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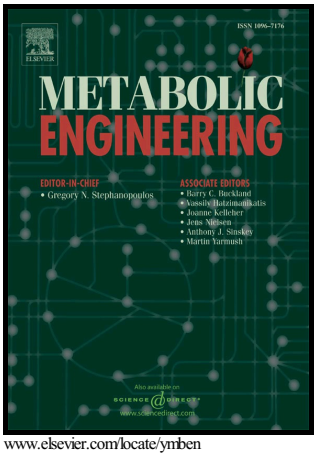

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Monica P. McNerney, Mark P. Styczynski

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Precise control of lycopene production to enable a fast-responding, minimal-equipment biosensor

Monica P. McNerney, Mark P. Styczynski*

School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA, USA

*Corresponding author. Address: 311 Ferst Drive NW, Atlanta, GA 30332-0100, USA; email: Mark.Styczynski@chbe.gatech.edu

Abstract

Pigmented metabolites have great potential for use in biosensors that target low-resource areas, since sensor output can be interpreted without any equipment. However, full repression of pigment production when undesired is challenging, as even small amounts of enzyme can catalyze the production of large, visible amounts of pigment. The red pigment lycopene could be particularly useful because of its position in the multi-pigment carotenoid pathway, but commonly used inducible promoter systems cannot repress lycopene production. In this paper, we designed a system that could fully repress lycopene production in the absence of an inducer and produce visible lycopene within two hours of induction. We engineered Lac, Ara, and T7 systems to be up to 10 times more repressible, but these improved systems could still not fully repress lycopene. Translational modifications proved much more effective in controlling lycopene. By decreasing the strength of the ribosomal binding sites on the *crtEBI* genes, we enabled full repression of lycopene and production of visible lycopene in 3-4 hours of induction. Finally, we added the mevalonate pathway enzymes to increase the rate of lycopene production upon induction and demonstrated that supplementation of metabolic precursors could decrease the time to coloration to about 1.5 hours. In total, this represents over an order of magnitude reduction in response time compared to the previously reported strategy. The approaches used here demonstrate the disconnect between fluorescent and metabolite reporters, help enable the use of lycopene as a reporter, and are likely generalizable to other systems that require precise control of metabolite production.

Keywords:
Lycopene; carotenoids; synthetic biology; biosensor; pathway engineering

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1. Introduction

The use of pigments as biosensor readouts holds great promise for emerging biosensor applications. When substituted for widely-used fluorescent reporters, pigment readouts can be interpreted without advanced equipment because they are visible to the naked eye, making them useable even in resource-poor settings. While there have been isolated examples of the use of colors as biosensor readouts (Pardee et al. 2014, Pardee et al. 2016), this approach is yet to be widely adopted.

One would expect the use of pigmented reporters to be a straightforward task given the breadth and depth of knowledge about pigment biosynthesis. Pigmented molecules are a common metabolic engineering target, whether due to their intrinsic value, their value as precursors, or just as a testbed for metabolic engineering techniques that enable simple screening (Rodrigues et al. 2013, Zhao et al. 2013, Fang et al. 2015, Zalatan et al. 2015, Fang et al. 2016). However, using metabolites as reporters requires more precise control than what is necessary for most metabolic engineering applications, since small amounts of enzyme can produce visible amounts of pigment and overproduction of certain metabolites can be toxic to the cell.

We have previously reported the ongoing development of a whole-cell biosensor that produces one of three pigmented metabolites—violacein (purple), lycopene (red), or β-carotene (orange)—based on extracellular zinc concentrations (Watstein et al. 2015). A whole cell biosensor using pigmented readouts could be especially useful in diagnostic tests for levels of micronutrients (vitamins and minerals) such as zinc. Micronutrient deficiencies are major public health problems in the developing world, but they are largely untreated because of a dearth of data on which areas are most affected (Bhutta et al. 2013). The cost and logistical challenges associated with current micronutrient diagnostic tests preclude effective population-level surveillance of micronutrient status. A biosensor that could be used in the field with minimal equipment could fill the need for a low cost, field-friendly micronutrient diagnostic tool (Garrett et al. 2011) and could be incorporated into the existing health surveillance pipeline.

In our initial zinc sensor prototype, the sensor cells always produced some pigment, a design selected for simplicity and to minimize ambiguity in readouts. However, this approach necessitates that the cells be grown from a very small inoculum so that coloration due to initial cell mass is minimal – thus requiring overnight assay times. To make the device more field-friendly, an important goal is to reduce the assay's time to coloration, which we are attempting to do by engineering cells that completely repress pigmentation during growth and quickly produce the appropriate pigment upon induction. This will enable sample addition to concentrated pre-cultured cells, making time to coloration only dependent on pigment production rates rather than coupled to the time required to grow a sufficiently large mass of cells.

In our previous work, we focused on the widely-studied and widely-engineered carotenoid pathway, which produces the red pigment lycopene. Overproduction strategies — including gene knockouts (Alper et al. 2005, Jin et al. 2007, Alper et al. 2008), precursor supplementation (Martin et al. 2003, Yoon et al. 2006, Pitera et al. 2007, Anthony et al. 2009, Ajikumar et al. 2010, Alonso-Gutierrez et al. 2013), and dynamic regulation of pathway genes (Farmer et al. 2000, Dahl et al. 2013) — have greatly improved lycopene yields and titers in previous

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metabolic engineering efforts. However, to use lycopene as a reporter in a biosensor, the primary goal must shift from maximizing yields and titers to maximizing response specificity.

To be effectively used as reporters, pigment production must be able to be turned completely off and on based on the state of the system. The need for tightly controlled metabolite production determined by the state of the system marks a departure from traditional metabolic engineering, in that maximizing product selectivity (rather than yield) is the primary concern (McNerney et al. 2015). Titer is important only in that a sufficient level (i.e., enough pigment to be visible) is produced; it is far more important to ensure that production is only turned on in specific conditions. Our previous efforts focused on controlling the transition between the red (lycopene) production state and the orange (β-carotene) state. We showed that a combination of transcriptional, translational, and post-translational regulation can be used to effect control over color production (Watstein et al. 2015). Here, we look instead to completely repress pigment production before inducing the biosynthetic pathway for downstream biosensor readout.

