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# An Engineered Strong Promoter for Streptomycetes

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**Well-characterized promoters are essential tools for metabolic engineering and synthetic biology. In *Streptomyces coelicolor*, the native *kasO*p is a temporally expressed promoter strictly controlled by two regulators, ScbR and ScbR2. In this work, first, *kasO*p was engineered to remove a common binding site of ScbR and ScbR2 upstream of its core region, thus generating a stronger promoter, *kasO*p$_3$. Second, another ScbR binding site internal to the *kasO*p$_3$ core promoter region was abolished by random mutation and screening of the mutant library to obtain the strongest promoter, *kasO*p* (where the asterisk is used to distinguish the engineered promoter from the native promoter). The activities of *kasO*p* were compared with those of two known strong promoters, *ermE*p* and SF14p, in three *Streptomyces* species. *kasO*p* showed the highest activity at the transcription and protein levels in all three hosts. Furthermore, relative to *ermE*p* and SF14p, *kasO*p* was shown to confer the highest actinorhodin production level when used to drive the expression of *actII*-ORF4 in *S. coelicolor*. Therefore, *kasO*p* is a simple and well-defined strong promoter useful for gene overexpression in streptomycetes.**

The genus *Streptomyces* is known for its ability to produce antibiotics and for its complex morphology (1, 2). It is not only an ideal model to study bacterial differentiation but is also considered a good host for antibiotic production. In the past decades, many genetic tools have been developed for streptomycetes (3, 4). They have greatly facilitated genetic manipulations of these organisms. However, for gene expression, only a limited number of promoters, such as the constitutive promoters *ermE*p* (where the asterisk signifies the presence of a one-base-pair mutation) and SF14p (5, 6) and the inducible promoters *tipA*p and *nitA*p (7, 8), are available. Among these promoters, only *ermE*p* is widely used for the overexpression of target genes. But even *ermE*p* has not been completely characterized and sometimes gives undesirable results in some *Streptomyces* species (9).

In modern metabolic engineering and synthetic biology practices, the fine-tuning of gene expression by well-characterized promoters is necessary (10, 11). In recent years, great efforts have been made to develop useful promoters or promoter libraries in several model organisms (12–14). However, for streptomycetes, such efforts and advances are lagging. This may be due to the intricate regulatory networks of streptomycetes, which contain hundreds of regulatory proteins and dozens of sigma factors (15, 16). This regulatory complexity is reflected by the degeneracy of reported promoter sequences (17). Among the 139 *Streptomyces* promoters previously compiled, only about 20% showed conserved core promoter sequences similar to those recognized by *Escherichia coli* $\sigma^{70}$ (18). Due to the lack of understanding of these diverse promoters in streptomycetes, few native promoters could be easily applied for controlled gene expression. The widely used *ermE*p*, which is a heterogenous promoter from *Saccharopolyspora erythraea*, has multiple −10 and −35 sites and bidirectional promoter activities (5). Such structural complexity hinders its characterization and evaluation (9, 19). Therefore, developing simple and well-characterized promoters is necessary for metabolic engineering in streptomycetes. *Streptomyces coelicolor* is the best genetically characterized species of streptomycetes. Among the 65 sigma factors in its genome, several have been characterized (20); for example, BldN is involved in the regulation of aerial growth (21) and WhiG is involved in spore formation and maturation (22). Other sigma factors are specifically used in response to various stresses, such as SigR, which is involved in the regulation of the oxidative stress response (23). Among them, HrdB was identified as the housekeeping sigma factor responsible for the transcription of essential genes (24). It recognizes promoters with the consensus sequence TTGACN (−35)—17 nucleotides (nt)—TAGAPuT (−10) (18). The strength of bacterial promoters is determined not only by the core −35 and −10 region but also by two flanking regions: 5′ of −35 and 3′ of the transcription start site (TSS) (25). However, the intrinsic strength of a promoter (transcription activity conferred by the core RNA polymerase in a manner independent of other transcriptional factors) is strongly correlated with the core promoter sequences which are recognized by the housekeeping or an alternative sigma factor in bacteria (26, 27). Hence, in *S. coelicolor*, promoters recognized by housekeeping sigma factor HrdB, which is normally highly expressed during growth, are the preferred candidates for promoter development.

