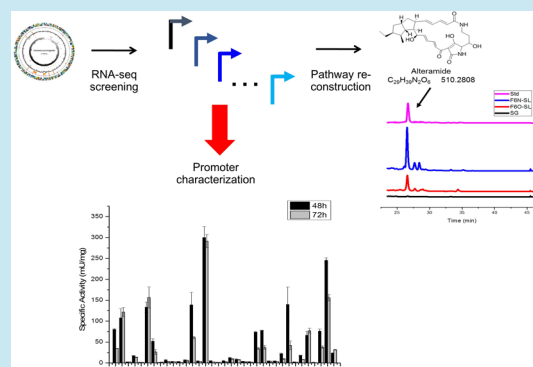


Systematic Identification of a Panel of Strong Constitutive Promoters from *Streptomyces albus*Yunzi Luo,^{†,‡} Lu Zhang,^{†,§,||} Katherine W. Barton,[⊥] and Huimin Zhao^{*,†,‡,⊥,||,#}[†]Department of Chemical and Biomolecular Engineering, [⊥]Department of Chemistry, [#]Departments of Biochemistry and Bioengineering, Center for Biophysics and Computational Biology, University of Illinois at Urbana–Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801, United States,[‡]Institute for Genomic Biology, University of Illinois at Urbana–Champaign, 1206 West Gregory Drive, Urbana, Illinois 61801, United States,[§]Key Laboratory of Systems Bioengineering (Ministry of Education), ^{||}SynBio Research Platform, Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, PR China

Supporting Information

ABSTRACT: Actinomycetes are important organisms for the biosynthesis of valuable natural products. However, only a limited number of well-characterized native constitutive promoters from actinomycetes are available for the construction and engineering of large biochemical pathways. Here, we report the discovery and characterization of 32 candidate promoters identified from *Streptomyces albus* J1074 by RNA-seq analysis. These 32 promoters were cloned and characterized using a streptomycete reporter gene, *xylE*, encoding catechol 2,3-dioxygenase. The strengths of the identified strong promoters varied from 200 to 1300% of the strength of the well-known *ermE**p in MYG medium, and the strongest of these promoters was by far the strongest actinomycete promoter ever reported in the literature. To further confirm the strengths of these promoters, qPCR was employed to determine the transcriptional levels of the *xylE* reporter. In total, 10 strong promoters were identified and four constitutive promoters were characterized via a time-course study. These promoters were used in a plug-and-play platform to activate a cryptic gene cluster from *Streptomyces griseus*, and successful activation of the target pathway was observed in three widely used *Streptomyces* strains. Therefore, these promoters should be highly useful in current synthetic biology platforms for activation and characterization of silent natural product biosynthetic pathways as well as the optimization of pathways for the synthesis of important natural products in actinomycetes.

KEYWORDS: *Streptomyces* promoters, actinomycetes, RNA-seq, *XylE* assay, qPCR, synthetic biology



Actinomycetes have been and continue to be excellent source organisms for the discovery and development of novel natural products that have found use as antibiotics, anticancer agents, anthelmintic agents, and insect control agents.^{1–3} Metabolic engineering and synthetic biology tools have been developed to engineer actinomycetes such as *Streptomyces coelicolor*, *Streptomyces lividans*, and *Streptomyces albus* for discovery and production of natural products.^{4–6} However, one of the key limitations in such studies is the lack of a panel of well-characterized strong promoters for precise control of gene expression. For example, only a limited number of promoters, such as the constitutive promoters *ermE**p⁷ and SF14p⁸ and the inducible promoters *tipAp*⁹ and *nitAp*,¹⁰ have been described and shown to be functional for expression of heterologous genes in streptomycetes. A constitutive promoter, *kasOp*, from *S. coelicolor* was engineered to generate a mutant that is stronger than *ermE**p and SF14p.¹¹ Recently, synthetic promoter libraries have been adopted as a means to obtain

useful promoters. A promoter library mimicking *Streptomyces* vegetative promoters with variable –10 consensus sequences was constructed, and a collection of promoters with varying strengths was obtained.¹² However, none of these promoters showed higher activity than that of *ermE**p. Similarly, a synthetic promoter library for actinomycetes based on the –10 and –35 consensus sequences of the *ermEp1* promoter was constructed (the *ermE* promoter region contains two different promoters, *ermEp1* and *ermEp2*; the *ermE**p promoter is a stronger variant containing the *ermEp2* and a TGG deletion in the –35 region of the *ermEp1* part),¹³ and promoters with strengths ranging from 2 to 319% compared with the *ermEp1* promoter were obtained. However, only two promoters showed larger than 2-fold enhancement relative to *ermEp1*, let alone

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Table 1. Selected 32 Promoter Regions from *S. albus* J1074

