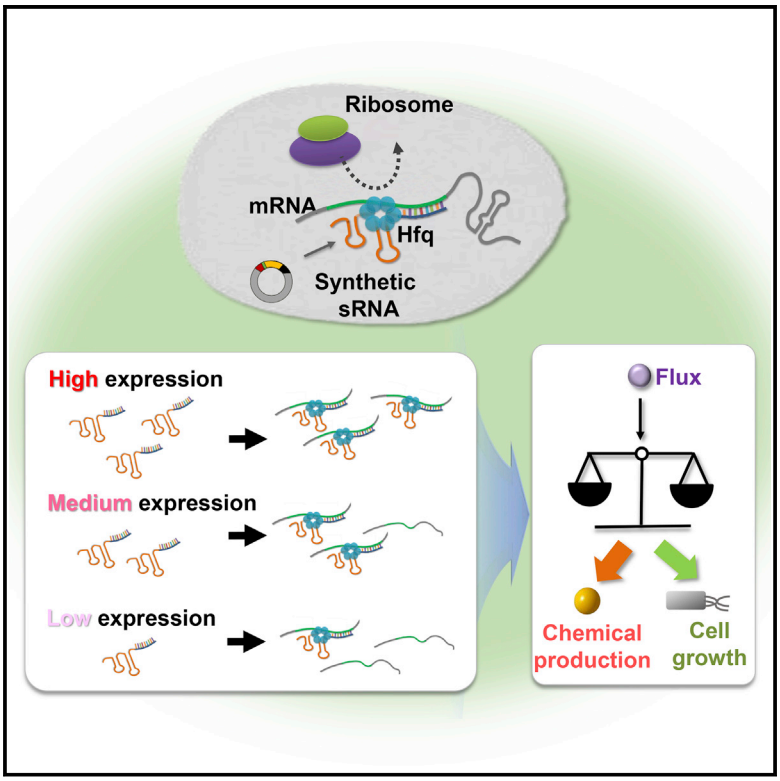
Cell Systems

Report

Gene Expression Knockdown by Modulating Synthetic Small RNA Expression in *Escherichia coli*

Graphical Abstract



Highlights

- Gene expression is fine-controlled by varying synthetic sRNA expression level
- Proportionality between sRNA expression level and target repression is observed
- Putrescine overproducing strain is developed by finely modulating sRNA expression
- L-Proline overproducer is rapidly developed by finely modulating sRNA expression

Authors

Minho Noh, Seung Min Yoo, Won Jun Kim, Sang Yup Lee

Correspondence

leesy@kaist.ac.kr

In Brief

A strategy of finely tuning expression of multiple genes by modulation of synthetic sRNA expression level was developed and used for constructing putrescine and L-proline overproducing strains.

Noh et al., 2017, Cell Systems *5*, 418–426
October 25, 2017
http://dx.doi.org/10.1016/j.cels.2017.08.016

CellPress

CellPress

Cell Systems
Report

Gene Expression Knockdown by Modulating Synthetic Small RNA Expression in *Escherichia coli*

Minho Noh,[1,3,4] Seung Min Yoo,[1,2,3,4] Won Jun Kim,[1,3] and Sang Yup Lee[1,2,3,5,*]
[1]Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical and Biomolecular Engineering (BK21 Plus Program), Korea Advanced Institute of Science and Technology (KAIST), Daejeon 34141, Republic of Korea
[2]BioProcess Engineering Research Center and Bioinformatics Research Center, KAIST, Daejeon 34141, Republic of Korea
[3]Institute for the BioCentury, KAIST, Daejeon 34141, Republic of Korea
[4]These authors contributed equally
[5]Lead Contact
*Correspondence: leesy@kaist.ac.kr
http://dx.doi.org/10.1016/j.cels.2017.08.016

SUMMARY

Escherichia coli gene expression knockdown using synthetic small RNA (sRNA) can be fine-tuned by altering sRNA sequences to modulate target mRNA-binding ability, but this requires thorough checking for off-target effects. Here, we present an sRNA gene expression knockdown system fine-tuned by using different promoters to modulate synthetic sRNA abundance. Our approach entails selecting knockdown target genes resulting from *in silico* flux response analysis and those related to product biosynthesis then screening strains transformed with a library of synthetic sRNA-promoter combinations for enhanced production. We engineered two *E. coli* strains, both utilizing fine-tuned repression of *argF* and *glnA* through our approach; one produced putrescine (42.3 ± 1.0 g/L) and the other L-proline (33.8 ± 1.6 g/L) by fed-batch culture. Fine-tuned gene knockdown by controlling sRNA abundance will be useful for rapid design of microbial strains through simultaneously optimizing expression of multiple genes at a systems level, as it overcomes the difficulties of constructing and testing many different sRNAs and checking their cross-reactivity.

INTRODUCTION

Fine-tuning gene expression is an essential step for achieving optimized performance of coordinated genetic and metabolic networks in fundamental biological studies as well as metabolic engineering. In metabolic engineering, fine-tuning gene expression enables microbial cell factories to avoid accumulation of toxic intermediates (Pitera et al., 2007; Martin et al., 2003) and allows optimization of metabolic fluxes toward better cell growth and enhanced synthesis of desired products (van Ooyen et al., 2012). For this reason, many engineering tools have been developed to achieve varied levels of gene expression through modification of regulation of transcription (Alper et al., 2005), mRNA stability and translation (Ferreira et al., 2013; Salis et al., 2009), and protein stability (Cameron and Collins, 2014). The expression levels of non-inherent heterologous genes cloned in plasmid vectors can be tuned quite efficiently using these tools and methods, but those of inherent chromosomal genes are still laborious and time consuming to manipulate.

To overcome these limitations of conventional gene manipulation strategies, researchers have developed *trans*-acting RNA-based knockdown tools, such as antisense RNA and CRISPRi (Qi et al., 2013). These tools have been widely used in metabolic engineering and synthetic biology (Yang et al., 2015; Lv et al., 2015). Despite these achievements, fine-tuning gene expression levels is still a time-consuming and labor-intensive process; this is because designing effective *trans*-acting RNAs for the fine-regulation of target genes requires repeated design, synthesis, and experimental validation.

We have recently reported development of another *trans*-acting RNA-based method for efficient gene expression knockdown using synthetic regulatory small RNAs (sRNAs) (Na et al., 2013; Yoo et al., 2013). In this method, expression of multiple genes of interest can be repressed to the desired extent by a plasmid-based expression of synthetic sRNAs designed to bind to the translation initiation regions (TIR) of target mRNAs. By simply altering the TIR-binding sequences of synthetic sRNAs, the expression levels of desired target genes can be easily modulated (Na et al., 2013; Yoo et al., 2013).

There are two basic strategies for fine-tuning gene expression using synthetic sRNA. One strategy is to modulate the target mRNA-binding ability of synthetic sRNA, which can be achieved by changing the sequence of the target-binding region within synthetic sRNA (Na et al., 2013). The other strategy is to vary the synthetic sRNA expression level (Sung et al., 2016) by employing promoters of different strengths and/or plasmids of different copy numbers. In the first strategy, synthetic sRNAs with different binding strengths can be designed based on calculation of the binding affinities of synthetic sRNAs using several programs, such as UNAfold (Markham and Zuker, 2008) and ViennaRNA (Lorenz et al., 2011). Although doable, one drawback of this approach is the requirement to design as many primers as needed for constructing various synthetic sRNAs with the desired binding strengths (Yoo et al., 2013). The potential cross-reactivity caused by increase or decrease of the target-binding region must also be considered (Na et al., 2013; Yoo

et al., 2013). On the other hand, the second approach allows repression of target gene translation without requiring any sRNA modifications. Once a synthetic sRNA expression platform is established through screening different promoter strengths or replication origins, many series of sRNAs can be easily constructed with the same number of primers for all the genes to be targeted and readily applied to large-scale identification of the target genes to be engineered and optimization of their expression levels. Thus, based on one's need, two approaches can be employed complementarily.

After successfully demonstrating the effectiveness of the first strategy previously (Na et al., 2013; Yoo et al., 2013), here we study the second strategy and report the development of a fine-tuned knockdown system through modulation of synthetic sRNA expression levels. First, the repression efficiencies of synthetic sRNAs expressed under the control of promoters with different strengths against two model proteins, a heterologous protein DsRed2 expressed from a plasmid and an inherent enzyme glucose-6-phosphate isomerase encoded in the chromosome, were evaluated. After finding that fine-tuned gene expression was possible with two model proteins, this strategy was employed for developing *Escherichia coli* strains capable of efficiently producing putrescine and L-proline as proof-of-concept applications to metabolic engineering. Knockdown target genes were rationally selected through pathway analysis and also by using *in silico* flux response analysis. Accordingly, a library of 75 synthetic sRNAs-promoter combinations was constructed and used to enhance putrescine production; repression of *glnA* and *argF* showed a synergistically positive effect and led to a substantial increase in putrescine production. As the majority of the L-proline biosynthesis pathway is common to the putrescine biosynthesis pathway, the strategy of *glnA* and *argF* repression was also applied to an L-proline-producing strain, which resulted in a dramatic increase in L-proline production. The fine-tuned gene expression strategy reported here through varying the expression levels of synthetic sRNAs allows the expression levels of multiple genes to be finely controlled simultaneously and is easily implementable because of its simple process of design and construction of sRNAs without laborious modifications.

