parameters f_{ss} and μ were measured separately for each member of the promoter library. P , in relative fluorescence units per absorbance unit per hour, was calculated from Eq. 1 for each clone. We performed duplicate cultures for each clone.

Transcriptional Analysis. Cultures inoculated as previously were grown for 3 h, and the total RNA was extracted from a 1.5-ml sample with a commercial kit (RNEasy, Qiagen, Valencia, CA). All samples were diluted to a final concentration of 20 μ g/ml and stored at -20°C. A commercial kit for RT-PCR (iScript One-Step

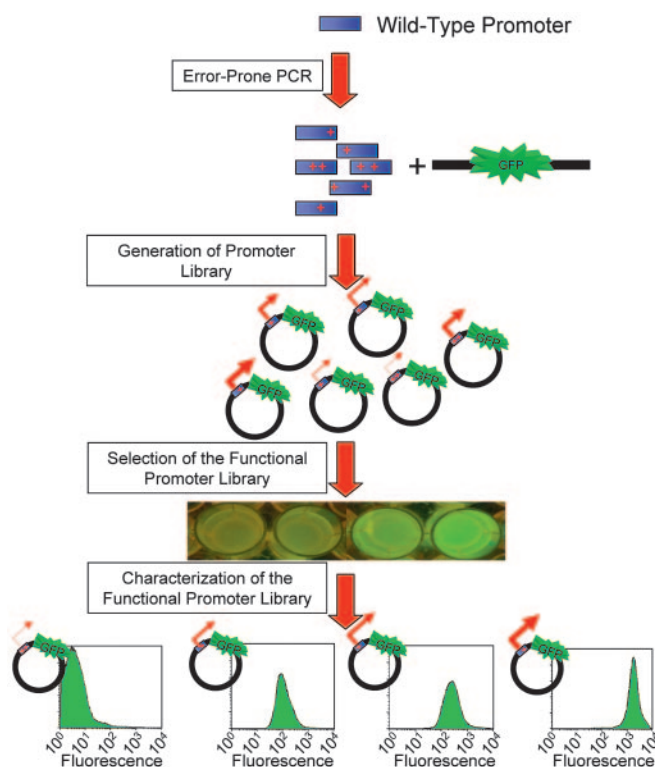


Fig. 1. Generation of the functional promoter library. A variant of the constitutive bacteriophage P_L- λ promoter was mutated through error-prone PCR, used in a plasmid construct to drive the expression of *gfp*, then screened based on fluorescence of colonies. The chosen constructs have a wide range of fluorescence both on a culture-wide and on a single-cell level, as illustrated by representative flow cytometry histograms at the bottom. All of the selected promoters have a uniform expression level on a single-cell level, as measured by GFP signal.

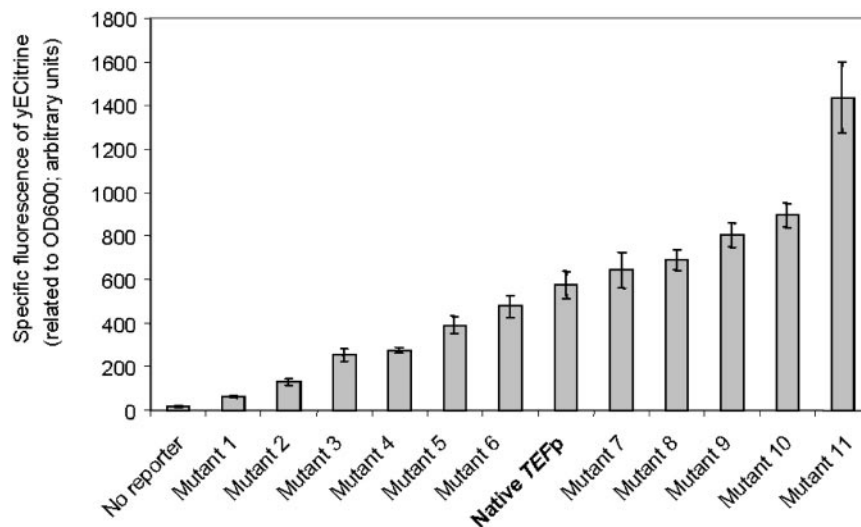


Fig. 4. Extension of promoter engineering to other systems. The basic concepts in this paper are further extended to a eukaryotic system (*S. cerevisiae*) by using the *TEF1* promoter. A similar wide range of yECitrine fluorescence is obtained from selected clones of the original promoter library. These results, along with other current work, indicate the ability to select for promoters responsible for tuning precise genetic control.

based measurements can lead to misclassification of the behavior of the promoter at the single-cell level and thus complicate quantitative gene expression studies, such as those performed in this study.