ID	name		CDS product	length of promoter (bp)	expression level
gene3583	XNR_3584	1	Cold shock domain-containing protein CspD	323	0.03%
gene3798	XNR_3799	2	Heat shock protein 60 family cochaperone GroES	695	0.12%
gene3710	XNR_3711	3	50S ribosomal protein L7/L12	111	0.13%
gene3722	XNR_3723	4	Elongation factor Tu 1	151	0.22%
gene3752	XNR_3753	5	50S ribosomal protein L36	64	0.25%
gene2392	XNR_2393	6	Heat shock protein 60 family chaperone GroEL	640	0.27%
gene3751	XNR_3752	7	Translation initiation factor IF-1 1	247	0.30%
gene2977	XNR_2978	8	30S ribosomal protein S6	303	0.35%
gene3709	XNR_3710	9	50S ribosomal protein L10	380	0.47%
gene3753	XNR_3754	10	30S ribosomal protein S13	221	0.51%
gene3742	XNR_3743	11	30S ribosomal protein S8	249	0.52%
gene3756	XNR_3757	12	50S ribosomal protein L17	386	0.61%
gene3727	XNR_3728	13	30S ribosomal protein S10	614	0.61%
gene3754	XNR_3755	14	30S ribosomal protein S11	65	0.66%
gene1699	XNR_1700	15	Peptide transport system secreted peptide-binding protein	517	0.67%
gene5229	XNR_5230	16	50S ribosomal protein L35	106	0.76%
gene3730	XNR_3731	S1	50S ribosomal protein L23	0	0.77%
gene2497	XNR_2498	17	Cold shock protein	591	0.79%
gene5230	XNR_5231	18	50S ribosomal protein L20	102	0.81%
gene3706	XNR_3707	19	50S ribosomal protein L11	190	0.83%
gene4882	XNR_4883	20	SSU ribosomal protein S 1p	284	0.83%
gene2188	XNR_2189	21	secreted protein	79	0.84%
gene3707	XNR_3708	22	50S ribosomal protein L1	100	0.86%
gene3728	XNR_3729	S2	50S ribosomal protein L3	14	0.86%
gene4374	XNR_4375	23	30S ribosomal protein S20	290	0.88%
gene3719	XNR_3720	24	30S ribosomal protein S12	589	0.89%
gene1216	XNR_1217	25	30S ribosomal protein S16	236	0.94%
gene4344	XNR_4345	S3	50S ribosomal protein L27	14	0.96%
gene3747	XNR_3748	S4	50S ribosomal protein L15	1	1.06%
gene1473	XNR_1474	26	50S ribosomal protein L31	318	1.11%
gene3015	XNR_3016	27	Membrane protein	326	1.13%
gene1130	XNR_1131	28	30S ribosomal protein S15	399	1.13%
gene2975	XNR_2976	29	30S ribosomal protein S18 2	66	1.15%
gene3744	XNR_3745	S5	50S ribosomal protein L18	3	1.16%
gene3586	XNR_3587	30	Hypothetical protein	206	1.16%
gene3743	XNR_3744	S6	50S ribosomal protein L6	21	1.20%
gene3169	XNR_3170	31	Chaperone protein DnaK	414	1.20%
gene3799	XNR_3800	32	Heat shock protein 60 family chaperone GroEL	157	1.28%
gene4918	XNR_4919	gapdhp	Glyceraldehyde-3-phosphate dehydrogenase	338	2.43%

*ermE**p. More recently, a plug-and-play strategy was developed to activate cryptic pathways in *S. lividans* by decoupling pathway expression from native sophisticated regulation cascades.^{4,5} In this method, well-characterized constitutive or inducible promoters from actinomycetes are used to drive the expression of individual genes, which turns on the entire gene cluster to activate synthesis of target compounds. A panel of 13 promoters from actinomycetes was characterized and used to refactor cryptic pathways. However, these promoters were mainly discovered and characterized under one growth condition and evaluated in one heterologous host, *S. lividans*. Moreover, for larger gene clusters containing more than 13 genes, additional strong constitutive promoters are needed.

To address these limitations, we sought to systematically discover a panel of constitutive promoters from *S. albus* J1074 using RNA-seq based transcriptional profiling and perform detailed biochemical characterization of these promoters. *S. albus* J1074 is a mutant strain from *S. albus* G defective in *Sall* restriction and modification.¹⁴ It has been successfully used to express a wide variety of natural product gene clusters, and in

some cases, it can lead to a better yield than *S. coelicolor* and *S. lividans*.^{15–20} In addition, nonstreptomycete actinomycete secondary metabolite gene clusters have been expressed in *S. albus* J1074.²¹ All of these advantages make this strain a promising heterologous host for secondary metabolite pathway expression; therefore, we sought to discover strong promoters that could work efficiently in this host.

In this work, 32 candidate promoters from *S. albus* J1074 were discovered based on RNA-seq data under two culturing conditions (MYG liquid medium and R2YE liquid medium). These 32 candidate promoters were subsequently cloned upstream of the *Pseudomonas* reporter gene *xylE*, encoding catechol 2, 3-dioxygenase. Ten out of the 32 promoters showed stronger activities than those of control promoters (the *ermE** promoter and our previously characterized strong promoters⁵) in *S. albus*, and four might be constitutively active. These promoters should facilitate metabolic engineering and, particularly, our recently developed plug-and-play system for natural product discovery and production in actinomycetes.

RESULTS AND DISCUSSION

Screening of Strong Constitutive Promoters in the *S. albus* Transcriptome via RNA-seq. The expression levels of all 5937 genes were sorted based on two target culturing conditions and two defined time points. The number of highly expressed genes was counted with different cutoffs (Figure S4). For each sample, the genes were ranked from the most highly expressed to the least highly expressed from their RPKM values. On the basis of all four criteria (two time points under two culturing conditions), there are 20 genes in the 1% cutoff, which means that 20 genes are in the top 1% of the most highly expressed genes based on RPKM values under all four conditions. Among these 20 genes, there are two genes with very short promoter regions or no promoter region (0 and 14 bp), which makes the corresponding promoters difficult to clone. Therefore, to satisfy all four conditions, there are only 18 genes to choose in the 1% cutoff. If we consider the top 1% of highly expressed genes under either the MYG or R2YE condition at two time points, then there are 28 genes in total (Figure S4). Among all 28 highly expressed genes, only 25 have promoter regions long enough for cloning. Therefore, to ensure successful identification of as many strong promoters as possible, the selection of promoters was expanded to the top 1.3% of the most highly expressed genes, which led to a total of 32 promoters (Table 1).

qPCR Analysis of the Selected Promoters in the Native Host. To assess the activity of the 32 selected promoters, qPCR primers were designed to amplify ~100–200 bp fragments for all of the 32 highly expressed genes plus *gapdh*, which is a previously identified gene that is typically highly expressed among most actinomycetes. Meanwhile, the ones that were highly expressed in the RNA-seq data but with no or short promoter regions (named S1–S6) were selected for qPCR analysis, too. Standard curves were made for these 39 pairs of primers using genomic DNA of *S. albus* as the template. The *rpoD* gene was chosen as the internal control, and its standard curve was made, as shown in Figure S5. qPCR was performed as described in the Methods.

Cells were grown for 72 h, and samples for RNA isolation were collected at three different time points: 24, 48, and 72 h. Total RNA was isolated, and cDNA was synthesized afterward. To check for genomic DNA carry-over, the cDNA synthesis reaction without reverse transcriptase was also carried out to serve as a negative control. As shown in Figure S6, generally, all 32 selected genes plus *gapdh* have expression levels higher than that of *rpoD* (which is set as 1). Therefore, we considered them as containing strong promoters and chose them all for cloning in the following experiments.

Cloning of the Constitutive Promoters. The promoter regions of the 32 highly expressed genes were PCR-amplified from the genomic DNA of *S. albus*, spliced with the amplified *xylE* gene, and inserted into a yeast–*Escherichia coli*–*Streptomyces* shuttle vector using the DNA assembler method (Figure S7). Each of the promoter regions that we selected to clone is the intergenic region between the highly expressed gene and its upstream gene. The assembled constructs were purified from *E. coli* and sequenced to confirm the identity of the cloned promoter regions.