RESULTS

Assessment of an SgrS-S Scaffold-Based Synthetic sRNA Expression System

Synthetic sRNAs are short RNAs that hybridize to the translation initiation region of a target mRNA, thereby preventing access of ribosomes and interfering with translation (Vogel and Wagner, 2007). Synthetic sRNAs are composed of two modules: an mRNA-binding module and an Hfq-binding module. RNA-binding Hfq protein protects sRNAs from degradation and facilitates the degradation of sRNA-bound mRNAs by RNase E (Moll et al., 2003).

For modulation of the synthetic sRNA expression level to systematically fine-tune the repression of target gene translation, we used SgrS-S, a 5′-truncated variant of Hfq-binding sRNA SgrS that retains the high capacity of the original SgrS, to repress the target gene (*ptsG*; Ishikawa et al., 2012), as a representative sRNA scaffold. Among the three best sRNAs (MicC, MicF, and SgrS; Na et al., 2013), SgrS-S was used in this study because it is the most compact (Figure S1A). Prior to finely controlling target gene expression using the SgrS-S scaffold, the knockdown activity of SgrS-S scaffold-based synthetic sRNA was examined by constructing pKKSDsRed2 containing the DsRed2 mRNA-targeting synthetic sRNA and measuring DsRed2 fluorescence (Figure S1B). When expressed under the control of the P_R promoter, a strong promoter from bacteriophage lambda, the anti-DsRed2 sRNA reduced the florescence intensity of DsRed2 by 99% (Figure S1C).

Quantitative Relationship between sRNA Expression Level and Target Gene Repression

Using the SgrS-S scaffold, the quantitative relationship between sRNA expression level and target gene repression was examined next. Modulation of sRNA expression levels was first performed by using different strength promoters (Figure 1A); the P_R promoter in pKKSDsRed2 was replaced with synthetic promoters from the Anderson promoter collection (MIT Registry, BBa_J23100–BBa_J23118), resulting in sRNA expression plasmid series pKK1XXSDsRed2 harboring promoter J231XX (Figure 1B; see STAR Methods). The knockdown efficiencies of synthetic sRNA were determined by measuring the decrease in red fluorescence intensity of cells expressing DsRed2. The quantitative relationship between the varied expression levels of sRNA and target protein shows threshold-linear behavior (Figure 1C); gene expression is silenced if enough sRNA molecules are made and their binding to the target mRNA is saturated, while residual-free mRNAs are translated when not enough sRNA molecules are bound to its target. This threshold-linear behavior was also observed for a target protein versus varying mRNA levels under fixed sRNA levels (Levine et al., 2007). This result suggests that the modulation of sRNA expression levels using different strength promoters can tune target gene expression as desired. In addition to DsRed2, expression level modulation of glucose-6-phosphate isomerase (*pgi*), a chromosomally encoded inherent model protein, was examined as a proof-of-concept example of modulating inherent protein expression level. A series of anti-*pgi* sRNA expression plasmids were constructed with five different strength promoters (J23117, J23115, J23105, J23107, and J23100; Figure S1D; see Table S1 for strengths and sequences of promoters; see STAR Methods) and their knockdown efficiencies were determined by measuring the expression of glucose-6-phosphate isomerase through western blot analysis (see STAR Methods); glucose-6-phosphate isomerase expression for cells expressing the anti-*pgi* sRNA under J23100, the strongest promoter from the Anderson collection, could not be measured due to severe growth retardation as reported previously (Charusanti et al., 2010). It was found that the promoter strength of anti-*pgi* variants was also correlated with the translation repression level of glucose-6-phosphate isomerase (Figure S1E). The above results suggest that a controllable sRNA expression level by different strength promoters can tune the expression of both exogenous and endogenous genes in *E. coli*.

Large-Scale Tuning of Gene Expression Levels to Enhance Putrescine Production

To evaluate the practical use of the above fine-tuned knockdown system in metabolic engineering, rapid development of an

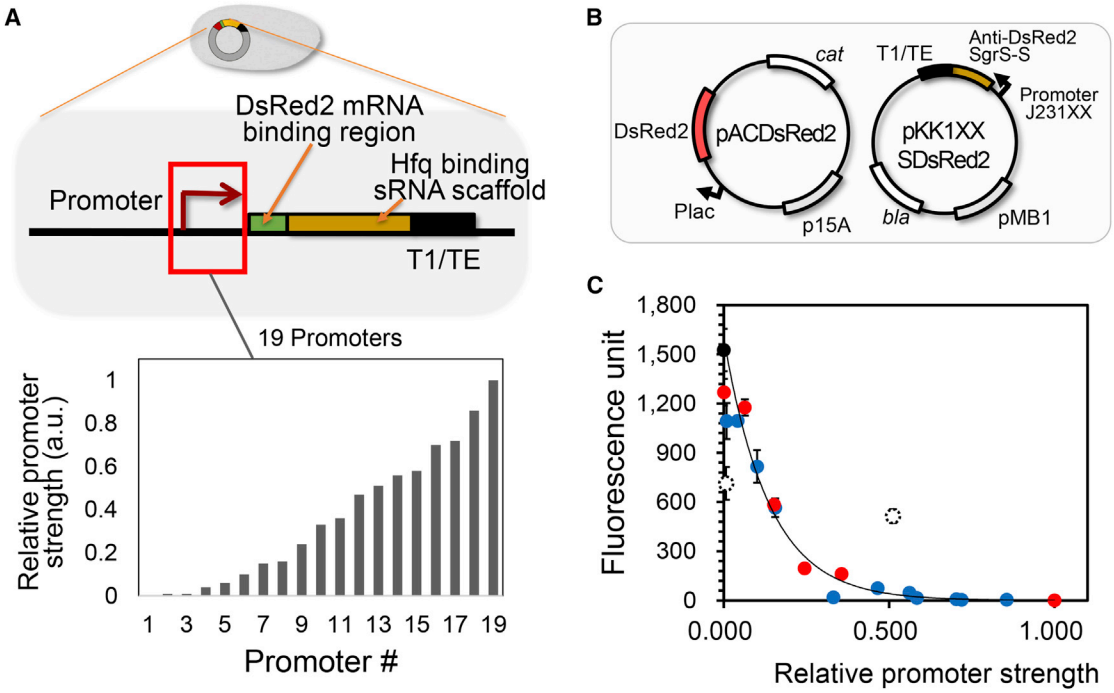


Figure 1. Quantitative Relationship between sRNA Expression Level and Gene Knockdown Efficiency

(A) Schematic showing the modulation of anti-DsRed2 sRNA expression level using different strength promoters. The promoter strengths were provided by the iGEM Registry website (http://parts.igem.org/Promoters/Catalog/Anderson).

(B) Plasmids used for evaluating knockdown efficiency of an anti-DsRed2 synthetic sRNA expressed under the control of different strength promoters.

(C) Quantitative relationship between the strengths of promoters expressing anti-DsRed2 sRNAs and red fluorescence intensities of each strain. Expression levels of sRNA were diversified using promoters from the Anderson promoter collection (blue dots, red dots, and dotted circles). Red dots indicate the promoters used in the following experiments for the production of putrescine and L-proline: J23112, J23117, J23115, J23105, J23107, and J23100 (in sequence from left to right). The spots produced using J23103 and J23108 promoters are represented by dotted circles. Control strain expressing DsRed2 without anti-DsRed2 sRNA is represented by the black dot. Mean values with SD from triplicate experiments are shown.

engineered *E. coli* strain capable of efficiently producing putrescine was attempted. Putrescine is a monomer of engineering plastics including nylon-4,6, which has high mechanical strength and strong resistance to solvents and heat. As a base strain, the previously constructed *E. coli* strain, XQ52 harboring p15SpeC (Qian et al., 2009), was employed. Plasmid p15SpeC harbors a gene encoding ornithine decarboxylase, which converts L-ornithine to putrescine. This strain is capable of producing 1.68 g of putrescine per liter in R/2 minimal medium containing 10 g/L glucose and 3 g/L ammonium sulfate (Qian et al., 2009).

To select target gene candidates for enhanced putrescine production, we predicted the effects of repressing central and putrescine biosynthetic pathways on putrescine production by using flux response analysis (Figure S1F; see STAR Methods). Based on this *in silico* genome-scale metabolic simulation, nine pathway reactions (*pfkA*, *pykF, aceF*, *poxB*, *sucA, aceA*, *glnA*, *proB*, and *gltX*) were selected as knockdown target genes for large-scale gene expression tuning (Figures 2B and S1F). In addition, six target genes were rationally selected (Figure 2B). Three genes (*pck*, phosphoenolpyruvate carboxykinase; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase) were selected to possibly increase the flux of the tricarboxylic acid (TCA) cycle. Based on the previous *in silico* simulation result showing that enolase expression level negatively correlates with L-glutamate (Nishio et al., 2013), the *eno* gene was chosen. Also, *pgi* (phosphoglucose isomerase) and *argF* (ornithine carbamoyltransferase chain F) were chosen to increase the availability of NADPH (Chemler et al., 2010) and L-ornithine, respectively.