Previous metabolic engineering work has demonstrated the difficulty of fully repressing lycopene production – a goal that is critical for biosensing applications. When the enzymes that produce lycopene were expressed from an IPTG-inducible promoter, high lycopene yields and titers were achieved without any IPTG induction, meaning that enough protein was produced even in the uninduced state to catalyze lycopene production (Yoon et al. 2006). In fact, IPTG induction actually reduced both cell growth and lycopene production, proposed to be due to toxicity effects. Since maximum pigment production does not correspond with maximum protein production, precise control of the induced expression level is thus necessary to completely control pigment production.

To deal with the known difficulties in controlling lycopene production, we sought to investigate the abilities of different transcriptional and translational control mechanisms to enable both complete repression of lycopene when uninduced as well as fast production of visible amounts of lycopene upon induction. We chose to explore three commonly used promoter systems — the Lac, Ara, and T7 systems — that are all considered very inducible (at least in terms of dynamic range) but have varying levels of uninduced leaky expression, though Ara is widely considered to be tightly repressible and not leaky (Guzman et al. 1995, Terpe 2006). We sought to characterize the levels of unwanted protein expression in these promoter systems and reduce this unwanted expression by manipulating *cis* regulatory elements. Once systems were developed that could fully repress lycopene, we compared the time to response of the three different promoter systems. Finally, we explored the effect of metabolic precursor supplementation on the time to visible lycopene production upon induction.

Here we present approaches that ultimately enabled full repression of lycopene in the absence of an inducer while also enabling production of visible amounts of lycopene within two hours of induction, a reduction of over an order of magnitude compared to the previously reported strategy. Promoter engineering, RBS strength modification, and pathway engineering were all explored, and the effectiveness of each approach is evaluated.

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2. Materials and Methods

2.1 Materials

T4 DNA ligase, T5 exonuclease, Taq ligase, Phusion polymerase, Q5 polymerase, and restriction endonucleases were purchased from New England Biolabs (Ipswich, MA, USA). E.N.Z.A. Plasmid Mini Kits were purchased from Omega Bio-tek (Norcross, GA, USA), and QIAquick PCR Purification Kits and QIAquick Gel Extraction Kits were purchased from QIAGEN (Valencia, CA, USA). A lycopene standard was purchased from Millipore Sigma (St. Louis, MO). Sudan I (95%) was purchased from TCI America (Portland, OR, USA).

2.2. Strains and plasmids

Escherichia coli K-12 DH10B (New England Biolabs, Ipswich, MA) was used for plasmid assembly and for all protein and metabolite production. The plasmid pSB3T5, with a p15A origin, was taken from the Standard Registry of Biological Parts. To better enable colony selection based on antibiotic resistance during construct assembly, we made variations of the pSB3T5 plasmid by replacing the tetracycline resistance gene with either a kanamycin or chloramphenicol resistance gene. Consistent protein expression across plasmids was verified with fluorescence (data not shown), and these plasmids were used for all heterologous protein production. The plasmid pSB6A1, which is a derivative of the pBR322 plasmid, was obtained from the Standard Registry of Biological Parts and used for expression of the mevalonate genes.

2.3. Cloning and construct assembly

All constructs were assembled with either Gibson assembly (Gibson et al. 2009) or restriction endonuclease digestion of components and subsequent ligation and transformation following the BioBricks idempotent standard assembly (Shetty et al. 2011). For fluorescent characterization, *mRFP* was obtained from the Registry of Standard Biological Parts (bba_E1010), and the barcode downstream of the coding sequence was removed. The genes *crtE*, *crtB*, and *crtI* were amplified from Part bba_k274100 of the Registry of Standard Biological Parts; they were originally isolated from *Pantoea ananatis*.

Mevalonate pathway experiments used genes from the plasmid pJBEI-6409 (Alonso-Gutierrez et al. 2013), which was obtained from Addgene (Cambridge, MA, USA). Mevalonate pathway genes were amplified from this plasmid, a ribosomal binding site with a putative translation rate of 500 as determined by the RBS Calculator (Salis et al. 2009) was added to each gene, and genes were placed under control of the arabinose-inducible promoter pBad and cloned into the plasmid pSB6A1.

2.4. Promoter assembly

The promoters pBad (bba_I0500) and pLac (bba_R0011) were obtained from the Registry of Standard Biological Parts and used as templates for promoter modifications. pLac is a pλ-based promoter with two operator sites for LacI repression. wpLac was purchased as a gene fragment from IDT (Coralville, IA). wpLac2 was assembled by adding an additional lac operator site to

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wpLac through Gibson assembly. All T7 promoters were assembled with Gibson assembly. The T7 RNA polymerase was taken from the Registry of Standard Biological Parts (bba_K145001) and placed under control of wpLac. In all Lac-controlled constructs, LacI was constitutively expressed from the promoter pλr (bba_R0051).

2.5 Cell culture

For RFP expression, freshly transformed DH10B colonies were inoculated in triplicate into 5 mL LB medium and grown at 37°C and 180 rpm for 18 hours. 1 μL of each culture was then added to 150 μL of medium containing the appropriate concentration of inducer (either IPTG or arabinose) in a 96-well plate, and the plate was incubated at 37°C and 180 rpm for 18 hours and then analyzed. For time course fluorescence experiments, 15 μL of overnight cultures was added to 135 μL of fresh media, and plates were incubated in a Biotek Synergy H4 plate reader at 37°C with medium-strength shaking and analyzed every 20 minutes.