As controls, three additional constructs were built. Previously, we identified the *gapdh* and *rpsL* promoters from actinomycetes as being highly active under normal laboratory culturing conditions (MYG medium).⁵ The *gapdh* promoter,

named *gapdh*p, is located upstream of the operon consisting of glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase (*pgk*), and triosephosphate isomerase (*tpiA*), the enzymes catalyzing the sixth, seventh, and fifth steps, respectively, in the glycolysis pathway. The *rpsL* promoter, named *rpsL*p, resides upstream of another operon consisting of 30S ribosomal proteins S12 and S7 and elongation factors G and Tu. On the basis of the RNA-seq analysis, the expression level of the gene encoding glyceraldehyde-3-phosphate dehydrogenase from *S. albus* J1074 ranked number 86. The promoter region of this gene was cloned to serve as a control as well (Gp). Among all 32 promoter regions selected, 20 resided upstream of 30S ribosomal proteins. Therefore, the previously characterized *rpsL*p from *Cellulomonas flavigena* was chosen as a positive control (Rp), as it is one of the strongest actinomycete promoters ever reported. The third control promoter was *ermE**p (Ep), which is the most often used strong promoter from *Saccharopolyspora erythraea*.^{22,23}

Characterization of the Chosen Promoters Using the *xylE* Reporter Gene. The verified plasmids were transformed into *Escherichia coli* WM6026, and the resulting transformants were used as the donors for conjugative transfer of the assembled plasmids to *S. albus* J1074 following a protocol described elsewhere.²⁴ To account for variations among different ex-conjugants, four different ex-conjugants were picked for screening in the XylE activity assay (data not shown). Ex-conjugants with the highest expression levels were selected for analysis in biological duplicates. The activities of these promoters are shown in Figure 1, which varied from 4 to 1300% of the activity of *ermE**p.

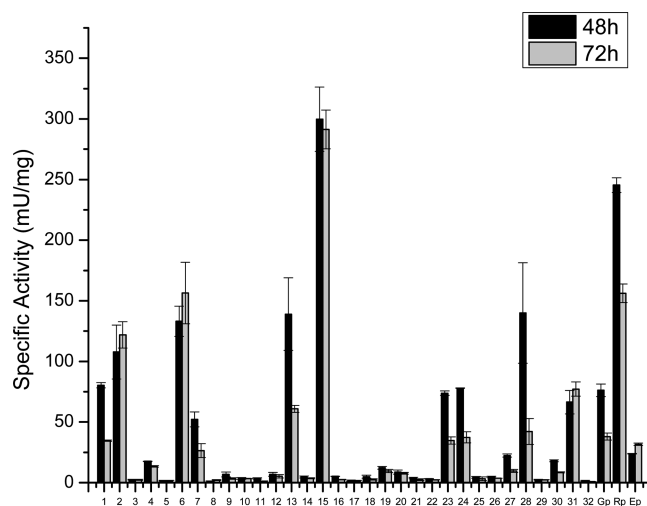


Figure 1. Characterization of all 32 identified promoters from *S. albus* J1074. Evaluation of the activities of the heterologous promoters using *xylE* as a reporter. Promoter *ermE**p is the most commonly used promoter reported in the literature. Gp: *gapdh*p; Rp: rCFp; Ep: *ermE**p.

In contrast with the RNA-seq data, some of the promoters seemed to be quite weak under the MYG culturing condition. To investigate the possible causes, we located these promoters with their downstream genes on the chromosome of *S. albus*. All of the 32 promoters that we selected could be classified into 18 clustered gene groups according to their locations on the genome (Table S3). Therefore, it is possible that several of the adjacent genes may be involved in operon structures, and the

high expression of these genes may be driven by only one promoter in front of the whole operon. Thus, we speculate that region nos. 4, 5, 10, 12, 14, and 32 displayed weak activities because they represent intergenic regions in an operon context and do not represent constitutive promoter sequences. Among the rest of the promoters, 10 promoters showed strong activities under this culturing condition. Comparing with *ermE**p, these 10 promoters (nos. 1, 2, 6, 7, 13, 15, 23, 24, 28, and 31) showed 2- to 10-fold enhancement based on the XylE activity assay.

To characterize these 10 strong promoters in detail, a time course (24, 48, 72 h) analysis was performed alongside the samples with control promoters (Figure 2). In general, these

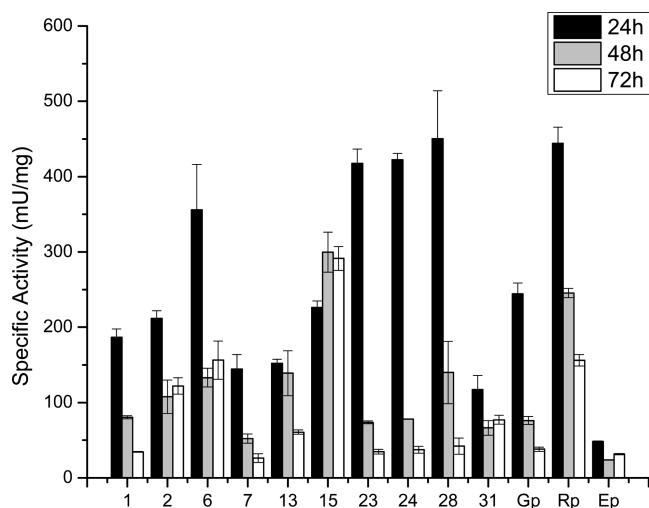


Figure 2. Characterization of the selected 10 strong promoters. Evaluation of the activities of the heterologous promoters using *xylE* as a reporter. Promoter *ermE**p is the most commonly used promoter reported in the literature. Gp: *gapdhp*; Rp: rCFp; Ep: *ermE**p.

strong promoters are highly active at 24 h, with the most active promoter showing nearly 10-fold enhancement compared with that of *ermE**p. *ermE**p is a constitutive promoter, as its activity does not change significantly at the 72 h time point compared to that at the 24 and 48 h time points. Similarly, promoter nos. 2, 6, 13, 15, and 31 retained a relatively high *xylE* activity level. Therefore, we considered them to be strong constitutive promoters under the chosen culturing condition in MYG medium. In agreement with previous observations on the strengths of *gapdhp* homologues,⁵ the *gapdhp* homologue in *S. albus* (Gp) showed strong activity under the chosen culturing condition.