According to previous research, *kasO* (also known as *cpkO* and *SCO6280*), encodes a SARP family regulator and is an activator of a cryptic type I polyketide synthase gene cluster responsible for coelimycin P1 production in *S. coelicolor* A3 (2, 28, 29). The upstream region of the *kasO* promoter (*kasO*p) spans nearly 400 bp of DNA, and its core promoter sequence is similar to the HrdB-recognized consensus sequence (30) (Fig. 1A). Our previous work indicated that *kasO*p is rigorously regulated by ScbR and ScbR2; the former is the γ-butyrolactone (GBL, SCB) receptor in *S. coelicolor*, whereas ScbR2 shows great similarity to ScbR but could not bind GBL and thus was called a "pseudo"-GBL receptor (31). *kasO* transcripts are undetectable during rapid growth, but the gene is sharply turned on and off in a small time window at the

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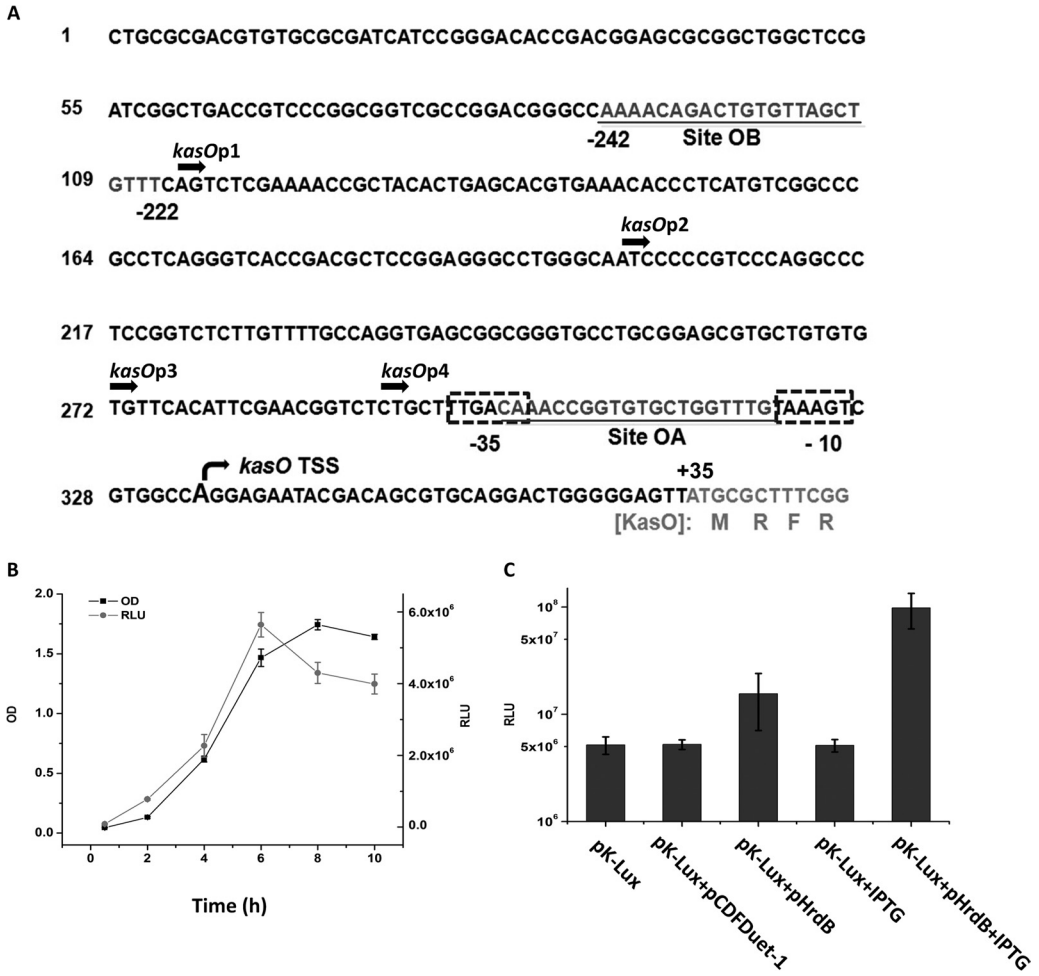


**FIG 1** Evaluation of *kasO*p activity in *E. coli*. (A) Nucleotide sequences of *kasO*p. The sequence is numbered on the left. The TSS is indicated by a bent arrow and bigger letters. The sequences of site OA and site OB are underlined. The putative −10 and −35 sites of *kasO*p are marked by dashed frames. The translation start codon of *kasO* is marked in gray letters. Translated amino acids are given below the nucleotide sequence. The truncated sites of *kasO*p$_1$, *kasO*p$_2$, *kasO*p$_3$, and *kasO*p$_4$ are indicated by thick arrows. (B) Growth ($OD_{600}$) and bioluminescence of *E. coli* DH5α containing pK-Lux. RLU, relative light units. (C) Increase of *kasO*p activity in response to induced expression of HrdB in *E. coli* BL21(DE3) containing pK-Lux and pHrdB. Data are expressed as average values obtained from three independent experiments. Error bars indicate means ± standard deviations (SDs).