For lycopene expression, freshly transformed DH10B colonies were inoculated in triplicate into 5 mL LB medium containing the appropriate inducer and grown at 37°C and 180 rpm for 18 hours. For time-course experiments, overnight cultures were diluted to an OD of 0.15 in 50 mL of fresh medium, the appropriate inducer was added, and the sample was aliquoted into culture tubes corresponding to different time points. OD and lycopene content were measured at the time of inoculation, every hour for six hours, and at 18 hours.

LB medium composed of 10 g L^{-1} NaCl, 5 g L^{-1} yeast extract, and 10 g L^{-1} tryptone was used in all experiments. Either IPTG or arabinose was used for induction, and the following antibiotics were used for appropriate selection: tetracycline (15 μg/mL), chloramphenicol (33 μg/mL), kanamycin (30 μg/mL), and carbenicillin (100 μg/mL).

2.7 Measurement of optical density and fluorescence

Optical density of samples was quantified by measuring the absorbance at 600 nm either in a ThermoFisher Genesys 20 spectrophotometer with a 10 mm path length or in a Biotek Synergy H4 plate reader. 1 mL of culture was used for spectrophotometer measurements, and 150 μL of culture in a 96 well plate was used for plate reader measurements. Calibration curves were made for each instrument and used for uniform reporting of optical density. All optical densities reported correspond with those taken in the spectrophotometer.

For RFP quantification, fluorescence at 585 nm excitation and 610 nm emission was measured on a Biotek Synergy H4 plate reader. All reported fluorescence values are background-subtracted and normalized to optical density.

2.8 Lycopene extraction

Either 250 μL, 500 μL, or 1 mL of bacterial culture was pelleted at 18,000 rcf for 5 minutes. Cell pellets were resuspended in 50 μL of ultrapure water. Lycopene was extracted with 1 mL of acetone at 50°C for 20 minutes. A heat block was used to maintain temperature, and extractions were vortexed every 5 minutes. Cellular debris was pelleted at 18,000 rcf for 5 minutes, and 500

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μL of supernatant was removed for analysis. Sudan I was used as an internal standard (Xu et al. 2006) and added to the acetone used for extractions at a concentration of 1 μg/mL.

2.9 HPLC analysis

All HPLC analysis was conducted on a Shimadzu Prominence UFLC using an Agilent C18 4.6 mm x 50 mm column with a 5 μm particle size and a Shimadzu photodiode array detector. A solvent ratio of 50:30:20 acetonitrile:methanol:isopropanol was used as the mobile phase (Lv et al. 2016) and run at a flow rate of 1 mL/min with a 25 μL sample injection volume. Absorption was detected at 471 nm. Retention times and peak intensities were compared to analytical standards spiked into control extractions from DH10B cells, and the internal standard Sudan I was used to account for acetone evaporation during the extraction protocol and for instrument drift.

3. Results

3.1 Commonly used inducible promoter systems cannot repress lycopene production

Three inducible promoter systems were tested for their ability to control lycopene production: pLac, pBad, and pT7. The promoter pLac is a synthetic pλ-based promoter with two operator sites for LacI repression, and the promoter pBad was originally isolated from the *E. coli* genome. LacI was constitutively expressed from the promoter pλr, and AraC was expressed from its native promoter that promotes transcription in the opposite direction of pBad. To confirm the inducibilty of each promoter, each was used to control RFP production (Supplementary Figure S1). The pLac and pBad systems were then used to control the *crtEBI* genes, which code for the enzymes that produce lycopene. Successful clones of the pT7 system controlling *crtEBI* could not be isolated, which may have been due to toxicity caused by higher baseline levels of *crtEBI*.

In both the pLac and pBad systems, cells had clearly visible lycopene production when no inducer was present (Figure 1A). The pBad system had a significant increase in lycopene production at $1x10^{-4}$ percent arabinose, but lycopene production decreased with further addition of inducer. The pLac system had consistent specific lycopene production at all inducer concentrations, though with an insignificant downward trend (Figure 1B). Both promoter systems exhibited reduced cell growth with addition of inducer: compared to the uninduced state, fully induced cultures had 50% fewer cells with pBad-regulated lycopene production and 98% fewer cells with pLac-regulated lycopene production (Figure 1C). DH10B cells with negative control pLac and pBad vectors showed only slight decreases (about 15%) in terminal OD when fully induced (Supplementary Figure S2) and produced no detectable lycopene.

Inability to repress lycopene and cell toxicity upon lycopene induction is consistent with previous reports of *crtEBI* expression from a Lac-inducible promoter (Yoon et al. 2006). These limitations and constraints pose a significant challenge to using lycopene as a reporter, motivating efforts to engineer more repressible promoter systems.

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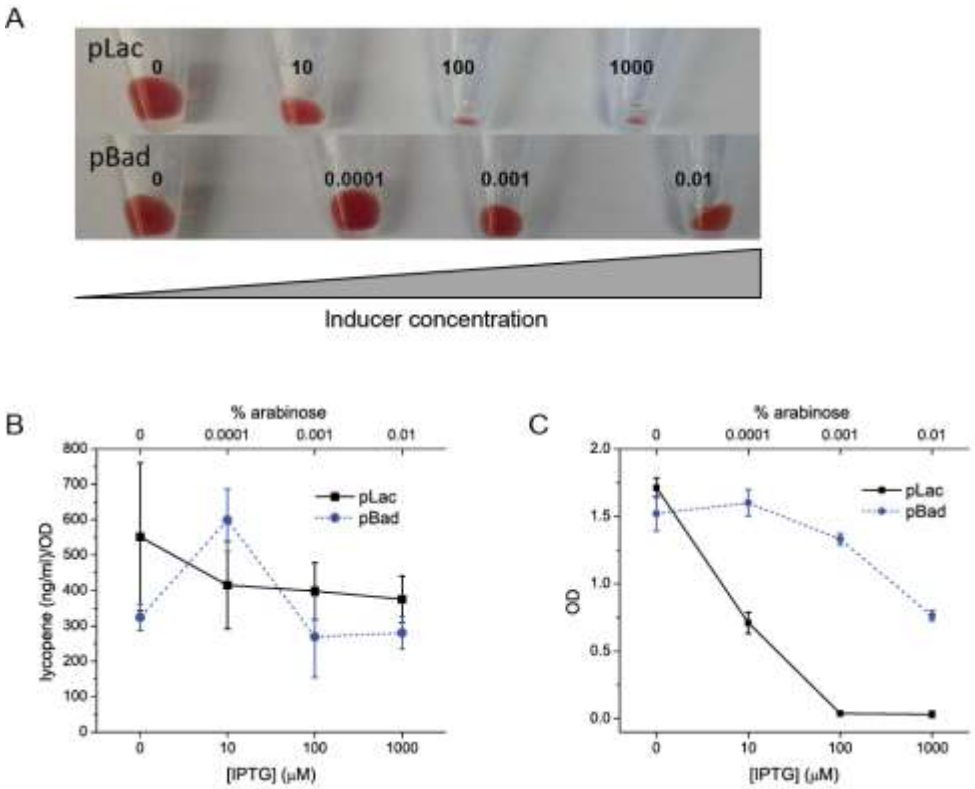