To perform large-scale expression tuning of selected target gene candidates using synthetic sRNAs, a library of 75 synthetic sRNAs was constructed to knock down 15 target genes by applying five different promoters of varying strengths (J23100, J23107, J23105, J23115, and J23117) in combination (Figure 2A and Table S1; see Table S3 for a description of the strains; see STAR Methods). Plasmids constructed to generate each of the 75 synthetic sRNAs were individually transformed into the base putrescine producer strain. All the strains transformed with sRNA plasmids were cultured to evaluate putrescine production levels in flasks (Figure 2A). Six synthetic sRNAs increased putrescine titers by more than 10% (>1.85 g/L; Figure 2B, red arrows) compared with the base strain without any synthetic sRNA expression. Interestingly, all synthetic sRNA variants that led to an increase in putrescine production, including anti-*pgi*, -*glnA*, and -*argF* sRNAs, were under the control of a medium-strength promoter or even a weak promoter (anti-*argF* sRNA). When driven by a strong promoter, the expression of eight synthetic sRNAs (anti-*poxB*, -*eno*, -*ackA*, -*pck*, -*glnA*, -*sucA*, -*argF*, and -*gltX* sRNAs) reduced putrescine titer compared with the base strain. It is noteworthy that putrescine production was increased or decreased depending on the expression levels

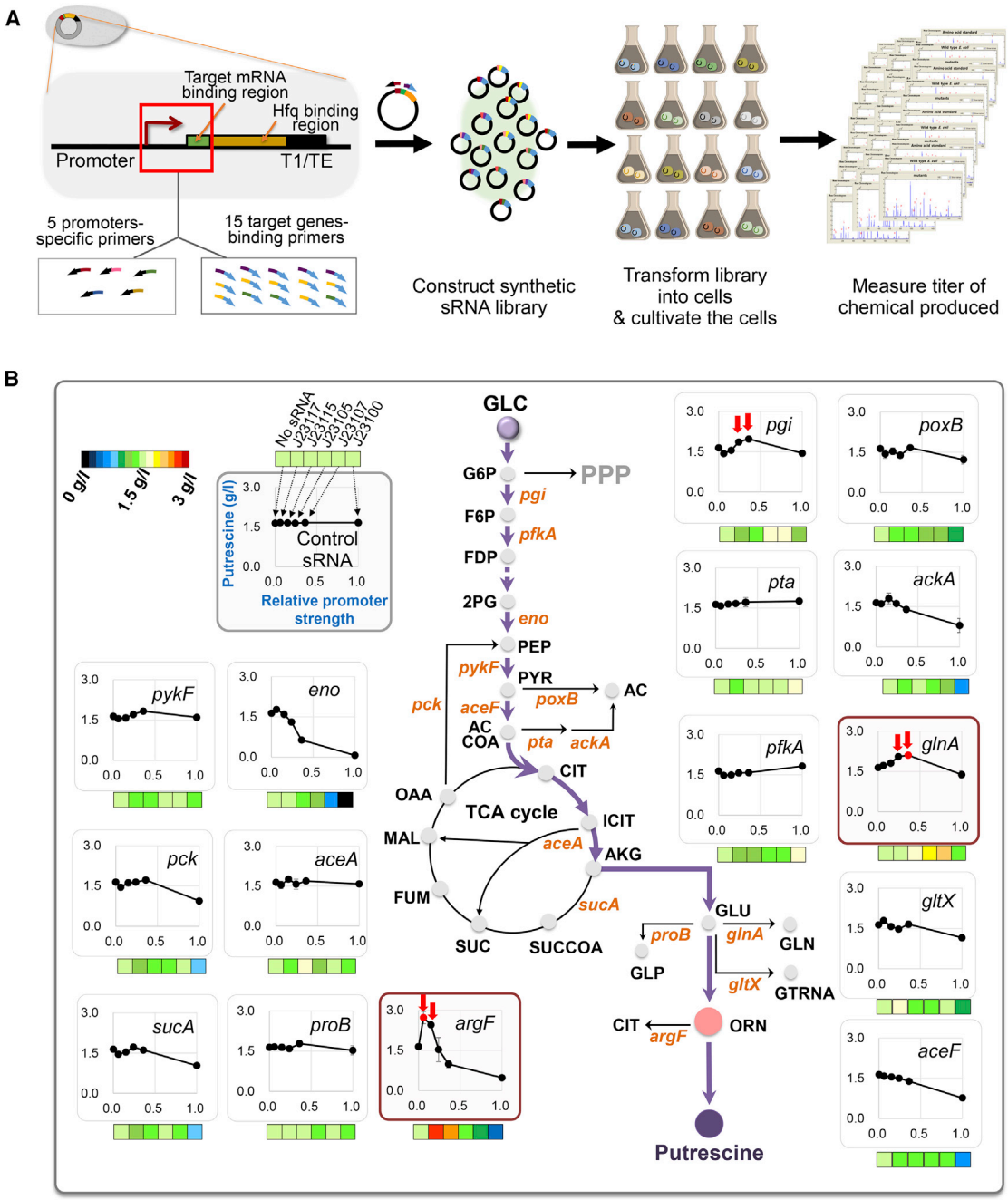


Figure 2. Synthetic sRNA-Based Knockdown Strategy for Enhanced Putrescine Production

(A) Library construction and the best producer screening by using different strength promoter-synthetic sRNA combinations. The library was constructed *via* inverse PCR using a promoter-specific primer and target mRNA-binding primer, and then each plasmid was transformed into base strain XQ52 harboring p15SpeC. All combinations of 5 promoter-specific primers and 15 target mRNA-binding primers were constructed to create a library of 75 synthetic sRNAs. The selected promoter strengths are shown in Figure 1C (red dots) and Table S1. Individual strains were separately cultivated in flasks, and their chemical producing capabilities were measured by liquid chromatography analysis.

(B) The putrescine biosynthesis pathway consists of glycolysis, partial TCA cycle, partial arginine production pathway, and single-step conversion from L-ornithine to putrescine. Candidates for screening were selected rationally or by *in silico* flux response analysis (see also Figure S1F). Putrescine titers obtained from individual strains are represented by colored boxes and by dots in the graph. Red arrows indicate more than 1.85g/L putrescine titer compared with the control strain. Red closed circles are the strongest putrescine producers from strains repressed two best knockdown targets, *argF* and *glnA*, respectively. Anti-DsRed2 sRNA was used as a control sRNA. Mean values with SD from triplicate experiments are shown. Abbreviations: 2PG, glycerate 2-phosphate; ACCOA, acetyl-CoA; AKG, α-ketoglutarate; CIT, citrate; CIT, L-citrulline.; F6P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; FUM, fumarate; G6P, glucose 6-phosphate; GLC, glucose; GLN, L-glutamine; GLP, L-glutamyl 5-phosphate; GLU, L-glutamate; GTRNA, L-glutamyl tRNA; ICIT, isocitrate; MAL, malate; OAA, oxaloacetate; ORN, L-ornithine; PEP, phosphoenolpyruvate; PYR, pyruvate; SUC, succinate; SUCCOA, succinyl-CoA.

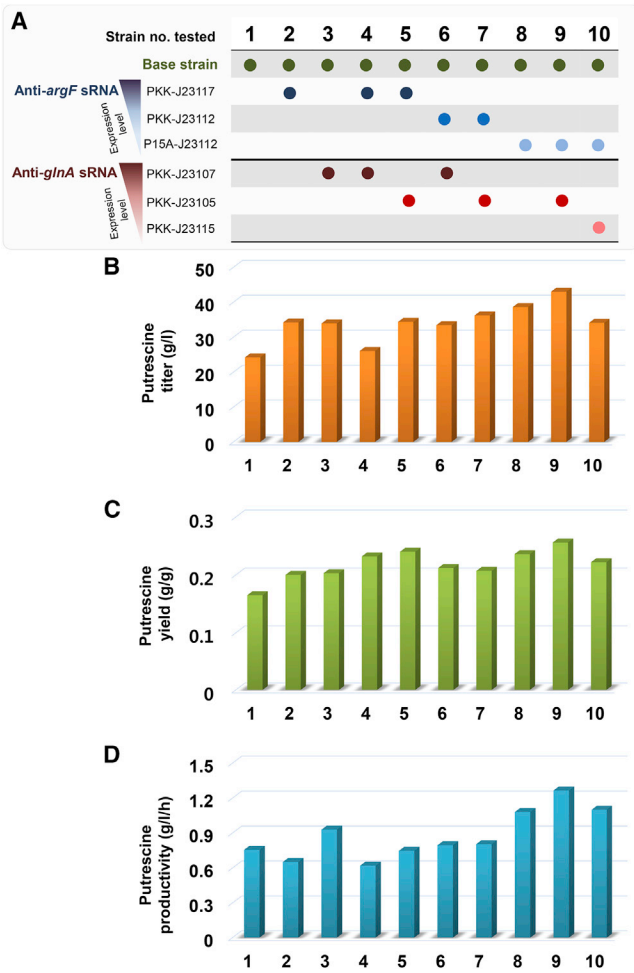


Figure 3. Fed-Batch Cultures of Ten Putrescine Producer Strains Expressing sRNAs to Different Levels

(A) Strains used in fed-batch cultures. From the two best putrescine producers isolated from tuned knockdown screening, strains were constructed by combining and modulating expression of sRNAs to obtain synergistic improvement of putrescine production. PKK-promoter, sRNA cassette in pMB1 origin-based plasmid; p15A-promoter, sRNA cassette in p15A origin-based plasmid.