Application of the Promoter Library. We applied the functional promoter library to introduce precise transcriptional control in the investigation of specific genetic effects on a cellular phenotype. We performed chromosomal promoter delivery into the region upstream of the targeted gene, replacing the native promoter and its inherent regulation modality.

The utility of the promoter library was tested by investigating the effect of two endogenous genes [phosphoenolpyruvate carboxylase (*ppc*) and *dxs*] on two divergent phenotypes, growth yield and lycopene production. First, we investigated the growth yield from glucose as a function of the expression level of the *ppc* gene in *E. coli*. *E. coli*'s native *ppc* promoter was replaced with varying-strength promoter-*ppc* constructs, and these mutants were cultured while biomass and glucose concentrations were periodically monitored. Fig. 3*a* presents the exponential-phase biomass yields as a function of the average promoter strength metric. Increasing *ppc* levels have a positive effect on the biomass yield only to a certain point. This increase reaches a plateau, and further increases in the *ppc* level have a negative effect on the biomass yield. These results illustrate an optimum in the expression level of *ppc* that is above that found from endogenous expression.

In this second case, volumetric productivity of lycopene accumulation in glucose medium was investigated as a function of the expression levels of the *dxs* gene in two different *E. coli* strains: the wild-type K12 strain and a previously engineered strain, which already produces lycopene in high titers (18). Fig. 3*b* shows the lycopene production in these *dxs* constructs in a wild-type (K12) background. Elevating *dxs* expression increases lycopene accumulation only until a certain point. Beyond this optimum, increased *dxs* expression is detrimental for lycopene production. Finally, the strength of the native *dxs* promoter can be inferred from this analysis, as is illustrated on the graph (Fig. 3*b*).

In contrast to the above results, a linear relationship was obtained when similar promoter-*dxs* constructs were placed in an engineered strain (18) overexpressing downstream genes in the isoprenoid pathway (*ispFD* and *idi*). Fig. 3*c* illustrates a nearly linear response of lycopene production to varying levels of *dxs* expression, suggest-

ing that in the new genetic background, *dxs* has become rate-limiting.

Extension of the Promoter Library. We applied the promoter engineering concept to *S. cerevisiae* as well. By screening a library of *TEF1* promoter mutants, also created by error-prone PCR, a promoter collection was obtained that drove a wide dynamic range of YFP production in *S. cerevisiae* (Fig. 4). Thus, the promoter engineering paradigm can yield libraries of promoter for precise genetic control despite the profound differences in bacterial and eukaryotic transcription mechanisms (32, 33).

Discussion

The nearly 200 random promoter mutants we screened varied widely in their expression strength and clonal expression heterogeneity. Screening for only those promoters that drive stable monovariate expression in culture by flow cytometry was critical for deployment of our promoter constructs in pathway analysis and expression optimization. Isolating only the homogeneous expressers allowed us to establish a well defined metric of promoter strength, which combined data from several experimental assessments of gene expression levels. Using only a single technique to assess promoter strength often resulted in a scattering of the data, confounding the analysis of gene expression studies. The reliance on bulk averages would obscure the underlying relationship between expression and phenotype. The use of an integrated system allowed us to bypass the instabilities and inherent mutation rates associated with the overexpression of endogenous genes by using plasmid-based systems (34). Furthermore, this and other promoter libraries appear to have a broad host range (11), perhaps due to construction based on a heterologous constitutive promoter and reliance on the general polymerase machinery in the cell. This is exemplified through the three different strain backgrounds used in this study.