Figure 1: pLac and pBad control of lycopene production. (A) Cells expressing *crtEBI* from either pLac or pBad that were grown for 18 hours in different concentrations of inducer. pLac (top) and pBad (bottom) had visible lycopene production in the uninduced state. Terminal cell desnities decreased with increasing inducer concentration, and cells in all states were visibly red. Numbers correspond to μM of IPTG for pLac and % arabinose (w/v) for pBad. (B) Lycopene production normalized to cellular density. pLac-regulated production was constant at all concentrations of IPTG. pBad-regulated production increased only at 0.0001% arabinose. (C) Optical density at 18 hours of cultures with *crtEBI* expressed from either pLac or pBad. Cell density decreased with increasing concentration of inducer. All error bars represent standard deviation.

3.2 Promoter engineering decreases leaky protein expression, but engineered systems cannot fully repress lycopene production.

Variants of three common promoter systems—Lac, Ara, and T7—were constructed with the goal of decreasing lycopene production in the uninduced state. The pLac and pBad promoters used previously were modified to create weaker versions by changing the -10 and -35 RNAP binding domains to those of J23113, a very weak promoter. An additional pLac variant was constructed by adding a third operator site for LacI downstream of the -10 RNAP binding domain. T7 promoter variants were built by adding operator sites for LacI downstream from the consensus T7 sequence. In all T7 constructs, T7 RNA polymerase was expressed on the same plasmid from the Lac-inducible promoter wpLac. Table S1 contains the description and sequences for all promoters constructed.

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Each promoter was used to control RFP, and fluorescence was measured in both the uninduced and fully induced states (Figure 2A) and across a range of inducer concentrations (Supplementary Figure S3). Decreasing the strength of the RNA polymerase binding domains reduced uninduced protein expression by about an order of magnitude in both the Lac and Ara systems. Addition of operator sites for LacI reduced uninduced protein expression in the T7 system by orders of magnitude but had no significant effect on uninduced protein expression from wpLac2 compared to wpLac.

Modifications to pBad made the promoter both more repressible and increased its dynamic range. pBad is already considered one of the most repressible promoters, and wpBad is over 30 times more repressible than pBad. Despite this dramatic difference, induced expression from pBad was only 3 times higher than from wpBad, which is likely because AraC recruitment of RNA polymerase largely compensates for the polymerase's decreased affinity for the -10 and -35 RNAP binding domains. wpBad has a dynamic range of over 5000, which is over ten times greater than any of the other promoters characterized.

The most repressible promoter from each category was tested for its ability to repress lycopene (Figures 2B and 2C), and lycopene repression and induction generally did not follow the fluorescent induction trends in Figure 2A. wpBad enabled repression and induction of lycopene, a marked improvement over pBad's constitutive expression of lycopene. However, when uninduced, cells with *crtEBI* controlled by wpBad still had slight, but visible, lycopene production. Cells with *crtEBI* controlled by wpLac and pT7lacO2 had obviously visible lycopene in the uninduced state, no change in OD normalized lycopene production with induction, and visible reductions in cell growth upon induction. Though promoters were engineered to be 10 times more repressible than commonly used inducible promoters, only one (wpBad) was able to repress lycopene production, and even then the repression was incomplete.

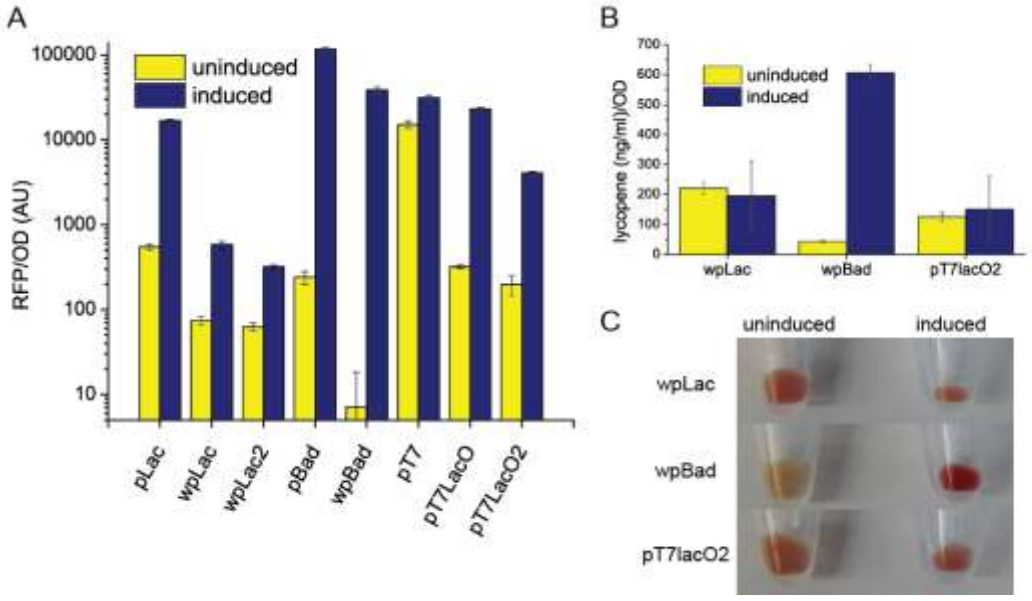

Figure 2: Discrepancy between fluorescent protein production and lycopene production from engineered promoter systems (A) Normalized RFP production from the variants of the Lac, Ara, and T7 promoter systems described in Table S1. (B)