transition phase (2, 30). The regulatory mechanism is most likely determined by the interplay between ScbR and ScbR2 (32). The onset of *kasO* transcription is probably initiated by SCBs by derepression of the GBL receptor protein ScbR (30). The pseudo-γ-butyrolactone receptor ScbR2 binds the upstream region of *kasO*p, at a time after ScbR derepression, to shut down *kasO* expression, thus giving rise to the pulse-expression pattern of *kasO* (32). The endogenous antibiotics actinorhodin (Act) and undecylprodigiosin could also play a role in the control of *kasO*p expression by interaction with ScbR2 (31). These known interactions between *kasO*p and its regulators make it an ideal candidate for promoter engineering.

Strong promoters could be obtained by many approaches. A simple method is to use a shotgun clone method with native promoters from genomic DNA. The promoter SF14p was discovered this way from a fragment of *Streptomyces ghanaensis* phage I19 (6). Recently, synthetic promoter libraries have been adopted as a means to obtain useful promoters and many synthetic promoters have been generated this way (12, 33, 34). Seghezzi et al. constructed a promoter library mimicking *Streptomyces* vegetative promoters with a relatively fixed −35 region (TTGACN) and a more variable −10 box(es) (TASVDT) and obtained a collection of promoters with various strengths (12). However, none of these promoters showed higher activity than *ermE*p*. The reasons for this are unknown (12). Promoter engineering is another strategy to develop and obtain desired promoters (35). In this work, we chose *kasO*p for rational engineering. After two engineering steps, a strong promoter, *kasO*p*, was obtained. The performance of this promoter was evaluated in *E. coli* and several *Streptomyces* strains by different methods. Our results indicate that it is the strongest simple promoter currently reported for streptomycetes.

## MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** The bacterial strains used in this study are listed in Table 1. *E. coli* strains were grown aerobically at 37°C in Luria-Bertani medium (36). For the spore suspension preparation and kanamycin resistance assay, *Streptomyces coelicolor* M145 and *Streptomyces avermitilis* NRRL8165 and their derivatives were culti-

**TABLE 1** Strains and plasmids used in this study

| Strain or plasmid | Relevant genotype[a] | Reference or source |
|---|---|---|
| Strains | | |
| *S. coelicolor* M145 | Prototrophic derivative of *S. coelicolor* A3(2) | 3 |
| *S. venezuelae* ISP5230 | Wild type | 28 |
| *S. avermitilis* NRRL 8165 | Wild type | ATCC |
| *S. coelicolor* actII-K*OE | pK*-actII-integrated *S. coelicolor* M145 | This study |
| *S. coelicolor* actII-SOE | pS-actII-integrated *S. coelicolor* M145 | This study |
| *S. coelicolor* actII-EOE | pE-actII-integrated *S. coelicolor* M145 | This study |
| *S. coelicolor* control | pDR4-integrated *S. coelicolor* M145 | This study |
| *E. coli* JM109 | General cloning host for plasmid manipulation | Novagen |
| *E. coli* ET12567(pUZ8002) | Donor strain for conjugation between *E. coli* and *Streptomycetes* | 3 |
| *E. coli* DH5α | Host for reporter system | Novagen |
| *E. coli* BL21(DE3) | Host for expression plasmids with T7-derived promoter | Novagen |
| Plasmids | | |
| pCS26-Pac | $Kan^r$, promoterless *luxCDABE* reporter | 33 |
| pCDFDuet-1 | $Sm^r$, containing two multiple cloning sites preceded by a T7*lac* promoter and ribosome binding site (rbs), CloDF13-derived CDF replicon | Novagen |
| pHrdB | $Sm^r$; insertion of *hrdB* gene into pCDFDuet-1 | This study |
| pK-Lux | $Kan^r$, pCS26-Pac containing the *kasO*p | This study |
| pK1-Lux | $Kan^r$, pCS26-Pac containing the $kasOp_1$ | This study |
| pK2-Lux | $Kan^r$, pCS26-Pac containing the $kasOp_2$ | This study |
| pK3-Lux | $Kan^r$, pCS26-Pac containing the $kasOp_3$ | This study |
| pK4-Lux | $Kan^r$, pCS26-Pac containing the $kasOp_4$ | This study |
| pACYC184 | $Cm^r$, $Tc^r$, $rep^{p15A}$ | NEB |
| pScbR2 | $Cm^r$; for ScbR2 expression in the reporter system | 27 |
| pET23b::*scbR* | For recombinant expression of ScbR protein | 27 |
| pET23b::*scbR2* | For recombinant expression of ScbR2 protein | 27 |
| pScbR | $Cm^r$; for ScbR expression in the reporter system | This study |
| pDR2 | Double-reporter vector containing a *xylE-neo* cassette | 34 |
| pDR3 | Multiple-cloning site inserted in pDR2 | This study |
| pIJ963 | Contains the hygromycin resistance gene | Presented by K. F. Chater |
| pDR4 | Apramycin resistance gene replaced by hygromycin resistance gene in pDR3 | This study |
| pDR4-K | pDR4 inserting *kasO*p | This study |
| pDR4-K1 | pDR4 inserting $kasOp_{314}$ | This study |
| pDR4-K* | pDR4 inserting $kasOp_{361}$ (*kasO*p*) | This study |
| pDR4-K3 | pDR4 inserting $kasOp_{382}$ | This study |
| pDR4-K4 | pDR4 inserting $kasOp_{3154}$ | This study |
| pDR4-E | pDR4 inserting *ermE*p* | This study |
| pDR4-S | pDR4 inserting SF14p | This study |
| pK*-actII | *actII-orf4* replacing *xylE-neo* in pDR4-K* | This study |
| pS-actII | *actII-orf4* replacing *xylE-neo* in pDR4-KS | This study |
| pE-actII | *actII-orf4* replacing *xylE-neo* in pDR4-KE | This study |