Enabled by a fully characterized library, we tested the promoter engineering concept for the analysis of two different phenotypes in *E. coli*. In the first, the expression of *ppc* was modulated to effect biomass yield from glucose. This gene expresses phosphoenolpyruvate (PEP) carboxylase, a key anaplerotic enzyme. A *ppc* knockout is lethal for *E. coli* in glucose minimal medium (35). Furthermore, overexpression of this gene has been shown to improve the growth yield on glucose (36). These data imply two possibilities: either biomass yield is a monotonically increasing

function of *ppc* expression, or there exists a particular *ppc* expression level that maximizes yield. Our data show that the latter is the case. Possible reasons why ever-increasing *ppc* levels lead eventually to a decrease in yield include the metabolic burden of severe overexpression of *ppc* or, more likely, the creation of a futile ATP-wasting cycle in metabolism, where PEP is converted to oxaloacetate by *ppc* and back again by *pck*, the gene for PEP carboxykinase.

In addition to the global pleiotropic phenotype of growth yield, we also used promoter engineering in the study of a single metabolic pathway, by modulating *dxs* expression and measuring lycopene biosynthesis. Kinetic control of metabolic pathways is often distributed and depends on the expression level of several genes within the pathway (37). The gene *dxs* represents the first committed step in isoprenoid synthesis in *E. coli* and has been implicated in control of lycopene production (38); however, the quantitative nature of this control was unclear, and promoter delivery experiments also allowed us to quantify this control in multiple backgrounds (Fig. 3 *b* and *c*). In the case of wild-type *E. coli*, an optimal *dxs* expression was again apparent. Past the optimum, increasing *dxs* expression lowers lycopene yield, presumably because of the inadequate activity of downstream enzymes in the isoprenoid pathway and resulting toxic buildup of DXP. In contrast, in a strain already engineered to overexpress *idi*, *ispF*, and *ispD*, downstream genes in lycopene biosynthesis, no maximum is apparent. A linear response to an enzyme concentration is expected for rate-controlling genes exhibiting a high flux control coefficient for a given pathway (39), suggesting that even at the highest expression levels examined in this study, the *dxs*-catalyzed reaction is rate-limiting for lycopene biosynthesis. We also note that cell density in both strains was greatly reduced in the constructs harboring low-strength promoters, which was expected, because *dxs* is an essential gene. A significant step in performing these quantitative functional genomics studies is creating a reliable characterized promoter library for which confidence in the cellular gene expression level may be placed. When this initial step is established, it is possible to quantitatively analyze the control a single enzyme exerts in a given pathway of interest, exemplified by the *dxs* example.

The creation of a library of promoter mutants in yeast illustrates the applicability of this approach in both prokaryotic and eukaryotic contexts. As with *E. coli*, flow cytometry allowed isolation of

only those promoters with relatively homogeneous reporter gene expression. It is possible to further extend and refine the selection process to create libraries of conditional promoters, active only under specified conditions. We have recently applied this selection methodology to create conditional genetic control elements that are responsive to environmental perturbations (e.g., oxygen concentration) (data not shown).

Additionally, the analysis of libraries of promoters may be studied to deduce a linkage between sequence and phenotype. To this end, it would be possible to create correlations between mutation sites and promoter metrics such as strengths or variability in gene expression (40). Further application and study of this promoter library can greatly facilitate efforts in synthetic biology aiming to create synthetic genetic operons. The cataloging of promoter sequences along with their behavior can help in the selection of components to be used in synthetic gene networks such as toggle switches (41) and for creating polygenic operons with prescribed ratios of gene expression.

Conclusion

We have created a general framework for the precise quantitative control of gene expression *in vivo*. Our strategy allows (i) achievement of any desired expression level for a specific gene, (ii) optimization of gene expression for maximal (or minimal) pathway function, and (iii) a means for the analysis of the distribution of genetic control on pathway behavior. In two disparate examples, we have shown that pathway function can exhibit well-defined extrema with respect to levels of gene expression. The existence of these extrema evinces the need for precise gene-dosage studies for the full understanding of pathway behavior. The creation and detailed characterization of a promoter library, as described here, are a facile and robust means to such an end.

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