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Lycopene concentration normalized to cellular density. The most repressible promoter systems were used to control *crtEBI* and lycopene was quantified. Only wpBad showed increased lycopene production with induction, likely due to wpBad's very low levels of uninduced protein expression. (C) Cells expressing *crtEBI* from either wpLac, wpBad, or pT7lacO2. Cells were grown for 18 hours with or without inducer. In the uninduced state, wpBad was the least colored, but still produced visible amounts of lycopene. In all experiments, cells were induced with either 1mM IPTG (for the Lac and T7 constructs) or with 0.01% (w/v) arabinose (for the pBad constructs). All error bars represent standard deviation.

3.3 Qualitative and quantitative evaluation of visible lycopene

Since the goal of our work is to have colorless cells that can be induced to be clearly colored, we needed to carefully consider our definition of what qualifies as "colorless" and "red". For example, is the uninduced lycopene production of wpBad in Figure 2C sufficiently low to call it colorless? To minimize potential subjectivity, we set thresholds for lycopene visibility based on the OD normalized lycopene concentration of cells considered universally "colorless" and universally "red" in a survey that asked 10 different people to match the perceived "redness" of ten different samples to different colors on a spectrum (Supplementary Figure S4). The lycopene levels of samples unanimously considered either "colorless" or "red" were then set as the thresholds: samples with OD normalized lycopene concentrations < 10.6 (ng/ml)/OD are considered colorless, and samples with OD normalized lycopene concentrations > 195 (ng/ml)/OD are considered fully red.

These thresholds will likely have to be adjusted to fit the final form factor of a biosensor device, since the media used (ie: LB vs. M9), culture volume, and cell density all affect the apparent "redness" of a sample, even if the lycopene concentration values are the same. However, these thresholds are useful for evaluating the effects of different genetic modifications and will be used throughout this work to quantify lycopene visibility.

3.4 Reduced ribosomal binding site strength enables lycopene repression and induction

Since rational promoter engineering was unable to prevent unwanted lycopene production, reduction in ribosomal binding site (RBS) strength was explored as another way to decrease uninduced protein expression. The *crtEBI* genes used here have been widely used elsewhere; they were originally taken from the Registry of Standard Biological Parts (bba_k274100) and already contained RBSs that were added to create a composite part. The RBS calculator (Salis et al. 2009) was used to determine the strength of the RBS on each gene, and the predicted strengths were comparable (on the same order of magnitude) to the predicted strength of bba_B0034 (B34), a commonly used strong RBS from the Registry of Standard Biological Parts. Since a strong RBS amplifies the effect of any unwanted transcription, reduction in RBS strength was considered a viable option for reduction of unwanted protein. As an initial proof of principle, repressible promoter systems were used to control RFP production with weaker RBSs, which reduced uninduced fluorescence to below the level of detection (Supplementary Figure S5), thus supporting RBS strength reduction as a viable strategy for reducing unwanted protein and pigment production.

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RBSs with a range of strengths were then tested for their ability to control lycopene production. Operons were assembled with a promoter from each group controlling the *crtEBI* genes with either the medium strength RBS bba_B0032 (B32) or the weak RBS bba_B0033 (B33) on all genes. The most repressible promoter from each group was chosen to control *crtEBI*, with the exception of the pLac variants: pLac was used for testing instead of wpLac or wpLac2 because of their very low dynamic ranges (less than 10). Cells were grown with different concentrations of inducer from small inocula, and levels of lycopene were determined both quantitatively and qualitatively after 18 hours. With RBSs of B32 and B33 on the *crtEBI* genes, cells contained no visible lycopene when uninduced (lycopene < 10.6 (ng/ml)/OD) and produced increased amounts of lycopene with addition of inducer. wpBad and pT7lacO2 with the RBS B32 had the greatest dynamic ranges, maintaining white cells when uninduced and dark red cells (lycopene > 450 (ng/ml)/OD) at full induction (Figure 3).

When a strong RBS is on the *crtEBI* genes, the pLac and pT7lacO2 systems are unable to induce lycopene production. These samples also have a lower final cell density, which suggests that overproduction of the CrtEBI proteins, lycopene, or an intermediate metabolite places some stress on the cell that reduces cell growth and prevents further lycopene production. In the pT7lacO2 system with a strong RBS and to a lesser extent in the pLac system with the medium-strength RBS, lycopene production increases with partial induction (10 μM IPTG) but then decreases with further induction. This peak at low levels of induction, combined with decreased cell growth at higher induction levels further supports that overproduction of lycopene can be toxic to the cell.

The induction profiles of RFP and lycopene showed notable differences. Though maximum RFP production varied by orders of magnitude across promoter systems (Supplementary Figure S5), maximum lycopene production in all promoter systems was similar (~600 (ng/ml)/OD). Differences in maximum RFP production could be attributed to variations in either promoter strength or effective RBS strength within a promoter system. Because of such drastic variation in protein production from each system, as represented by RFP levels, it is surprising that maximum lycopene production is so similar across promoter systems. The comparable amounts of lycopene produced suggest that either fluorescent proteins are not adequate indicators of the amounts of CrtEBI proteins actually produced, or that there is an upper limit on the amount of lycopene that cells can accumulate, and increased protein production does not necessarily correspond with increased lycopene production.