Analysis of the Identified Promoters Using qPCR. To quantify gene expression levels, quantitative real-time PCR (qPCR) was employed. The RpoD (HrdB) protein is the principal sigma factor in *Streptomyces* strains,²⁵ which is encoded by the *rpoD* (*hrdB*) gene.²⁶ The *rpoD* gene was chosen as the internal control, and its transcription signal remained comparable in all samples. Primers were designed to amplify ~100–200 bp fragments for the *rpoD* gene and the *xylE* gene from the cDNA library. Standard curves were made first for these two pairs of primers using genomic DNA of *S. albus* harboring the no. 1-promoter–*xylE* cassette as the template (Figure S5). qPCR was performed as described in the Methods.

Cells were grown for 72 h, and samples for RNA isolation were collected at two different time points: 48 and 72 h. Total RNA was isolated, and cDNA was synthesized afterward. To check for genomic DNA carry-over, the cDNA synthesis reaction without reverse transcriptase was also carried out to serve as a negative control. To be consistent with the XylE activity assay, the ex-conjugant with the highest XylE activity out of the four different ex-conjugants was selected to perform RNA isolations in biological duplicates. As shown in Figure 3,

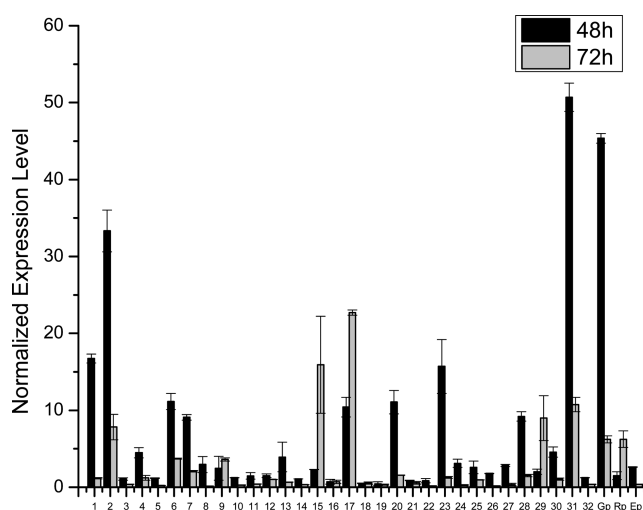


Figure 3. Promoter characterization via qPCR analysis. Transcription of the *xylE* gene under different promoters in *S. albus* was quantified at different time points. The y-axis scale represents the expression value relative to that of *rpoD*, a commonly used housekeeping sigma factor, which was set to 1. Gp: *gapdhp*; Rp: rCFp; Ep: *ermE**p. The *rpoD* gene was used as the internal control for *S. albus*, and its expression level was set as 1.

all 35 promoters (32 selected ones plus three controls) were analyzed under the chosen growth condition (MYG medium). The gene expression levels from different time points were compared. The 10 strong promoters characterized from the XylE activity assay also showed high expression levels in the qPCR experiments, which further confirmed the strengths of these promoters. Interestingly, promoter nos. 4, 8, 9, 17, 20, 29, and 30 also showed moderately strong activity, although they did not appear as obviously in the XylE assay. We suspect that, as we did not experimentally determine the ribosomal binding site (RBS) for each promoter, the activity profile that we observed actually encompasses the effects from both the promoter and the RBS, whereas the expression profile obtained from qPCR analysis reflects the impact of the corresponding promoter only. Therefore, the weak performance of these promoters in the enzymatic assays may be attributed to the strength of the corresponding RBS.

Similarly, a time course (24, 48, 72 h) analysis was performed for detailed characterization of these 10 strong promoters, and the three controls were also included (Figure 4). In general, these strong promoters are highly active at 24 h, with the most active one showing a nearly 35-fold enhancement in its expression level. The results obtained correlate well with the data collected from the XylE assay. Meanwhile, promoter nos. 2, 6, 15, and 31 retained a relatively consistent transcription level of the downstream gene. Therefore, we considered them to be strong constitutive promoters under the chosen culturing condition.

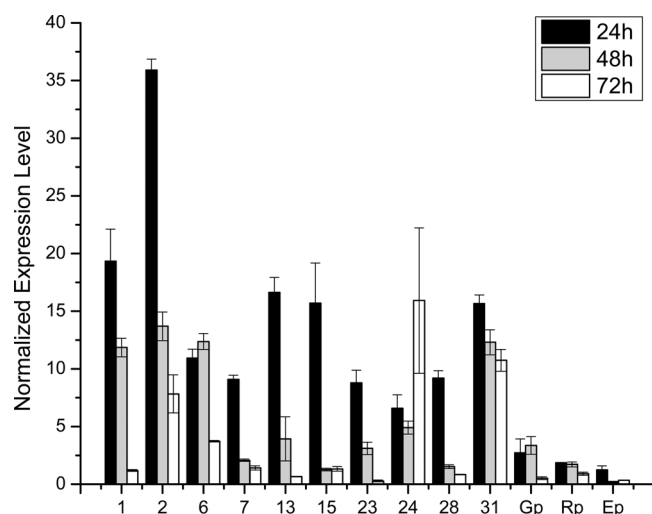


Figure 4. Characterization of the selected 10 strong promoters. Evaluation of the activities of the promoters using qPCR. Promoter *ermE**p is the most commonly used promoter reported in the literature. Gp: *gapdhp*; Rp: *rCFp*; Ep: *ermE**p. The *rpoD* gene was used as the internal control for *S. albus*, and its expression level was set as 1.

Comparison of Polycyclic Tetramate Macrolactam (PTM) Production Using Newly Identified Strong Promoters versus Previously Identified Promoters. To demonstrate an application of the above-characterized strong promoters, five out of the 10 strong promoters were selected to activate a cryptic natural product biosynthetic pathway. As proof of concept, we chose the previously activated PTM gene cluster from *S. griseus* as the target, which includes a polyketide synthetase/nonribosomal peptide synthetase (PKS/NRPS) hybrid enzyme and a cascade of modification enzymes.⁴ We amplified the open reading frames from the genomic DNA and refactored the target gene cluster using the newly identified promoters. The resulting construct, named F6N, contains five strong promoters identified from this study and *rCFp*, from our previous study, as a control (Figure S8). In parallel, the target gene cluster was refactored using previously identified strong

promoters⁴ and named F6O (Figure S8). Both F6O and F6N were transformed into three different *Streptomyces* hosts (*S. lividans* 66, *S. albus* J1074, and *S. coelicolor* M1146).