[a] Cm, chloramphenicol; Kan, kanamycin; Sm, spectinomycin; Tc, tetracycline.

vated on mannitol soy flour medium (MS) agar plates, whereas *Streptomyces venezuelae* strains were cultivated on a malt extract-yeast extract-maltose medium (MYM) agar plate (3). To isolate RNA and to assay XylE activity, *S. coelicolor* strains were cultivated in liquid minimal medium supplemented with 0.2% Casamino Acids (SMM), while *S. avermitilis* and *S. venezuelae* were cultivated in yeast extract-malt extract (YEME) medium at 28°C and 250 rpm (3, 32). For Act production in *S. coelicolor*, both SMM and R2YE were used (3). Antibiotics were added appropriately as follows: kanamycin at 50 μg/ml, hygromycin at 50 μg/ml, ampicillin at 50 μg/ml, and chloramphenicol at 25 μg/ml.

**Evaluation of *kasO*p activity in *E. coli*.** All primers and synthetic oligonucleotides used in this work are listed in Table S1 in the supplemental material. Standard techniques for nucleic acid manipulation were used as described by Sambrook and Russell (36). The *kasO*p-driving Lux reporter plasmid was constructed as follows. Promoter *kasO*p was amplified from genomic DNA of *S. coelicolor* M145 by the use of primers kasOpF and kasOpR (Table S1). The PCR product was digested by XhoI and BamHI and then inserted into pCS26-Pac (37) digested with the same enzymes to get pK-Lux. The growth and bioluminescence of an *E. coli* DH5α transformant with pK-Lux were measured simultaneously. Growth was measured as optical density at 600 nm ($OD_{600}$), and the bioluminescence was measured using the same culture and a 20/20-n single tube luminometer (Turner Biosystems). For the construction of pHrdB, *hrdB* was amplified with primers HrdB-F and HrdB-R (Table S1) from genomic DNA of *S. coelicolor* M145. The PCR product and pCDFDuet-1 were both digested by NdeI and BglII and then ligated to create pHrdB. It was used to express HrdB upon isopropyl-β-D-thiogalactopyranoside (IPTG) induction. For evaluating the effect of HrdB on *kasO*p activity, pHrdB and pK-Lux were transformed into *E. coli* BL21(DE3). Bioluminescence was measured as described above.