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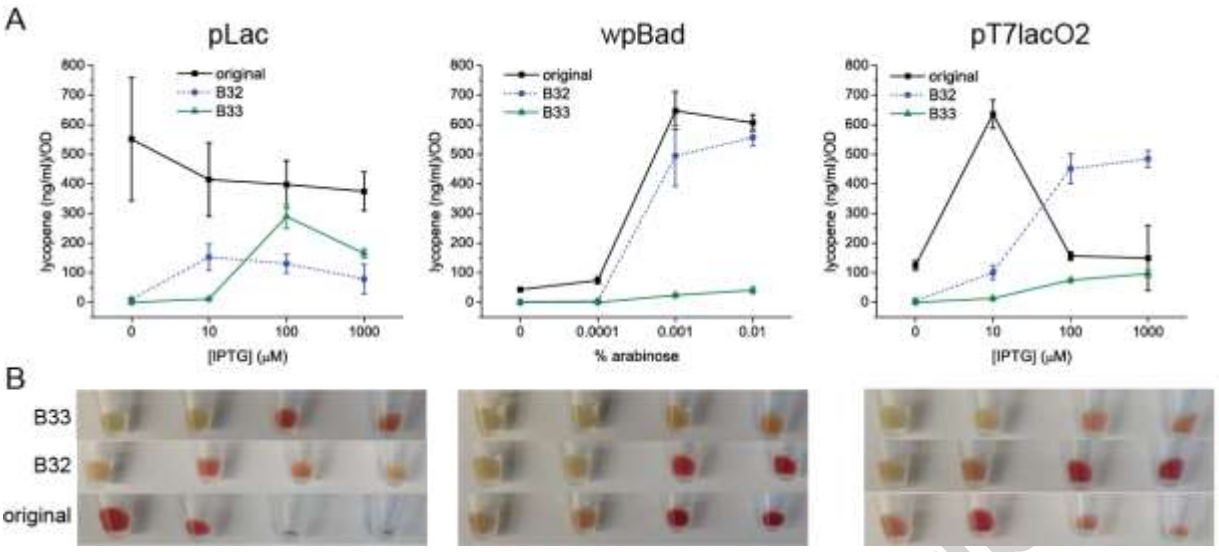

Figure 3: Effect of RBS strength on lycopene production across different promoter systems. (A) Lycopene production from each promoter system with three different RBSs on the *crtEBI* genes: original (strong), B32 (medium), and B33 (weak). Weakening the RBS decreased uninduced lycopene production. The effect of RBS strength on induced lycopene production varied by promoter system. The pLac system had maximal lycopene production (in terms of dynamic range) when B33 was on the *crtEBI* genes, and the wpBad and pT7lacO2 systems had maximal dynamic range when B32 was on the *crtEBI* genes. Error bars represent standard deviation. (B) Cells expressing *crtEBI* with different RBSs and inducer concentrations. In all systems, when B32 and B33 were on the *crtEBI* genes, uninduced cells were colorless. The visible redness upon induction corresponds with the lycopene production in panel A.

3.5 The pLac system produces visible lycopene most quickly

By enabling full repression of pigment production, the time to visible pigmentation is determined by the rate of pigment production upon induction, rather than the time for growth of large numbers of cells. The RBS and promoter modifications were thus next tested for their time to lycopene production upon induction.

The three different promoter systems, each with the medium-strength RBS B32 on the *crtEBI* genes, were tested for their time to visible lycopene production upon induction. All systems reached the previously established threshold for visible lycopene within four hours, and the pLac system had slightly higher lycopene levels than the pBad and pT7lacO2 systems at all time points (Figure 4A). This relative ranking of systems is consistent with the relative time to half-maximum fluorescence of each system (Supplementary Figure S6), which gives a reasonable approximation for how quickly each system responds to addition of inducer molecules.

The pLac system produced the most lycopene at all time points, with a final lycopene production of 950 (ng/ml)/OD (Figure 4A), ten times more than what was produced after 18 hours when a small inoculum was used (Figure 3A), which prompted exploration of the effects of different inoculum densities. Cultures were inoculated to starting optical densities between 0.01 and 0.4

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and induced. Cultures inoculated with more cells had higher final lycopene levels, though the effects plateaued above an initial inoculum density of OD 0.05 (Supplementary Figure S7). Cultures inoculated to optical densities above 0.05 all reached the threshold for visible lycopene at approximately the same time (Supplementary Figure S7).