(B–D) Putrescine titers (B), glucose yields (C), and productivities (D) obtained for ten engineered strains. Numeric values are listed in Table S2. For the best-performing strain (no. 9), duplicate fed-batch cultures were performed and fermentation results were reproducible (no. 11). See also Figure S3.

of an identical synthetic sRNA, as observed with the anti-*glnA* or anti-*argF* sRNAs. Knocking down *argF* and *glnA* using sRNAs under a strong promoter significantly retarded cell growth, causing decreased putrescine production (Figure S2A).

The recombinant *E. coli* strain with *argF* knockdown using the anti-*argF* sRNA under J23117 promoter produced putrescine to the highest titer of 2.71 g/L, which is 61.3% higher than that obtained without sRNA-based gene repression. Since J23117 is the weakest of the five promoters used here, the use of anti-*argF* sRNA under J23112 promoter, the weakest promoter from Anderson promoters (Table S1), was further examined to see if putrescine production can be further increased. The recombinant strain with *argF* knockdown using anti-*argF* sRNA under J23112 promoter produced 2.52 g/L of putrescine, which is lower than the best strain (Figure S2B). Thus, the anti-*argF* sRNA expression level obtained under the J23117 promoter was found to be optimal for enhanced putrescine production. Knocking down the *glnA* gene using anti-*glnA* sRNA under a medium-strength J23107 promoter (Table S1) also enhanced putrescine production to 2.10 g/L, which is 25.0% higher than that obtained without sRNA expression.

To confirm the repression of target genes by synthetic sRNAs, six genes that showed increase (n = 2), decrease (n = 2), or no change (n = 2) in putrescine production were selected from 15 knockdown targets. Six genes were 10× His-tagged at the C termini (Figure S2C), and the expression levels of the respective proteins encoded by these genes were examined using western blot analysis (see STAR Methods). As the stronger promoter was employed for synthetic sRNA expression, the repression of target gene expression became greater (Figure S2D). These results suggest that the fine-tuned knockdown system described here can be used to identify engineering target genes and to determine their gene expression levels, yielding the highest production of a desired chemical.

Combinatorial Gene Knockdown for Increased Putrescine Production in Fed-Batch Culture

Since bio-based production of chemicals is often performed in fed-batch mode, we performed fed-batch cultures of the best producer strains expressing anti-*argF* under J23117 (Figure 3, strain 2, and Figure S3A) and anti-*glnA* under J23107 (Figure 3, strain 3, and Figure S3B). These two strains produced 34.2 and 33.9 g/L putrescine, respectively, corresponding to 41% and 40% higher values than that (24.2 g/L; Figure 3, strain 1) obtained with the control strain, XQ52 harboring p15SpeC (Qian et al., 2009).

Since these two knockdown strains showed increased putrescine production, we next performed fed-batch culture of the strain expressing both sRNA combinations, anti-*argF* sRNA under J23117 and anti-*glnA* sRNA under J23107. We located two sRNA expression cassettes about 0.7-kb apart in the plasmid to avoid the homologous recombination problem caused by repetitive sRNA expression cassettes. Unexpectedly, this strain showed severely impaired cell growth (final OD_{600} = 20.4) and putrescine production; the putrescine titer decreased to 26.0 g/L (Figure 3, strain 4, and Figure S3C). This result suggests that simultaneous repression of *argF* and *glnA* is not beneficial but rather stressful to cells. Indeed, *argF* became an essential gene in the putrescine-producing base strain in which another gene with the same function, *argI*, was already disrupted; thus, L-arginine cannot be produced without *argF* expression (Qian et al., 2009). The *glnA* is also essential for the L-glutamine biosynthetic pathway and cell growth in minimal media (Patrick et al., 2007). Furthermore, biosynthesis pathways leading to the production of L-arginine, L-glutamine, and putrescine share L-glutamate as a common precursor. Thus, it seems that a delicate balance among these three pathways is required to obtain a synergistic increase in putrescine production through combined repression of *argF* and *glnA*.

To determine whether weaker knockdown of *argF* and *glnA* would increase putrescine titer in fed-batch culture without causing cell growth defects, we cloned dual-knockdown sRNA

variants combinatorially expressing anti-*argF* sRNA under J23117 or J23112, and anti-*glnA* sRNA under J23107 or J23105. The relative strengths of the promoters are: J23117, 0.064; J23112, 3.93×10^{-4}; J23107, 0.356; J23105, 0.245 (Table S1). When anti-*glnA* sRNA was expressed under the control of the weaker J23105 promoter while maintaining the expression of anti-*argF* sRNA (anti-*argF* sRNA under J23117 and anti-*glnA* sRNA under J23105), putrescine production was recovered (34.4 g/L), but cell growth was still low (final OD_{600} = 30.3; Figure 3, strain 5, and Figure S3D). On the other hand, when anti-*argF* sRNA was expressed under the control of the weaker J23112 promoter while maintaining the expression of anti-*glnA* sRNA (anti-*argF* sRNA under J23112 and anti-*glnA* sRNA under J23107), both putrescine production (33.4 g/L) and cell growth (final OD_{600} = 38.1) were recovered (Figure 3, strain 6, and Figure S3E). Furthermore, when the expression of sRNAs for both target genes was weakened (anti-*argF* sRNA under J23112 and anti-*glnA* sRNA under J23105), putrescine production was further increased (36.2 g/L; Figure 3, strain 7, and Figure S3F). Based on these results, we further reduced the expression level of sRNAs. Since the J23112 promoter is the weakest among the Anderson collection, the expression level of anti-*argF* sRNA was further reduced by lowering sRNA dosage through transferring the sRNA-expressing cassette from plasmid pKKKS (15–20 copies per cell) to plasmid p15SpeC (10–12 copies per cell). By doing so, the putrescine titer, yield of glucose, and productivity were all enhanced (Figure 3, strain 8, and Figure S3G). Combining this lowest anti-*argF* sRNA-expressing construct with anti-*glnA* sRNA under J23105 yielded a strain that showed a dramatic increase in titer, yield, and productivity (Figure 3, strain 9, and Figure S3H); the resulting putrescine titer was 43.0 g/L, which is 77.7% higher than that obtained with the control strain. The yield of glucose and productivity of putrescine were 0.256 g/g and 1.265 g/L/hr, respectively, which are the highest values obtained. Further reducing the expression level of anti-*glnA* sRNA in this best producer strain using the J23115 promoter reduced putrescine production (Figure 3, strain 10, and Figure S3I). The fed-batch fermentation of the best-performing strain (Figure 3, strain 9) was independently performed to check the reproducibility (no. 11 in Table S2); the average putrescine titer obtained was 42.3 ± 1.0 g/L. The putrescine production performances of all engineered strains are summarized in Table S2.

These results together suggest that the fine-tuned knockdown system based on modulation of synthetic sRNA expression level can be a powerful tool for developing producer strains through simultaneous modulation of multiple gene expression.

Application of Fine-Tuned Gene Expression to Develop an L-Proline Overproducing *E. coli* Strain

As another example of developing a producer strain by sRNA knockdown modulation, development of L-proline overproducing strain was attempted next. First, an L-proline-producing base strain (NMH26, Table S3) was constructed by additional engineering of the putrescine-producing strain XQ39 (Table S3; Figure 4A). The L-proline utilization pathway was removed by deleting the *putA* gene that encodes L-proline dehydrogenase. Also, the L-proline importers (*proP* and *putP*) were deleted. The branched pathway in which L-ornithine is converted to putrescine was removed by deleting *speC* and *speF* genes (ornithine decarboxylase and its isozyme). Then, an alternative pathway using *Pseudomonas putida* ornithine cyclodeaminase (OCD; Jensen and Wendisch, 2013), converting L-ornithine to L-proline, was introduced; this is different from the *E. coli* L-proline biosynthetic pathway producing L-proline from glutamate involving *proB*, *proA*, and *proC* (Csonka and Leisinger, 2007).

In the newly constructed base strain, the L-proline biosynthetic pathway shares L-ornithine as a precursor with the putrescine biosynthetic pathway. Thus, it was expected that anti-*argF* and anti-*glnA* sRNAs would also enhance the production of L-proline. Each of the plasmids harboring anti-*argF* and *glnA* synthetic sRNAs was transformed into the L-proline base strain. Expression of these sRNAs led to significant increases in L-proline titer, showing 229.6% (0.89 g/L) and 174.1% (0.74 g/L) increases in strains expressing anti-*argF* under J23100 and anti-*glnA* sRNA under J23100, respectively, compared with that (0.27 g/L) obtained with the base strain (Figure 4B). Differently from putrescine, however, the increase in L-proline production was maximal when the expression of sRNAs was performed using the strongest J23100 promoter. To examine whether stronger sRNA expression further increases L-proline production, we additionally constructed anti-*argF* and anti-*glnA* synthetic sRNAs under the trc and P_R promoters (relative strengths of J23100, trc, and P_R promoters are displayed in Figure S4A; see STAR Methods). As a result, the maximum production of L-proline could be achieved in both strains harboring anti-*argF* sRNA under the trc promoter and anti-*glnA* sRNA under the P_R promoter; both strains coincidently produced the same concentration (1.14 g/L) of L-proline, which is 322% higher than that obtained with the base strain (Figure 4B).