**Serial truncation of *kasO*p and evaluation of *kasO*p variants in *E. coli*.** Promoters $kasOp_1$, $kasOp_2$, $kasOp_3$, and $kasOp_4$ were amplified with the corresponding forward primers kasOp1F, kasOp2F, kasOp3F, and kasOp4F and the same reverse primer, kasOpR (see Table S1 in the supplemental material), from genomic DNA of *S. coelicolor* M145. Similar to *kasO*p, they were introduced to XhoI-BamHI-cut pCS26-Pac to yield

plasmids pK1-Lux, pK2-Lux, pK3-Lux, and pK4-Lux, respectively. For the construction of pScbR, an *scbR* gene with an introduced Shine-Dalgarno sequence was amplified from *S. coelicolor* genomic DNA by using a pair of primers, scbRF and scbRR (Table S1). The BamHI-digested *scbR* was then inserted into pACYC184 digested with EcoRV and BamHI to obtain pScbR. To analyze the repression effect of ScbR and ScbR2 on engineered *kasO*p$_3$ *in vivo*, pScbR and the previously constructed pScbR2 (31) were transformed into DH5α bearing pK3-Lux, respectively. After 12 h of incubation, the bioluminescence of *E. coli* cultures was measured.

**Constructing and screening of a random site OA library of *kasO*p$_3$.** To construct a site OA mutant library of *kasO*p$_3$, overlap extension PCR was used with the degenerated primers kasOp3nF and kasOp3nR (see Table S1 in the supplemental material). The PCR fragments were digested by XhoI and BamHI and inserted into the promoterless Lux reporter plasmid pCS26-Pac to obtain the site OA mutant library. Afterwards, the site OA mutant library was transformed into DH5α bearing pScbR. Clones that bioluminesce similarly to DH5α harboring only pK3-Lux were selected and further sequenced.

**Electrophoretic mobility shift assay of engineered *kasO*p promoters.** His$_6$-tagged ScbR2 and ScbR were purified from *E. coli* BL21(DE3) harboring pET23b::scbR2 and pET23b::ScbR, as described by Xu et al. (31). Promoters *kasO*p$_3$, *kasO*p$_{314}$, *kasO*p$_{361}$, *kasO*p$_{382}$, and *kasO*p$_{3154}$ were amplified with the same pair of primers (KF and kasOpR) (see Table S1 in the supplemental material) using their corresponding templates, respectively. The subsequent binding experiments were performed using a modified gel mobility shift assay described previously (32). The DNA probe (5 ng) was incubated with various concentrations of purified ScbR2 or ScbR at 25°C for 30 min in 20 μl of buffer containing 20 mM Tris base (pH 7.5), 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mg/ml calf bovine serum albumin (BSA), and 5% (vol/vol) glycerol. After incubation and electrophoresis, the nondenaturing 4% (wt/vol) polyacrylamide gels were stained with SYBR Gold nucleic acid gel stain (Invitrogen) for 30 min in TBE (89 mM Tris base, 89 mM boric acid, 1 mM EDTA, pH 8.0) buffer and photographed under a UV transilluminator using a Bio-Rad Gel Doc XR system.

**Evaluation of engineered *kasO*p promoters in streptomycetes by a double-reporter method.** The *xylE-neo* double-reporter cassette was cloned into pDR2 previously in our laboratory (38). Plasmid pDR3 was derived from pDR2 by inserting the synthetic multiple cloning site MCS1 (Table S1) into the NotI-digested and blunt-ended site. Following that, plasmid pDR4 was derived from pDR3 by replacing the original apramycin resistance gene with a hygromycin resistance gene. The 1,883-bp hygromycin resistance gene was amplified from pIJ963 with a pair of primers, hygF and hygR (see Table S1 in the supplemental material). The amplified PCR products were then digested by appropriate enzymes and inserted between ApaLI and NheI sites of pDR3 to generate pDR4.

To evaluate the activities of different promoters in streptomycetes, promoters *kasO*p$_{314}$, *kasO*p$_{361}$, *kasO*p$_{382}$, and *kasO*p$_{3154}$ were all amplified with the primer pair KF and KR (see Table S1 in the supplemental material), whereas the native *kasO*p was amplified with the primer pair KOF and KR (Table S1). The PCR products were digested with BamHI and SpeI and then inserted into the corresponding site of pDR4 to create pDR4-K1, pDR4-K*, pDR4-K3, pDR4-K4, and pDR4-K, respectively. The five resulting plasmids were transformed into *E. coli* ET12567 (pUZ8002) and stably integrated into the chromosome of *S. coelicolor* M145 by site-specific recombination at the phage C31 attachment site (attB) to obtain the promoter-reporter strains via conjugation.