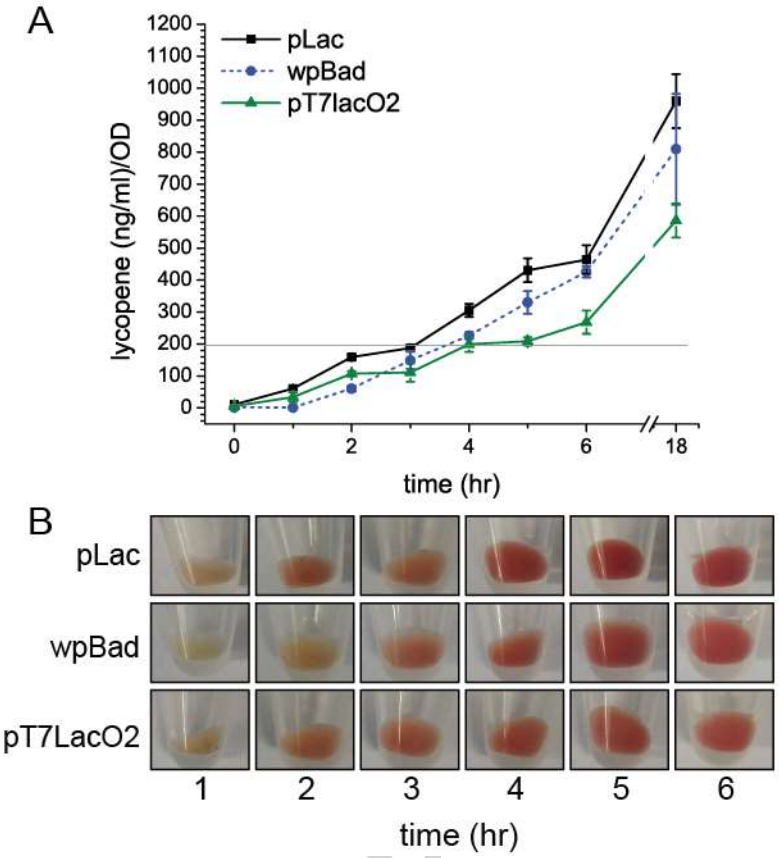

Figure 4: Time to response of three different promoter systems. (A) Lycopene produced from each promoter system with respect to time. All systems reached the 195 (ng/ml)/OD threshold (marked with gray line) within four hours, and the pLac system reached it the most quickly. Error bars represent standard deviation. (B) Cells from each system at different time points.

3.6 Precursor supplementation reduces time to visible lycopene but also increases uninduced lycopene production

To attempt to further decrease the time to visible lycopene production, metabolic precursor supplementation was explored. Farnesyl diphosphate (FPP) is the substrate for CrtE, the first enzyme in the lycopene-production pathway, and FPP is produced by either the nonmevalonate (dxp pathway) or the mevalonate pathway. The dxp pathway naturally occurs in *E. coli*, and the mevalonate pathway can be heterologously expressed in *E. coli*. We have previously shown that addition of the mevalonate pathway increases end-point lycopene production by a factor of three

(Watstein et al. 2015), and we hypothesized that its addition could decrease the time to visible lycopene.

The fastest responding system (the pLac crtEBI system) was cotransformed with either a plasmid containing the mevalonate genes under pBad control (pBad-MEV) or an empty control vector (pBad ∅). pBad-MEV supplementation decreased the time to visible lycopene (lycopene > 195 (ng/ml)/OD) to about 1.5 hours (Figure 5A). Interestingly, the cells produced lycopene faster and produced more total lycopene when the mevalonate pathway was induced at the same time as the *crtEBI* pathway, rather than when the mevalonate pathway was induced during the growth stage to potentially accumulate precursors ready for induction of *crtEBI*. Of note, cells cotransformed with an empty vector had a longer time to visible lycopene production than when just the pLac crtEBI vector was transformed (about 5.5 hours compared to 3 hours), suggesting that maintenance of a second plasmid can significantly decrease pigment production from the first plasmid.

Though mevalonate supplementation decreased the time to visible lycopene, it also increased the amount of unwanted lycopene in the uninduced state. When pBad-MEV was induced in the growth stage, cells contained about 14 times more lycopene compared to cells without pBad-MEV, and even when pBad-MEV was repressed in the growth stage, cells still contained over 3 times more lycopene, pushing the lycopene concentration just over the preferred threshold for colorless cells (Figure 5B).

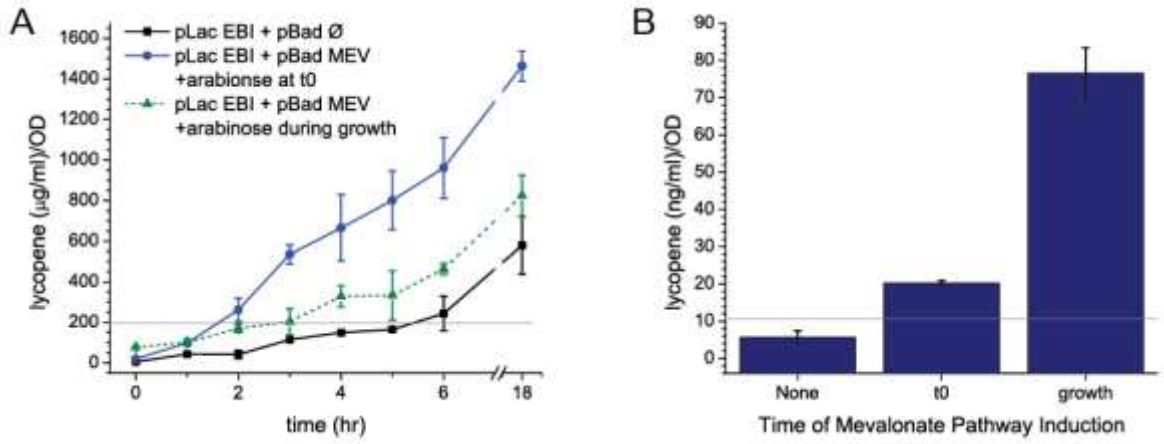

Figure 5: Effects of precursor supplementation on lycopene production. (A) Normalized lycopene production from the pLac system cotransformed with either an empty vector (pBad ∅) or pBad-MEV, with arabinose induction either during the growth phase or at the time of IPTG induction. Addition of the mevalonate pathway decreases the time to visible lycopene to 1.5 hours, with the greatest reduction occurring when the mevalonate pathway is repressed during the growth stage and induced at the same time as the *crtEBI* pathway. The gray line marks the threshold for visibility. (B) Lycopene produced in the growth stage for each system. In the uninduced state (no IPTG), lycopene production increased with the presence of pBad-MEV and further increased when pBad-MEV was induced with arabinose. The gray line marks the threshold for perceived colorlessness. All error bars represent standard deviation.

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4. Discussion

In this work, we engineered metabolic pigment pathways to harness them for use in a fast-responding whole cell biosensor. Using pigments as reporters can better enable sensor use in low-resource settings, since sensor output can be interpreted by the naked eye, without any equipment. By engineering cells to be initially colorless, a large inoculum of precultured cells can be added to the sample, and the time to pigmentation will be determined by the rate of metabolite production rather than the rate of cell growth. However, fully repressing pigment production to achieve an initial "off" state poses unique challenges, since small amounts of unwanted protein can catalyze the production of large, visible amounts of pigment. Commonly used inducible promoter systems could not adequately repress lycopene, which necessitated exploring engineering modifications at the transcriptional and translational level to eliminate unwanted pigmentation. When exploring transcriptional modifications, we generated a mutated pBAD with 30 times lower uninduced levels and a 10 times greater dynamic range, which could be of use in a broad range of applications.