Expecting synergistic enhancement by dual repression of *argF* and *glnA*, the effects of varying fluxes of ornithine carbamoyltransferase (OCBT) and glutamine synthetase (GLNS) on the rate of L-proline production were examined by using *in silico* response analysis. It was predicted that L-proline production is the highest when the fluxes of both reactions are set to zero, because the pathways of L-arginine and L-glutamine synthesis are competitive against the putrescine biosynthesis pathway (Figure S4B). However, the activities of both reactions in this engineered strain are essential for cell growth as mentioned above. Since cell growth is important for achieving an overall high performance, the cell growth rate and the L-proline production rate were combined to give an objective function with the same weight during *in silico* simulation to examine the overall L-proline production efficiency (Figure S4C). The simulation results suggested that for the increased production rate, the activities of the GLNS and OCBT should be decreased, but not too much.

Based on the *in silico* simulation results, the expression of *glnA* and *argF* was knocked down with 25 synthetic sRNA-promoter combinations (5 × 5 combinations using five promoters: J23105, J23107, J23100, trc, and P_R; Figure 4C). Among 25 sRNA combinations tested, the one in which the expression of both sRNAs was driven by the trc promoter produced L-proline to the highest titer (2.02 g/L), while the other strains produced L-proline to 0.44–1.96 g/L (Figure 4C). This dramatically improved L-proline titer corresponds to more than 600% increase compared with that obtained with the base strain

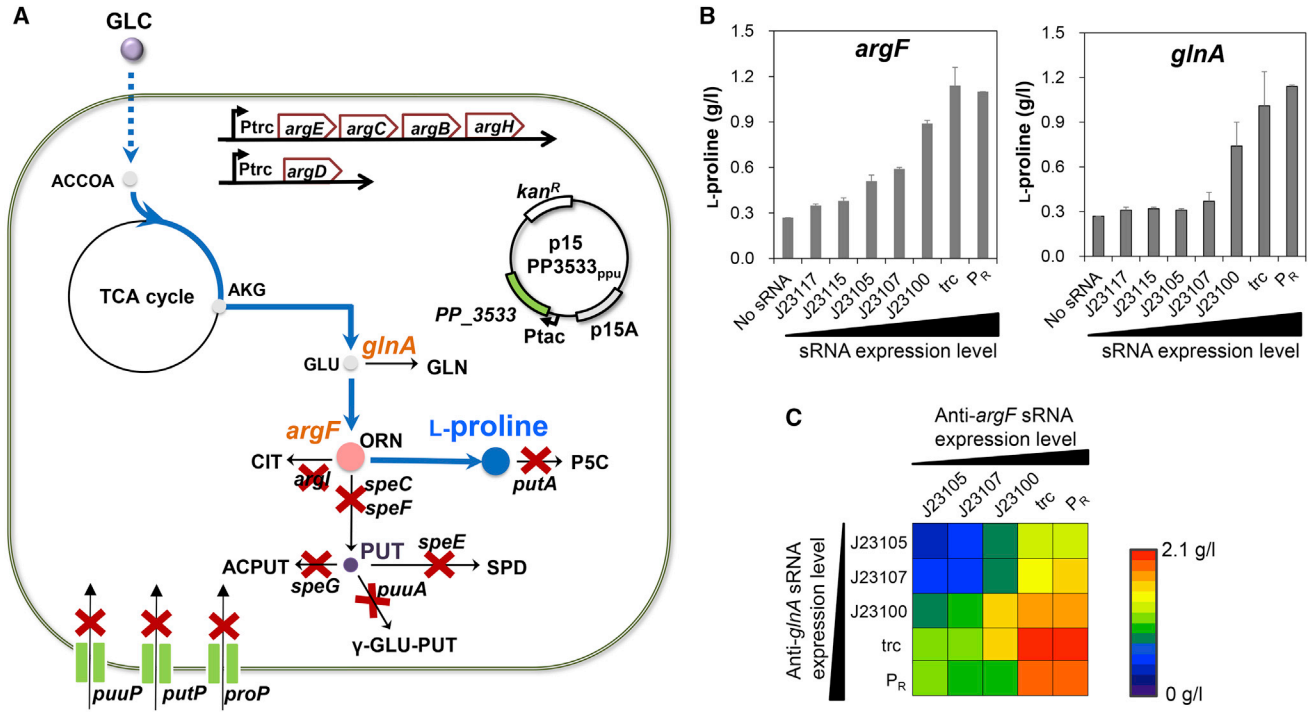


Figure 4. Synthetic sRNA-Based Knockdown Strategy for Enhanced L-Proline Production

(A) Metabolic pathways associated with L-proline biosynthesis in *E. coli*, and metabolic engineering approaches applied to overproduce L-proline. Abbreviations: ACCOA, acetyl-CoA; ACPUT, N-acetyl-putrescine; AKG, α-ketoglutarate; CIT, L-citrulline; GLC, glucose; GLN, L-glutamine; GLU, L-glutamate; ORN, L-ornithine; P5C, 1-pyrroline-5-carboxylate; PUT, putrescine; SPD, spermidine; γ-GLU-PUT, γ-glutamyl-L-putrescine.

(B) Production of L-proline by introduction of anti-*argF* and anti-*glnA* synthetic sRNAs with different strength promoters into the base strain (NMH26 p15PP3533). Mean values with SD from duplicate experiments are shown. See also Figure S4A and Table S1 for strengths of promoters.

(C) Effects of combinatorial synthetic sRNA knockdown on L-proline production. See also Figure S4C for the result of *in silico* metabolic response analysis and Figure S4D for the results of fed-batch culture.

(NMH26 harboring p15PP3533). It should be noted that the use of the trc promoter, which is strong enough, but not as strong as the P_R promoter, was best as predicted from *in silico* simulation when the objective function considering both cell growth and L-proline production was used. Fed-batch culture with the best producer strain resulted in production of 32.7 g/L L-proline (Figure S4D), which is the highest reported titer of L-proline in *E. coli.* When the strain with the strongest repression of both genes (anti-*glnA* under P_R promoter and anti-*argF* under P_R promoter) was cultured under the same fed-batch condition, 16.6 g/L L-proline was produced with reduced cell growth (Figure S4D). The fed-batch fermentation of the best-performing strain was independently performed to check the reproducibility (Figure S4E); the average L-proline titer obtained was 33.8 ± 1.6 g/L. This result demonstrates that *in silico* simulation-based forward design of sRNA knockdown expression control is possible.

DISCUSSION

Fine-tuning of gene expression levels is essential for constructing optimal cell factories and genetic circuits. In metabolic engineering, modulation of chromosomal gene expression is often required to construct a strain for the efficient production of desired products. However, manipulation of chromosomal gene expression often requires much time and effort, despite the recent development of various tools including the CRISPR-Cas9 system. Thus, the main challenge in this field is developing a facile approach for finely controlling target gene expression. To address these challenges, we developed a complementary method of a fine-tuned knockdown system based on the modulation of synthetic sRNA expression levels.

In developing the putrescine producer strain, the repression rate of anti-*aceA* sRNA was less than 50%, differently from knocking down other genes (Figure S2D). This relatively low repression seems to be due to the presence of *aceA* after *aceB* in an operon, causing less efficient sRNA binding to the targeted *aceA* gene; the secondary structure of *aceB-aceA* might have affected the binding of sRNA differently. In case of L-proline production, the same sRNAs (anti-*argF* and anti-*glnA*) were employed as in the putrescine producer, but the levels of sRNA-based knockdown were quite different; e.g., relatively low expression of sRNAs for putrescine overproduction and relatively high expression of sRNAs for L-proline production. This is notable because two products largely share the same metabolic pathways up to ornithine formation. One major difference between the putrescine- and L-proline-producing base strains is the absence (in the putrescine producer strain) or the presence (in L-proline producer strain) of the *rpoS* gene, encoding the stress-responsive RNA polymerase sigma factor, RpoS. Since

RpoS is an important global transcription regulator controlling about 10% of *E. coli* genes, the metabolic engineering strategies for their efficient production can vary. This is another important point that, regardless of genetic background, strains can be engineered to overproduce desired bioproducts through sRNA-based knockdown modulation.

The use of inducible promoter to control the expression levels of sRNAs allows precisely timed repression of target genes. However, the use of an inducer is generally discouraged as induced expression can complicate the bioprocess and may result in two subpopulations, fully induced and uninduced cells (Khlebnikov and Keasling, 2002), making it difficult to finely control the metabolic fluxes. For research purposes, the inducible promoters giving expression levels proportional to the concentrations of the inducer can extend the use of sRNA for time-controlled and tunable gene repression.