The resulting *Streptomyces* mutants were cultured in MYG medium for 4 days. Meanwhile, native *S. griseus* was cultured under the same condition (MYG medium), and no production of any related compounds was detected (Figure 5). Compared with the F6O construct, the F6N construct resulted in a roughly 1.8-fold enhanced production of the target compound in *S. coelicolor* M1146, a 1.2-fold enhanced production of the target compound in *S. lividans* 66, and similar production in *S. albus* J1074 (Figure 5). This confirms that all of the newly identified promoters are strong promoters and that they are active not only in *S. albus* J1074 but also in other *Streptomyces* hosts as well.

The 72 h samples of all of the *Streptomyces* mutants harboring either F6N or F6O constructs were taken, and total RNA was isolated for qPCR analysis. The gene *SGR815* was designed to be under the control of *rCFp* (Rp) in both F6O and F6N constructs to monitor the growth condition of both strains. Similar expression levels of *SGR815* suggested that the two strains harboring F6O and F6N were at the same growth stage (Figure 6). Comparison of the expression levels of all of the other genes is shown in Figure 6; the promoters identified in this study worked best in the *S. coelicolor* M1146 strain,²⁷ a derivative of wild-type strain A3(2) lacking four endogenous secondary metabolite gene clusters encoding for the production of actinorhodin, prodiginine, CPK, and CDA. Overall, most of the promoters performed well in all three *Streptomyces* strains, and particularly for the F6O construct, gene 812 was under the control of *ermE**p and its expression level was very low under all conditions, whereas in the F6N construct, the expression level of gene 812 was increased by several folds.

Notably, a total of 100 promoters in *E. coli* were characterized,²⁸ and, similarly, a total of 14 constitutive promoters from *S. cerevisiae* were cloned and characterized using green fluorescent protein (GFP) as a reporter.²⁹ However, no such well-characterized native promoter library of *Streptomyces* is available. To further explore the application of actinomycete promoters, we have successfully chosen 32

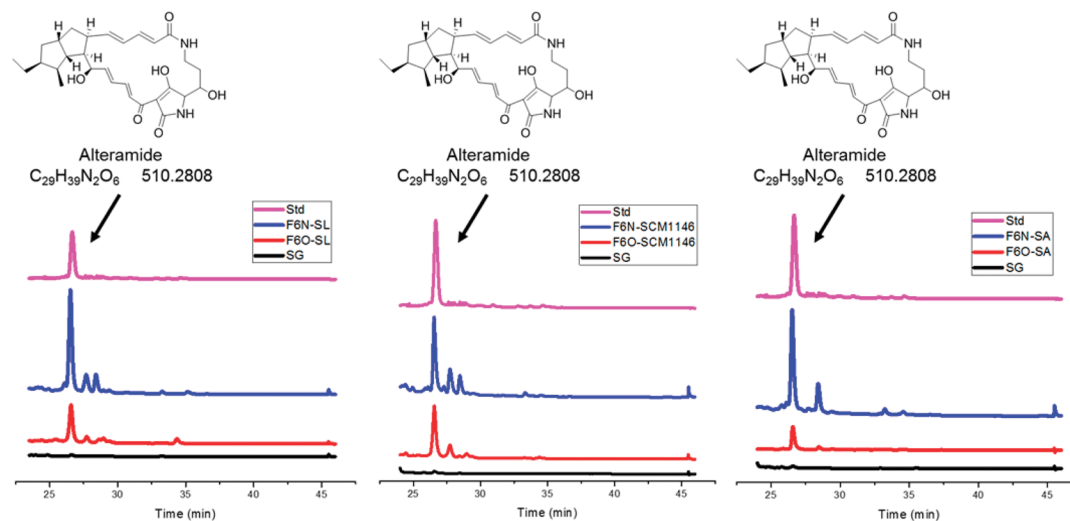


Figure 5. HPLC analysis of the refactored PTM pathway. SG: *Streptomyces griseus*. F6O-SCM1146: F6O in *S. coelicolor* SCM1146. F6N-SCM1146: F6N in *S. coelicolor* SCM1146. F6O-SL: F6O in *S. lividans* 66. F6N-SL: F6N in *S. lividans* 66. F6O-SA: F6O in *S. albus* J1074. F6N-SA: F6N in *S. albus* J1074. Std: Alteramide compound.