When characterizing the effects of genetic modifications on RFP and lycopene production, we repeatedly encountered the problem that low levels of fluorescent protein expression did not correspond with visibly low levels of pigment production. Reducing fluorescence by orders of magnitude still yielded promoters with visible uninduced lycopene and induced lycopene levels that changed by much smaller factors. The systems that could adequately repress lycopene had uninduced fluorescent levels below the plate reader's limit of detection, emphasizing the need for much tighter regulation when using pigments instead of fluorescent reporters. When using fluorescent reporters, the value of the baseline "off" level is inconsequential as long as the "on" value is distinctly higher. However, when using pigment reporters, the enzymatic amplification of low levels of expression in the "off" state can confound sensor output, which necessitates focusing on reduction of absolute (rather than relative) protein expression. Transcriptional modifications made modest improvements in decreasing uninduced lycopene expression, but only when the RBS strength of the *crtEBI* genes was reduced could each system adequately control lycopene production. This suggests that translational modifications are perhaps the best starting point in combatting unwanted expression.

Differences in RFP and fluorescent induction profiles accentuate the limitations in using fluorescent reporters to characterize metabolic pathways. In many systems, as inducer was added, RFP production increased (as expected), but lycopene production decreased. This reduction in lycopene production upon induction is likely because of the added complexities of using metabolite reporters, specifically metabolic precursor limitations and toxicity effects at high levels of induction. These differences necessitate that when using metabolites as outputs or reporters, all final levels of tuning and system characterization must be performed with the final metabolite of interest as the reporter. However, despite the limitations of fluorescent reporters, they are useful for first approximations of system behavior, especially when assaying for metabolite levels is time-intensive and costly.

Lycopene production varied greatly based on the inoculum density: higher initial inocula resulted in higher final titers of lycopene. Previous work in the area of dynamic metabolic engineering has demonstrated the usefulness of separating the growth stage and metabolite production stage

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(Solomon et al. 2012, Soma et al. 2014, Brockman et al. 2015b). Many of the approaches used in dynamic metabolic engineering (Brockman et al. 2015a, Venayak et al. 2015) can be applied to our biosensor design, since cells must switch from a growth to a production phase. However, the requirement of a completely colorless preculture necessitates elimination of baseline or leaky pigment expression during the growth stage, which is often permitted and can even be beneficial in dynamic metabolic engineering efforts.

When optimizing time to visible lycopene upon induction, we encountered a tradeoff between low levels of lycopene during the growth stage and fast production of lycopene upon induction. The pLac system reached the visibility threshold the most quickly; however, the pLac system also had the highest level of uninduced lycopene at the time of induction, which may partly account for the faster time to visible pigmentation. Similarly, supplementation of metabolic precursors further reduced the time to visible lycopene production, but also further increased unwanted lycopene produced during the growth stage to a level above the colorless threshold. Though these effects could likely be somewhat mitigated by using a more repressible promoter to control the mevalonate pathway genes (i.e.: wpBad instead of pBad), since the pLac system already has uninduced lycopene levels just below the color threshold, it is likely that any level of precursor supplementation will push uninduced levels past the threshold.

In exploring the effect of metabolic precursor supplementation, we expressed the *crtEBI* genes and the mevalonate pathway genes on separate plasmids. Just by addition of a second plasmid (an empty control plasmid), the rate of lycopene production significantly decreased, suggesting that addition of a second plasmid places a stress on the cell that can alter protein and metabolite production. When the mevalonate genes were expressed on the second plasmid, lycopene production dramatically increased despite the limitations of a two plasmid system, which emphasizes the power of precursor supplementation. Combining the two pathways onto one plasmid would likely further improve the effects of precursor supplementation.

Though the systems developed should be useful in any biosensing application that uses lycopene as a reporter, we acknowledge that the *crtEBI* genes used in this study are not the only ones used in metabolic engineering applications. In all of our experiments, we used *crtEBI* genes isolated from the bacterium *Pantoea ananatis*. Most previous work to engineer *E. coli* cells to produce lycopene use either these genes or those from *Pantoea agglomerans*. Though some of the problems we encountered—specifically, high levels of uninduced expression and toxicity effects at high levels of induction—could be specific to the *P. ananatis* proteins, work using *P. agglomerans* genes (Yoon et al. 2006) has demonstrated similar problems. We also recognize that the visibility thresholds used will likely need to be changed based on the form factor of the final application device, but similar approaches can be used to develop systems that meet the visibility requirements of different devices.

Here we have improved the response time of a previously reported biosensor framework by an order of magnitude. We have demonstrated ways to control production of the metabolite lycopene, and the approaches used should be generalizable to any other metabolite of interest. As the applications of metabolic engineering expand beyond traditional chemical production, tight control of output in response to the external environment will be necessary. Cells are already being engineered to specifically deliver drugs to tumors (Forbes 2010, Din et al. 2016,

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Hosseinidoust et al. 2016) and to produce different drugs based on what is needed in low-resource settings (Choi et al. 2014, Perez-Pinera et al. 2016). For both of these applications, it is necessary that cells produce the desired compound only in the desired condition—specificity of response based on environment is critical. Here, we have demonstrated the ways that transcriptional and translational control can be used in combination to engineer response specificity and the ways that pathway engineering can increase the rate and intensity of the metabolite produced in the "on" state. These same approaches can be used to control production of other metabolites and will likely help to broaden the scope of metabolic engineering applications.

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Highlights

Engineering *E. coli* to effectively use lycopene as a biosensor reporter
Transcriptional and translational control mechanisms enable full lycopene repression
Precursor supplementation decreases time to visible lycopene
Approach generalizable to systems that necessitate tight control of metabolism

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