The physical interactions between synthetic sRNAs and Hfq are important for the gene repression function of synthetic sRNAs. Thus, overexpression of synthetic sRNAs results in reduction of available Hfq proteins, which can consequently perturb the inherent sRNA gene regulation system. Our fine-tuned gene repression system has an advantage as sRNA can be expressed to the right amount, which results in overproduction of desired product without perturbing the sRNA gene regulation system much. Although not observed in this study, Hfq can be overexpressed if one encounters perturbed cell physiology caused by sRNA overexpression and consequent Hfq depletion.

In summary, a fine-tuned knockdown system based on modulation of synthetic sRNA expression levels enabled identification and fine-tuned combinatorial knockdown of target genes. As demonstrated for two products, this strategy will be useful for developing strains for the efficient production of chemicals, fuels, and materials.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Strains, Antibiotics, and Culture Conditions
- METHOD DETAILS
  - Plasmid Construction
  - Deletion and Tagging of Chromosomal Genes
  - Knockdown Activity Measurement
  - Preparation of Cell Extract and Western-Blot Analysis
  - *In Silico* Analysis
  - Analytical Methods
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY
  - Software

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, four tables, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.cels.2017.08.016.

AUTHOR CONTRIBUTIONS

S.Y.L. and M.N. designed the research. M.N. performed experiments. W.J.K. performed the *in silico* metabolic simulation. M.N., S.M.Y., and W.J.K. analyzed the data and generated the figures. M.N., S.M.Y., and S.Y.L. wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by grants from the Technology Development Program to Solve Climate Change on Systems Metabolic Engineering for Biorefineries from the Ministry of Science and ICT (MSIT) through the National Research Foundation (NRF) of Korea (NRF-2012M1A2A2026556 and NRF-2012M1A2A2026557) and the Intelligent Synthetic Biology Center through the Global Frontier Project (2011-0031963) of MSIT through the NRF of Korea. This work was further supported by Hanwha Chemical through the KAIST-Hanwha Chemical Future Technology Institute. S.Y.L., S.M.Y., and M.N. declare that they have conflict of interest as this technology has been filed as a patent (Method for minutely regulating gene expression using synthesis-regulating sRNA; no. WO2015190627) and is of commercial interest.

Received: May 21, 2017
Revised: July 30, 2017
Accepted: August 24, 2017
Published: September 27, 2017

REFERENCES

Alper, H., Fischer, C., Nevoigt, E., and Stephanopoulos, G. (2005). Tuning genetic control through promoter engineering. Proc. Natl. Acad. Sci. USA *102*, 12678–12683.

Cameron, D.E., and Collins, J.J. (2014). Tunable protein degradation in bacteria. Nat. Biotechnol. *32*, 1276–1281.

Charusanti, P., Conrad, T.M., Knight, E.M., Venkataraman, K., Fong, N.L., Xie, B., Gao, Y., and Palsson, B.Ø. (2010). Genetic basis of growth adaptation of *Escherichia coli* after deletion of *pgi*, a major metabolic gene. PLoS Genet. *6*, e1001186.

Chemler, J.A., Fowler, Z.L., McHugh, K.P., and Koffas, M.A. (2010). Improving NADPH availability for natural product biosynthesis in *Escherichia coli* by metabolic engineering. Metab. Eng. *12*, 96–104.

Csonka, L.N., and Leisinger, T. (2007). Biosynthesis of proline. EcoSal Plus *2*, http://dx.doi.org/10.1128/ecosal 3.6.1.4.

Ebrahim, A., Lerman, J.A., Palsson, B.Ø., and Hyduke, D.R. (2013). COBRApy: COnstraints-based reconstruction and analysis for python. BMC Syst. Biol. *7*, 74.

Fabiani, A., Versari, A., Parpinello, G.P., Castellari, M., and Galassi, S. (2002). High-performance liquid chromatographic analysis of free amino acids in fruit juices using derivatization with 9-fluorenylmethyl-chloroformate. J. Chromatogr. Sci. *40*, 14–18.

Ferreira, J.P., Overton, K.W., and Wang, C.L. (2013). Tuning gene expression with synthetic upstream open reading frames. Proc. Natl. Acad. Sci. USA *110*, 11284–11289.

Hädicke, O., and Klamt, S. (2010). CASOP: a computational approach for strain optimization aiming at high productivity. J. Biotechnol. *147*, 88–101.

Ishikawa, H., Otaka, H., Maki, K., Morita, T., and Aiba, H. (2012). The functional Hfq-binding module of bacterial sRNAs consists of a double or single hairpin preceded by a U-rich sequence and followed by a 3′ poly (U) tail. RNA *18*, 1062–1074.

Jensen, J.V.K., and Wendisch, V.F. (2013). Ornithine cyclodeaminase-based proline production by *Corynebacterium glutamicum*. Microb. Cell Fact. *12*, 63.

Khlebnikov, A., and Keasling, J.D. (2002). Effect of *lacY* expression on homogeneity of induction from the P_{tac} and P_{trc} promoters by natural and synthetic Inducers. Biotechnol. Prog. *18*, 672–674.

Kim, J.M., Lee, K.H., and Lee, S.Y. (2008). Development of a markerless gene knock-out system for *Mannheimia succiniciproducens* using a temperature-sensitive plasmid. FEMS Microbiol. Lett. *278*, 78–85.

Lee, K.H., Park, J.H., Kim, T.Y., Kim, H.U., and Lee, S.Y. (2007). Systems metabolic engineering of *Escherichia coli* for L-threonine production. Mol. Syst. Biol. *3*, 149.

Lee, S.Y. (1996). High cell-density culture of *Escherichia coli*. Trends Biotechnol. *14*, 98–105.

Lee, S.Y., and Chang, H.N. (1993). High cell density cultivation of *Escherichia coli* W using sucrose as a carbon source. Biotechnol. Lett. *15*, 971–974.

Levine, E., Zhang, Z., Kuhlman, T., and Hwa, T. (2007). Quantitative characteristics of gene regulation by small RNA. PLoS Biol. *5*, e229.

Lorenz, R., Bernhart, S.H., Höner Zu Siederdissen, C., Tafer, H., Flamm, C., Stadler, P.F., and Hofacker, I.L. (2011). ViennaRNA package 2.0. Algorithms Mol. Biol. *6*, 26.

Lv, L., Ren, Y.L., Chen, J.C., Wu, Q., and Chen, G.Q. (2015). Application of CRISPRi for prokaryotic metabolic engineering involving multiple genes, a case study: controllable P (3HB-co-4HB) biosynthesis. Metab. Eng. *29*, 160–168.

Markham, N.R., and Zuker, M. (2008). UNAFold: software for nucleic acid folding and hybridization. Methods Mol. Biol. *453*, 3–31.

Martin, V.J., Pitera, D.J., Withers, S.T., Newman, J.D., and Keasling, J.D. (2003). Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat. Biotechnol. *21*, 796–802.

Moll, I., Afonyushkin, T., Vytvytska, O., Kaberdin, V.R., and Blasi, U. (2003). Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. RNA *9*, 1308–1314.

Na, D., Yoo, S.M., Chung, H., Park, H., Park, J.H., and Lee, S.Y. (2013). Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs. Nat. Biotechnol. *31*, 170–174.

Nishio, Y., Ogishima, S., Ichikawa, M., Yamada, Y., Usuda, Y., Masuda, T., and Tanaka, H. (2013). Analysis of L-glutamic acid fermentation by using a dynamic metabolic simulation model of *Escherichia coli*. BMC Syst. Biol. *7*, 92.

Onal, A. (2007). A review: current analytical methods for the determination of biogenic amines in foods. Food Chem. *103*, 1475–1486.

Orth, J.D., Conrad, T.M., Na, J., Lerman, J.A., Nam, H., Feist, A.M., and Palsson, B.Ø. (2011). A comprehensive genome-scale reconstruction of *Escherichia coli* metabolism—2011. Mol. Syst. Biol. *7*, 535.

Patrick, W.M., Quandt, E.M., Swartzlander, D.B., and Matsumura, I. (2007). Multicopy suppression underpins metabolic evolvability. Mol. Biol. Evol. *24*, 2716–2722.

Pitera, D.J., Paddon, C.J., Newman, J.D., and Keasling, J.D. (2007). Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. Metab. Eng. *9*, 193–207.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., and Lim, W.A. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell *152*, 1173–1183.

Qian, Z.G., Xia, X.X., and Lee, S.Y. (2009). Metabolic engineering of *Escherichia coli* for the production of putrescine: a four carbon diamine. Biotechnol. Bioeng. *104*, 651–662.

Salis, H.M., Mirsky, E.A., and Voigt, C.A. (2009). Automated design of synthetic ribosome binding sites to control protein expression. Nat. Biotechnol. *27*, 946–950.

Song, C.W., and Lee, S.Y. (2013). Rapid one-step inactivation of single or multiple genes in *Escherichia coli*. Biotechnol. J. *8*, 776–784.

Sung, M., Yoo, S.M., Jun, R., Lee, J.E., Lee, S.Y., and Na, D. (2016). Optimization of phage λ promoter strength for synthetic small regulatory RNA-based metabolic engineering. Biotechnol. Bioproc. Eng. *4*, 483–490.

van Ooyen, J., Noack, S., Bott, M., Reth, A., and Eggeling, L. (2012). Improved L-lysine production with *Corynebacterium glutamicum* and systemic insight into citrate synthase flux and activity. Biotechnol. Bioeng. *109*, 2070–2081.