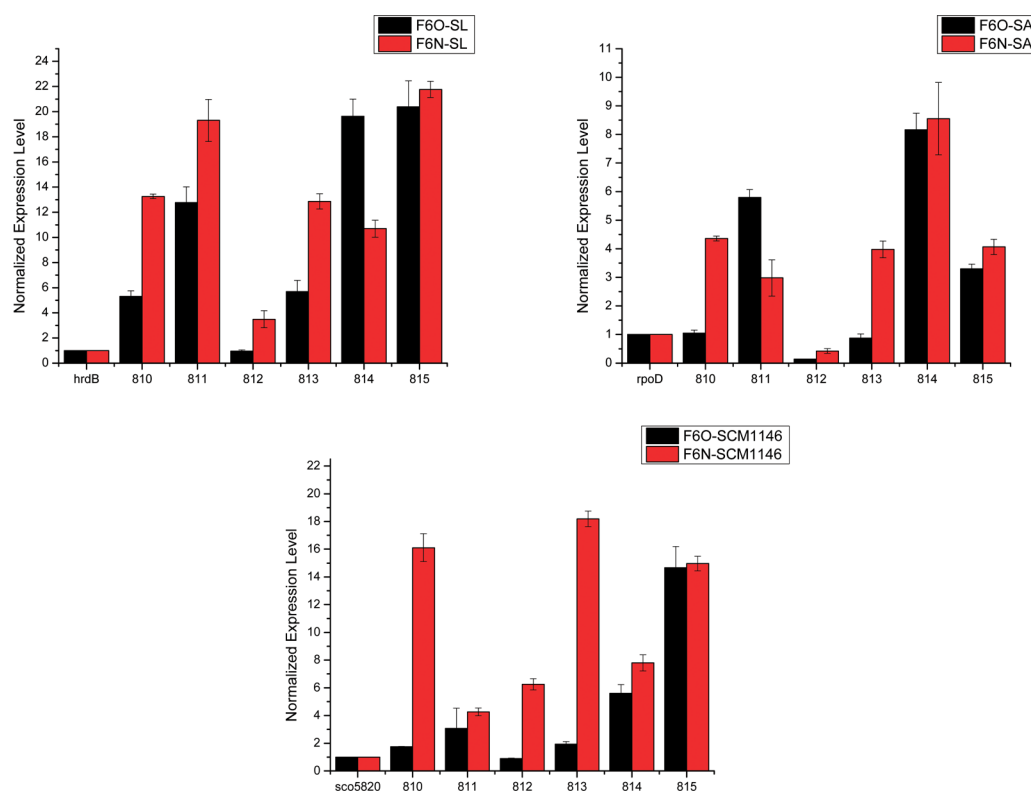


Figure 6. qPCR analysis of the refactored PTM pathway harboring previously identified and newly identified promoters. F6O-SL: F6O in *S. lividans* 66. F6N-SL: F6N in *S. lividans* 66. F6O-SA: F6O in *S. albus* J1074. F6N-SA: F6N in *S. albus* J1074. F6O-SCM1146: F6O in *S. coelicolor* SCM1146. F6N-SCM1146: F6N in *S. coelicolor* SCM1146. *hrdB* was used as the internal control for *S. lividans*, *rpoD*, for *S. albus*, and *sco5820*, for *S. coelicolor*. Their expression levels were set as 1.

promoters from *S. albus* J1074 via RNA-seq analysis and identified 10 promoters with at least 2-fold higher activity than that of *ermE**p. A time-course study of the selected 10 promoters further revealed that several of them may serve as strong constitutive promoters. Here, we did not experimentally determine the ribosomal binding site (RBS) for each promoter and assumed that it is located 6–10 bp upstream of each start codon.

The promoters identified in this study can potentially be used for many different applications. For single-gene analysis or protein expression, strong constitutive promoters could be used for the overexpression of a gene of interest. Previously, *ermE**p-based vectors have been used for protein expression in *Streptomyces* hosts.³⁰ With our stronger constitutive promoters, we would expect a higher protein expression level in *Streptomyces* hosts, which would be particularly beneficial for the characterization of large polyketide synthetases/non-ribosomal peptide synthetases/fatty acid synthetases (PKSs/NRPSs/FASs) proteins in *Streptomyces* that are usually hard to be expressed in *E. coli*.

Moreover, the promoters could be applied in synthetic biology toolkit development. For example, they could be used in our plug-and-play platform to replace the native promoters and therefore to drive the expression of heterologous pathway genes, as shown in our activation of the PTM biosynthetic pathway. This could benefit the field of natural product discovery as a simple approach for cryptic pathway activation. As the promoters were identified under two commonly used actinomycete culturing conditions and they were proven to be functional in different host strains, they should be more generally applicable for studies of actinomycete pathways.

In addition, the panel of promoters would be especially useful for metabolic engineering of actinomycetes to over-produce natural products by manipulating the expression of the rate-limiting steps.³¹ Fine-tuning gene expression has been demonstrated to be very useful for pathway optimization. Thus, the promoter library could be employed in fine-tuning and balancing specific gene expression.^{32,33} Additionally, the strong constitutive promoters that we identified could serve as templates for the construction of a synthetic promoter library with a greater dynamic range than that of those previously reported.¹³ Furthermore, the strategy employed here could be applied for promoter library construction for any genetically tractable organism of interest. This would benefit the field of synthetic biology, as it provides a simple means of creating well-characterized parts.

Conclusions. In summary, by taking advantage of RNA-seq, we were able to identify 32 candidate promoter regions from *S. albus*. Among these 32 promoters, 10 showed greater strength than that of the widely used constitutive promoter *ermE**p according to both the enzymatic activity assay and transcriptional analysis. Most of the identified strong promoters are from housekeeping ribosomal proteins or heat/cold shock proteins, which means that they should be common for most streptomycetes or even most actinomycetes. Therefore, they may become universal tools for gene engineering in those different hosts. To demonstrate their utility, five out of these 10 promoters were used to successfully activate a cryptic PTM biosynthetic pathway. In particular, higher expression levels of the pathway genes and higher production of the target compound were achieved when compared with those using previously identified strong promoters. This study not only

provides a useful toolkit for the synthetic biology field but also offers a general method for discovery and characterization of strong constitutive promoters under target conditions.

METHODS

Strains and Reagents. *S. cerevisiae* HZ848 (*MAT α* , *ade2-1*, Δ *ura3*, *his3-11, 15*, *trp1-1*, *leu2-3, 112*, and *can1-100*)⁴ was used as the host for DNA assembly. *S. albus* J1074³⁴ was a gift from Professor Wenjun Zhang (University of California, Berkeley, CA). *S. coelicolor* M1146³⁵ and *E. coli* strain ET12567/pUZ8002³⁵ were gifts from Professor Eriko Takano (The University of Manchester, Manchester, United Kingdom). Plasmid pAE4³⁶ and *E. coli* strains WM6026²⁴ and BW25141²⁴ were gifts from Professor William Metcalf (University of Illinois at Urbana–Champaign (UIUC), Urbana, IL). Nalidixic acid was obtained from Sigma-Aldrich (St. Louis, MO). ISP2, agar, beef extract, yeast extract, malt extract, and other reagents required for cell culture were obtained from Difco (Franklin Lakes, NJ). All restriction endonucleases and Q5 DNA polymerase were purchased from New England Biolabs (Beverly, MA). Failsafe PCR 2 \times PreMix G was purchased from Epicentre Biotechnologies (Madison, WI). SYBR Green PCR master mix was purchased from Applied Biosystems (San Francisco, CA). The QIAprep spin plasmid mini-prep kit, QIAquick PCR purification kit, QIAquick gel extraction kit, and the total RNA isolation mini kit were purchased from Qiagen (Valencia, CA). All primers were synthesized by Integrated DNA Technologies (Coralville, IA). Yeast YPAD medium (1% yeast extract, 2% peptone, and 2% dextrose supplied with 0.01% adenine hemisulfate) was used to grow *S. cerevisiae* strains. Synthetic complete drop-out medium lacking uracil (SC-Ura) was used to select transformants containing the assembled constructs of interest. MYG medium (4 g/L yeast extract, 10 g/L malt extract, and 4 g/L glucose) and R2YE medium³⁷ were used for growing *S. albus* strains.