Vogel, J., and Wagner, E.G. (2007). Target identification of small noncoding RNAs in bacteria. Curr. Opin. Microbiol. *10*, 262–270.

Yang, Y., Lin, Y., Li, L., Linhardt, R.J., and Yan, Y. (2015). Regulating malonyl-CoA metabolism via synthetic antisense RNAs for enhanced biosynthesis of natural products. Metab. Eng. *29*, 217–226.

Yildirim, H.K., Uren, A., and Yucel, U. (2007). Evaluation of biogenic amines in organic and non-organic wines by HPLC OPA derivatization. Food Technol. Biotechnol. *45*, 62–68.

Yoo, S.M., Na, D., and Lee, S.Y. (2013). Design and use of synthetic regulatory small RNAs to control gene expression in *Escherichia coli*. Nat. Protoc. *8*, 1694–1707.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-6X His tag antibody (HRP)	Abcam	Cat# ab1187; RRID: AB_298652
Anti-groEL antibody [9A1/2]	Abcam	Cat# ab82592; RRID: AB_1658428
Bacterial and Virus Strains		
DH5α	Lab Stock	N/A
XQ52	Lab Stock	N/A
XQ39	Lab Stock	N/A
NMH26	This study	N/A
Chemicals, Peptides, and Recombinant Proteins		
Tryptone	BD Biosciences	Cat# 211705
Yeast extract	BD Biosciences	Cat# 212750
NaCl	Junsei	Cat# 19015S0350
Agar	Junsei	Cat# 24440S1201
Ampicillin	Bio Basic	Cat# AB0028
Kanamycin	Bio Basic	Cat# KB0286
Chloramphenicol	Bio Basic	Cat# CB0118
Glucose	Junsei	Cat# 64220-0650
Ammonium sulfate	Junsei	Cat# 83110S0350
Diammonium hydrogen phosphate	Junsei	Cat# 84115S0350
Citric acid	Sigma-Aldrich	Cat# 251275
Magnesium sulfate heptahydrate	Junsei	Cat# M2773
Hydrochloride	Junsei	Cat# 20010S0350
Iron(II) sulfate heptahydrate	Sigma-Aldrich	Cat# F8633
Zinc sulfate heptahydrate	Sigma-Aldrich	Cat# Z0251
Copper(II) sulfate pentahydrate	Sigma-Aldrich	Cat# C8027
Manganese sulfate pentahydrate	Junsei	Cat# 83590-0301
Sodium tetraborate decahydrate	Sigma-Aldrich	Cat# B3545
Calcium chloride dihydrate	Sigma-Aldrich	Cat# C5080
Ammonium molybdate tetrahydrate	Sigma-Aldrich	Cat# 09878
L-citrulline	Sigma-Aldrich	Cat# C7629
Cellytic B cell lysis reagent	Sigma-Aldrich	Cat# B7435
o-Phthaldialdehyde	Sigma-Aldrich	Cat# P0657
Sodium phosphate dibasic anhydrous	Sigma-Aldrich	Cat# S7907
Sodium tetraborate decahydrate	Sigma-Aldrich	Cat# S9876
Sodium azide	Sigma-Aldrich	Cat# S2002
9-fluorenylmethyl-chloroformate	Sigma-Aldrich	Cat# 23186
Methanol	Burdick & Jackson	Cat# AH230-4
Acetonitrile	Avantor	Cat# 9017-03
Oligonucleotides		
Primers used in this study are listed in Table S4.	This study	N/A
Recombinant DNA		
Plasmid used in this study are listed in Table S3.	This study	N/A
Software and Algorithms		
Python	Python Software Foundation	https://www.python.org/
Flux response analysis	Lee et al., 2007	In silico analysis.py
Entire Python Resources for *in silico* Flux Response Analysis	This paper	Data S1

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Sang Yup Lee (leesy@kaist.ac.kr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Strains, Antibiotics, and Culture Conditions

The *E. coli* DH5α strain was used for gene cloning and plasmid preparation. This strain is cultured in Luria-Bertani (LB; 1% tryptone, 0.5% yeast extract and 1% NaCl) broth or on LB plates (1.5%, w/v, agar) at 37°C in the presence of appropriate antibiotics. When required, antibiotics were added with following concentration: 50 μg/mL of ampicillin, 25 μg/mL of kanamycin, and 17.5 μg/mL of chloramphenicol.

For putrescine production, *E. coli* XQ52 p15SpeC was used as a base strain (Qian et al., 2009). XQ52 p15SpeC harboring pKKS derivatives was cultured in LB medium with appropriate antibiotics at 37°C until stationary phase. This culture was inoculated to 300 mL baffled flask containing 50 mL of fresh R/2 medium supplemented with 10 g/L of glucose and 3 g/L of $(NH_4)_2SO_4$. The R/2 medium (pH 6.8) contains 2 g/L of $(NH_4)_2HPO_4$, 6.75 g/L of KH_2PO_4, 0.85 g/L of citric acid, 0.7 g/L of $MgSO_4 \cdot 7H_2O$ and 5 mL/L of trance metal solution. The trace metal solution contains 5 M HCl, 10 g/L $FeSO_4 \cdot 7H_2O$, 2.25 g/L $ZnSO_4 \cdot 7H_2O$, 1 g/L $CuSO_4 \cdot 5H_2O$, 0.5 g/L $MnSO_4 \cdot 5H_2O$, 0.23 g/L $Na_2B_4O_7 \cdot 10H_2O$, 2 g/L $CaCl_2 \cdot 2H_2O$, and 0.1 g/L $(NH_4)_6Mo_7O_{24}$ (Lee and Chang, 1993). Flask cultures were carried out for 24 hours in a shaking incubator at 37°C and 200 rpm. The culture of L-proline producer strains, *E. coli* NMH26 p15PP3533 and its derivatives, were performed using the same method as the above. For fed-batch culture, flask culture (4 flasks, 200 mL, early stationary phase for putrescine and exponential phase for L-proline) was inoculated into a 6.6 L jar fermentor (Bioflo 3000; New Brunswick Scientific Co., Edison, NJ) containing 1.8 L of R/2 culture medium supplemented with 10 g/L of glucose and 3 g/L of $(NH_4)_2SO_4$. To maintain pH 6.8, 14% ammonia solution was automatically added. Dissolved oxygen concentration was kept at 40% of air saturation automatically by adjusting the agitation speed and supplying of pure oxygen gas. A nutrient feeding solution was added by using the pH-stat feeding strategy (Lee, 1996) when the pH rose greater than set point (pH 6.8) by 0.02 due to carbon source depletion. The feeding solution contained: 522 g/L of glucose, 170 g/L of $(NH_4)_2SO_4$, and 8 g/L of $MgSO_4 \cdot 7H_2O$ for putrescine production; 650 g/L of glucose, 85 g/L of $(NH_4)_2SO_4$, and 8 g/L of $MgSO_4 \cdot 7H_2O$ for production of L-proline. Samples were harvested for the measurements of OD_{600} and the product concentrations were determined using the culture supernatant after centrifugation at 14000 rpm for 5 minutes.

METHOD DETAILS

Plasmid Construction

Primers used in plasmids construction are listed in Table S4. Plasmid pKKKS was constructed as an sRNA expression vector. pKK223-3 (Pharmacia, Uppsala, Sweden) was digested by *Hind*III and *Nae*I, then sticky end of *Hind*III site was filled by Klenow fragment. Multiple cloning site of pBluescript II KS (Stratagene, La Jolla, CA) was amplified using primers, MCSF_PvuII and MCSR_NaeI, and digested by *Pvu*II and *Nae*I. pKK223-3 fragment and digested multiple cloning site were ligated to construct pKKKS. Construction of pACKS was performed same way using pACYC184 (New England Biolabs, Beverly, MA) but enzyme digestion was done by *Xba*I instead of *Hind*III. Cassettes expressing sRNA and DsRed2 were amplified from plasmids of previous study (Na et al., 2013) using sRNA_NotI, sRNA_BamHI, PlacDsRed2-ApaI, and PlacDsRed2-SalI primers and inserted into pKKKS or pACKS by enzyme digestion and ligation (pKKMDsRed2 and pACDsRed2, respectively). pKKSDsRed2, a plasmid expressing DsRed2 targeting SgrS-S sRNA, was constructed by site directed mutagenesis of pKKMDsRed2 using SgrS-S F and SgrS-S R primers. Following modifications of sRNA expression cassettes were performed based on pKKSDsRed2 plasmid using site directed mutagenesis method. pKKSO, mRNA binding region-excluded sRNA expression plasmid, was constructed using iPr and iSO primers. Plasmids expressing sRNA under the control of Anderson promoter collection (J23100–J23118) were constructed using J23100–J23118 F and R primer pairs. The target gene of sRNA was changed by site directed mutagenesis using promoter binding primers (iJ23100, iJ23105, iJ23107, iJ23112, iJ23115, and iJ23117), target binding region harboring primers (iaceA, iaceF, iackA, iargF, ieno, iglnA, igltX, ipck, ipfkA, ipgi, ipoxB, iproB, ipta, ipykF, and isucA) and anti-DsRed2 sRNA plasmid harboring each promoter as templates. Plasmids expressing sRNA under the control of trc promoter and P_R promoter were constructed in the same way using iPr-full, iPtrc-full, iargF, and iglnA. Plasmids harboring two sRNAs were constructed by Gibson assembly method using primer pairs, sRNA-2 sRNAF and R pair and sRNA-2 vectorF and R pair. Those primer pairs were used to amplify plasmid backbone harboring sRNA and an additional sRNA to be inserted into backbone DNA fragment. J23112-SargF sRNA expression cassette was amplified using primer pair, sRNA_BamHIF and sRNA_NaeIR, digested, and ligated to construct p15SpeC-S112SargF. *Pseudomonas putida* ornithine cyclodeaminase, PP3533, was amplified from *P. putida* gDNA using primer pair, PP3533F and PP3533R and inserted into pTac15K *Sac*I-*Xba*I site by using restriction enzyme digestion and ligation to construct p15PP3533. DsRed2 was amplified from pACDsRed2 using PrApaIF, PtrcApaIF, J23100ApaIF, and DsRed2XhoIR primers and inserted into pACKS plasmid by enzyme digestion and ligation to construct pACPRDsRed2, pACtrcDsRed2, and pAC00DsRed2. These three DsRed2 expressing plasmids were used to examine the relative strength of J23100, trc, and P_R promoters.

Deletion and Tagging of Chromosomal Genes

Putrescine producing XQ39 strain was employed as a start strain to construct an L-proline production strain. This strain was further engineered by disrupting *putAP*, *proP*, *speC*, and *speF*. Linear gene disruption fragments harboring selection marker and 100-bp long gene-specific homologous region were amplified *via* two sequential PCR reactions using pECmulox (Kim et al., 2008) as a template. Used primers are listed in Table S4. Amplified fragments were electroporated into *E. coli* strain harboring pCW611 helper plasmid (Song and Lee, 2013) using Gene Pulser II (Bio-Rad, Hercules, CA; 2.5 kV of voltage, 200 Ω of resistance, and 25 μF of capacitance; 1-mm electroporation gap cuvettes). The helper plasmid pCW611 enables DNA fragment incorporation into chromosome and marker excision by expressing λ-Red and Cre recombinase. The detailed gene disruption procedure is described previously (Song and Lee, 2013). The deletion of each gene was confirmed by using colony PCR.

The His tag was attached to chromosomal genes, *pgi*, *argF*, *glnA*, *pykF*, *aceA*, *gltX,* and *eno*, for expression level determination using western-blotting in the presence of different levels of sRNA expression. The His tag-attaching linear DNA fragments harboring selection marker and 100-bp long gene-specific homologous region were amplified *via* two sequential PCR reactions using plasmid pECmulox (Kim et al., 2008) as a template. The following procedures were similar to the deletion procedures of chromosomal genes. The sequences of His tag were confirmed by sequencing of colony PCR products.

Knockdown Activity Measurement

Knockdown activity of sRNA was measured by using DsRed2, as a reporter gene. Briefly, DH5α strains harboring pACDsRed2 and pKKS derivatives were cultured in LB medium with appropriate antibiotics at 37°C until stationary phase. This culture was inoculated to 24-well plate containing 1 mL of LB medium. After culture for 24 hours at 37°C in shaking incubator, cultured cells were diluted to be OD_{600}=1 and fluorescence intensities were measured by using a multi-detection microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA). The measured intensities were modified by subtracting the fluorescence emitted from cells without DsRed2. When required, these modified intensities were normalized to the fluorescence intensity measured from cells expressing DsRed2 without synthetic sRNA. The measurement of promoter strengths were performed in the similar way.

Preparation of Cell Extract and Western-Blot Analysis

XQ52 strains with a His-tagged gene (*pgi*, *glnA*, *argF*, *gltX*, *eno*, *aceA,* or *pykF*) and their derivatives harboring sRNA-expressing plasmids were inoculated into LB medium of test tube and cultured overnight at 37°C in shaking incubator. Saturated culture was inoculated into 300 mL baffled flask containing 50 mL of fresh R/2 medium supplemented with 10 g/L of glucose and 3 g/L of $(NH_4)_2SO_4$. Citrulline of 2 mM was supplemented to culture of XQ52 *argF*-His strain which showed growth defect in R/2 minimal medium. Cells were harvested when OD_{600} of culture reached over 0.5 and resuspended in CelLytic™ B Cell Lysis Reagent (Sigma–Aldrich, Dorset, UK). The cells were lysed by sonication, and lysates were centrifuged at 4°C for 10 min at 14000 rpm. Supernatants were mixed with 5x SDS sample buffer (300 mM Tris-HCl pH 8.0, 10% SDS, 25% β-mercaptoethanol, 50% glycerol, 20 mM EDTA, and 0.05% bromophenol blue), boiled, and followed by running SDS-PAGE and western-blot analysis using antibody against His tag (ab1187 used at 1/5,000 dilution, Abcam) and GroEL (9A1/2, ab82592 used at 1/5,000 dilution, Abcam). The expression levels of each protein were estimated by measuring intensity of immunoblot bands and normalized by band intensity of GroEL or the gel stained by Coomassie blue.

In Silico Analysis

In silico flux response analysis was performed to identify the reactions that negatively correlated with putrescine production using the genome-scale metabolic model of *E. coli* iJO1366 (Orth et al., 2011; Lee et al., 2007). As the fluxes of central and putrescine biosynthesis reactions were gradually increased from the minimum to maximum values, the putrescine production rate was maximized as an objective function. The reactions that negatively related with putrescine production rate were selected as knockdown candidates. Also, the effects of varying the fluxes of glutamine synthetase (GLNS) encoded by *glnA* and ornithine carbamoyltransferase (OCBT) encoded by *argF* on L-proline production and growth rate were further analyzed. Since both GLNS and OCBT are essential enzymes for the cell growth, we introduced the artificial reaction *Rv* that simultaneously accounts for both cell growth and L-proline production (Hädicke and Klamt, 2010):

$$Rv = \alpha \cdot \text{Growth rate} + (1- \alpha) \cdot \text{target chemical production rate}, \alpha \in [0, 1]$$

The parameter α assigns proportion of the growth rate and target chemical production rate as varied from 0 to 1. The artificial reaction *Rv* was maximized as an objective function while the fluxes of GLNS and OCBT increased from the minimum to the maximum values combinatorially. As the parameter α was varied, the simulation results showed similar patterns except for the parameter $\alpha = 0$ which considers only chemical production rate. Throughout the flux response analysis, glucose uptake rate was set to 10 mmol/gDCW/h as a constraint. All simulations were conducted in Python environment with Gurobi Optimizer 6.0 and GurobiPy package (Gurobi Optimization, Inc., Houston, TX). Reading, writing, and manipulation of the COBRA-compliant SBML files were implemented using COBRApy (Ebrahim et al., 2013).

Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) with an Ultrospec 3000 spectrophotometer (Amersham Biosciences, Uppsala, Sweden). Concentrations of putrescine and L-proline were measured by high performance liquid chromatography (1100 Series HPLC, Agilent Technologies, Palo Alto, CA). Putrescine samples were filtered by 0.22-μm PVDF syringe filters (FUTECS, Korea), derivatized with *o*-phthaldialdehyde (OPA) using the previously described method (Qian et al., 2009; Onal, 2007; Yildirim et al., 2007). An Eclipse XDB-C18 LC-Column (5 μm, 4.6 mm x 15 cm; Agilent Technologies, Palo Alto, CA) was used and operated at 25°C for HPLC. The mobile phase was run at a flow rate of 1.2 mL/min; the mobile phase was consisted of solvent A (1.4 g/L Na_2HPO_4, 3.8 g/L $Na_2B_4O_7 \cdot 10H_2O$, and 8 mg/L NaN_3, adjusted to pH 7.2 with HCl) and solvent B (45% acetonitrile, 45% methanol and 10% distilled water, in v/v). The following gradient was applied: 0–0.5 min, 0% solvent B; 0.5–18 min, a linear gradient of solvent B from 0% to 57%; 18–26 min, a linear gradient of solvent B from 57% to 100%; 26–34 min, 100% solvent B; 34–35 min, a linear gradient of solvent B from 100% to 0%; and 35-38 min, 0% solvent B. L-proline samples were analyzed by same methods with following modification. L-proline samples were derivatized with 9-fluorenylmethyl-chloroformate (FMOC-Cl) using the method reported previously (Fabiani et al., 2002) and 100% acetonitrile was used as solvent B.

QUANTIFICATION AND STATISTICAL ANALYSIS

Graphs describe mean values with standard deviations except for fed-batch culture data.

DATA AND SOFTWARE AVAILABILITY

Software

All simulations were conducted in Python environment with Gurobi Optimizer 6.0 and GurobiPy package (Gurobi Optimization, Inc., Houston, TX). Reading, writing, and manipulation of the COBRA-compliant SBML files were implemented using COBRApy (Ebrahim et al., 2013). Custom scripts are included in Data S1.