***Streptomyces albus* Cultivation and RNA Extraction.** Wild-type *S. albus* culture was grown in both MYG medium and R2YE medium at 30 °C with constant shaking (250 rpm) until stationary phase was reached, at which time it was inoculated into fresh MYG and R2YE media at a ratio of 1:100. Cells were collected from 1 mL of culture in each medium at 24 and 72 h. Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA). RNA preparations were treated with Turbo DNA-free kit (Life Technologies, Carlsbad, CA) to remove contaminating DNA.

RNA-Seq Analysis. For the analysis and quantitation of total RNA samples, an Agilent RNA 6000 Nano kit from Agilent Technologies (Santa Clara, CA) was used to determine the RNA integrity number (RIN) of the samples. Qualified samples with RIN larger than 8 were submitted to the Roy J. Carver Biotechnology Center at UIUC, where rRNA was removed using a Ribozero Bacteria kit (Illumina, CA) and bar-coded libraries were made with TruSeq stranded RNA sample prep kit (Illumina, CA). The eight samples were pooled and sequenced on one lane of an Illumina HiSeq 2500 using a TruSeq SBS sequencing kit (v3, Illumina, CA), generating a total of 211 927 955 single-end reads of 100 bases. Libraries were constructed and sequenced at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana–Champaign. The sequencing data were submitted to the High-Performance Biological Computing group at UIUC. Raw FASTQ data were trimmed for sequencing adapters and low-quality bases using Trimmomatic v. 0.30³⁸ in single-end mode,

retaining reads with a minimum Phred quality score of 30 and minimum length of 36. The specific parameters used were “SE -phred33 ILLUMINACLIP: Huimin_Zhao_adapters.fa: 2:30:10 LEADING: 30 TRAILING: 30 MINLEN: 36.” The adapter sequence used was AGATCGGAAGAGCACACGTCTGAACTCCAGTCACNNNNNNATCTCGTATGCCGTCTTCTGCTTG. To verify that trimming improved sequence quality, FASTQC v. 0.10.1 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was run on the trimmed and the raw FASTQ data. Bowtie2 v. 2.1.0³⁹ was used for alignment of the trimmed data against the *S. albus* J1074 reference genome (GenBank accession CP004370.1) using $-k$ 1 and $-N$ 1 options (report 1 alignment per read and allow only 1 mismatch in a seed alignment during multiseed alignment, respectively). Raw read counts were generated using htseq-count from HTSeq v. 0.5.4⁴⁰ with the NCBI GFF gene model file for GenBank accession CP004370.1. The htseq-count parameters used were “-i Parent -m intersection-nonempty -s reverse.”

Expression Analysis. The raw read counts were input into R v3.0.2⁴¹ for QC and data preprocessing. RPKM (reads per kilobase of gene model per million mapped reads) values were calculated using the *rpkm* function from edgeR v3.4.0.⁴² QC analysis of the proportion of reads aligned to the genome but not within a gene and the distribution of RPKM values indicated that sample SA2_M72 likely had genomic DNA that was sequenced; hence, it was removed from further analysis. The RPKM values were used to rank the genes within each sample from highest to lowest expression, which were then converted to percentages by dividing by the number of genes ($n = 5937$). A gene was counted in the top $X\%$ of a group only if both replicates had percentages $\leq X$.

Promoter Cloning. The promoters of 32 strongly expressed genes under both culturing conditions at the two defined time points were selected and cloned into the pAE4 plasmid harboring the *xylE* reporter gene using the DNA assembler method.⁴³ *S. albus* was grown in liquid MYG medium at 30 °C with constant shaking (250 rpm) for 2 days. The genomic DNA was isolated from *S. albus* using the Wizard genomic DNA isolation kit from Promega (Madison, WI). Pairs of primers were designed with the sequences shown in Table S2. Promoters of all 32 chosen regions were amplified from the genomic DNA of *S. albus*. Generally, the PCRs were carried out in a 100 μ L reaction mixture consisting of 50 μ L of Failsafe PCR 2 \times PreMix G from Epicentre Biotechnologies (Madison, WI), 2.5 pmol of each primer, 0.5 μ L of genomic DNA, and 2.0 units of Q5 DNA polymerase for 35 cycles on a PTC-200 thermal cycler (MJ Research, Watertown, MA). Each cycle consisted of 20 s at 98 °C, 30 s at 58 °C, and 30 s at 72 °C, with a final extension of 8 min. The *S. cerevisiae* helper fragment was amplified from the commercial vector pRS416, and the *E. coli* and *S. albus* helper fragments were amplified from the *Streptomyces*–*E. coli* shuttle vector pAE4. Following electrophoresis, the PCR products were individually gel-purified from 1.0% agarose gels using the Qiagen gel purification kit. One-hundred nanograms of each individual fragment was mixed and precipitated with ethanol. The resulting DNA pellet was air-dried, resuspended in 4 μ L of Milli-Q double-deionized water, and subsequently electroporated into *S. cerevisiae* using a protocol reported elsewhere.

Verification of the Assembled Gene Clusters. Yeast plasmids were transformed to *E. coli* strain BW25141 and selected on Luria Broth (LB) agar plates supplemented with 50

$\mu\text{g/mL}$ apramycin. Colonies were inoculated into 5 mL of LB media supplemented with 50 $\mu\text{g/mL}$ apramycin, and plasmids were isolated from the liquid culture using the plasmid miniprep kit from Qiagen. Plasmids isolated from *E. coli* were then submitted to ACGT, Inc. (Wheeling, IL) for sequencing.

Heterologous Expression in *S. albus*. The verified clones were transformed to *E. coli* WM6026²⁴ and selected on LB agar plates supplemented with 19 $\mu\text{g/mL}$ 2,6-diaminopimelic acid and 50 $\mu\text{g/mL}$ apramycin. The resulting transformants were then used as donors for conjugative transfer of the assembled plasmids to *S. albus* following a protocol described elsewhere.²⁴ *S. albus* ex-conjugants were picked and restreaked on ISP2 plates supplemented with 50 $\mu\text{g/mL}$ apramycin and grown for 2 days. For each construct, four different single colonies were picked and inoculated into 2 mL of MYG liquid medium supplemented with 50 $\mu\text{g/mL}$ apramycin in a 14 mL Falcon tube. One milliliter of each saturated culture was inoculated into 50 mL of MYG medium supplemented with 50 $\mu\text{g/mL}$ apramycin in a 250 mL shake-flask (3 mm glass beads were added to improve liquid mixing and aeration). Samples were taken for further analysis at different time points: 24, 48, and 72 h.

Promoter Characterization via the XylE Assay.

Catecho1 2,3-dioxygenase, the product of the *xylE* gene from the *Pseudomonas* TOL plasmid, converts colorless catechol into yellow 2-hydroxyomuconic semialdehyde. Aliquots of 10 mL of the cell culture at different time points were collected and centrifuged at 4000 rpm for 10 min to remove the supernatant. Five milliliters of sample buffer (100 mM phosphate buffer, pH 7.5; 20 mM Na-EDTA, pH 8.0; 10% v/v acetone) was added to resuspend the cell pellets followed by sonication on ice. 0.1% Triton X-100 solution was added into the samples, which were then incubated on ice for 15 min. Then, the samples were centrifuged for 5 min (40 000g, 4 °C), and the cell lysates were transferred to fresh tubes. Assay buffer (900 μL) (10 mM phosphate buffer, pH 7.5; 0.2 mM catechol 2,3-dioxygenase) was added to 100 μL of the cell lysate, and the change in absorbance at 375 nm (A_{375}) was followed. The concentration of the total protein was determined by the Bradford method.⁴⁴ The slope of the linear part of the spectrophotometric output was used to calculate the specific activity as follows: $\text{mU catechol dioxygenase} [\text{nmol min}^{-1}] = 30.03 \times \Delta A_{375} / t [\text{min}]$.

Promoter Characterization via qPCR Analysis. Cells from 1 mL of culture were collected at 24, 48, and 72 h. Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA). RNA preparations were treated with the Turbo DNA-free kit (Life Technologies, Carlsbad, CA) to remove contaminating DNA. Reverse transcription was carried out using the ProtoScript first strand cDNA synthesis kit (New England Biolabs, Beverly, MA). Real-time PCR was performed with SYBR Green PCR master mix on a 7900HT fast real-time PCR system (Applied Biosystems, Carlsbad, CA). Primers were designed using the online tool provided by Integrated DNA Technologies (<https://www.idtdna.com/scitools/Applications/RealTimePCR/>). Ten microliters of 2 \times SYBR Green Mix, 1 μL of cDNA, 1 μL of each primer at a concentration of 10 pmol/ μL , and 7 μL of ddH₂O were mixed gently in each well of the Applied Biosystems MicroAmp Optical 384-well reaction plate. Reactions were performed using the following program: 2 min at 50 °C and 10 min at 95 °C for one cycle followed by 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C for 40 cycles, with a final cycle of 10 min at 72 °C. The endogenous gene *rpoD*, encoding an RNA polymerase sigma factor, was used as the internal

control for promoter characterization. The expression levels of the *xylE* gene under different promoters were normalized by the expression of the internal control. Data was analyzed using SDS2.4 software (Applied Biosystems, Carlsbad, CA).

PTM Gene Cluster Reconstruction, Heterologous Expression, and HPLC Analysis. Gene cluster fragments were amplified from the genomic DNA of *S. griseus*. The assembly followed a protocol reported elsewhere.⁴³ The verified clones were transformed to *E. coli* ET12567/pUZ8002 and selected on LB agar plates supplemented with 25 $\mu\text{g/mL}$ kanamycin, 25 $\mu\text{g/mL}$ apramycin, and 12.5 $\mu\text{g/mL}$ chloramphenicol. The resulting transformants were then used as donors for conjugative transfer of the assembled plasmids to *S. lividans* 66, *S. albus* J1074, and *S. coelicolor* M1146 following a protocol described elsewhere.²⁷ Ex-conjugants were picked and restreaked on ISP2 plates supplemented with 25 $\mu\text{g/mL}$ apramycin and grown for 2 days. A single colony was inoculated into 2 mL of MYG liquid medium supplemented with 25 $\mu\text{g/mL}$ apramycin, and after 72 h, the seed culture was transferred at a 1:50 ratio to 50 mL of fresh MYG medium in a 125 mL shake-flask (3 mm glass beads were added to improve liquid mixing and aeration). After culturing for 4 days at 30 °C, the liquid was extracted by ethyl acetate at a ratio of 1:1 twice, concentrated 1000-fold, and subjected to HPLC analysis. HPLC was performed on the Agilent 1100 series LC/MSD XCT plus ion trap mass spectrometer with a Phenomenex Luna C18 reverse-phase column (3.0 \times 150 mm, 3.5 μm). HPLC parameters were as follows: solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile; gradient, 10% B for 5 min to 100% B in 25 min, maintain at 100% B for 10 min, return to 10% B in 1 min, and finally maintain at 10% B for 9 min; flow rate, 0.3 mL/min; detection by UV spectroscopy at 280 nm.

■ ASSOCIATED CONTENT

● Supporting Information

Figure S1: Bioanalyzer traces of total RNA samples. Figure S2: Breakdown of total reads per sample in RNA-seq analysis. Figure S3: Distribution of gene expression levels per sample. Figure S4: Selection of top hits. Figure S5: Standard curves for qPCR. Figure S6: qPCR analysis of all the 32 selected genes plus the *gapdh* gene and S1–S6 in MYG and R2YE medium. Figure S7: Vector map of the promoter-*xylE* cassettes for promoter 1. Figure S8: Vector map of the refactored PTM gene cluster using previously identified promoters and newly identified promoters. Table S1: Total RNA samples submitted for RNA-seq analysis. Table S2: Primers used to clone the 32 putative strong promoters and the control promoter. Table S3: Gene clusters identified for the 32 promoters chosen. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00016.

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Author Contributions

Y.L. and H.Z. designed the study, analyzed data, and wrote the manuscript. Y.L., L.Z., and K.W.B. performed the experiments.

Notes

The authors declare no competing financial interest.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on May 7, 2015, with errors in Table S2 in the Supporting Information. The corrected version was reposted on May 19, 2015. The Supporting Information file was replaced with corrections to Figure 8 on September 1, 2015.