To compare the activities of promoters *kasO*p*, SF14p, and *ermE*p* in streptomycetes, SF14p and *ermE*p* were amplified with two pairs of primers, primer pair SF and SR and primer pair EF and ER (see Table S1 in the supplemental material). The PCR products were digested by the corresponding enzymes listed in Table S1 and inserted into pDR4 digested with the same enzymes to construct pDR4-S and pDR4-E, respectively. The two plasmids along with pDR4-K* were then introduced into *S. coelicolor* M145, *S. venezuelae* ISP5230, and *S. avermitilis* NRRL 8165 by *E. coli*/*Streptomyces* conjugation to obtain the respective promoter-reporter strains.

**RNA isolation and real-time PCR.** The promoter-reporter strains were cultivated as described above and sampled at different time points for the *S. coelicolor* strains (24, 36, 48, and 60 h), *S. venezuelae* strains (12, 24, and 36 h), and *S. avermitilis* strains (48, 72, 96, and 120 h). Total RNA was isolated per a standard procedure (3). The RNA samples were then treated with RNase-free DNase (Progema) and checked by PCR to eliminate the possibility of chromosomal DNA contamination.

First-strand cDNA synthesis was carried out using 2 μg of each RNA sample and a SuperScript III cDNA synthesis kit (Invitrogen), following the manufacturer's instructions. Real-time PCR was performed using selected genes and an LC-480 II real-time PCR detection system (Roche) and an Ultra SYBR mixture (with Rox). Ten percent of the cDNA synthesis reaction mixture was used as a template for each subsequent PCR using primers KMF and KMR (see Table S1 in the supplemental material) for the detection of the kanamycin restriction gene (*neo*), primers hrdBFv and hrdBRv (Table S1) for the detection of the *hrdB* gene in *S. venezuelae*, and primers hrdBFc and hrdBRc (Table S1) for the detection of the *hrdB* gene in *S. coelicolor* and in *S. avermitilis*. Real-time quantitative PCR (qPCR) parameters were set as follows: 95°C for 10 min followed by 40 two-step amplification cycles consisting of 15 s of denaturation at 95°C and 60 s of annealing and extension at 60°C. The results were analyzed by the use of LC-480 II software v2.0.1, and the relative expression levels of target genes were normalized internally to the *hrdB* level. Relative transcript levels were quantified by the $2^{-\Delta\Delta CT}$ method (39) and are shown as relative fold changes. All sample assays were conducted in triplicate.

**Evaluation of kanamycin resistance level and XylE activity.** About $10^5$ spores of promoter-reporter strains were spread on plates containing 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml, 600 μg/ml, and 800 μg/ml kanamycin and 50 μg/ml hygromycin and then incubated at 28°C for 4 days. The kanamycin resistance levels were chosen in accordance with the kanamycin concentration that resulted in a plate with just no perceptible growth where the plate corresponding to the next-lowest kanamycin concentration showed poor growth.

Quantitative measurement of total catechol-2,3-dioxygenase activity (XylE) in cell extracts was performed by a method described previously (40). The XylE activity was tested at the stationary phase: 48 h for *S. coelicolor*, 24 h for *S. venezuelae*, and 96 h for *S. avermitilis*. The XylE activity was calculated as the rate of change in optical density at 375 nm per minute per milligram of protein.

**Measuring the Act production level.** The 768-bp *actII*-ORF4 fragment was amplified with primers ActF and ActR (see Table S1 in the supplemental material) and digested by SpeI and KpnI. The reporter plasmids pDR4-K*, pDR4-S, and pDR4-E were cut by SpeI and KpnI to remove the *xylE-neo* reporter cassette and used as a backbone. The digested *actII*-ORF4 fragment was then joined with the three plasmid backbones mentioned above to create *actII*-ORF4 overexpression plasmids pK*-*actII*, pS-*actII*, and pE-*actII*, respectively. The three plasmids along with pDR4 were conjugated into *S. coelicolor* M145 as mentioned above to generate strains *S. coelicolor* actII-K*OE, *S. coelicolor* actII-SOE, *S. coelicolor* actII-EOE, and an *S. coelicolor* control, respectively.

About $4 \times 10^8$ spores of the *actII*-ORF4 overexpression strains and the control strain which contained pDR4 were incubated in 250-ml flasks containing 100 ml SMM at 28°C and 250 rpm for 60 h. In parallel, about $2 \times 10^8$ spores were cultured in 250-ml flasks containing 100 ml R2YE liquid medium at 28°C and 250 rpm for 48 h when Act but not Red was produced. Act production levels under both sets of growth conditions were assayed as described by Hopwood et al. (3).

## RESULTS

***kasO*p is recognized by HrdB in *E. coli*.** The putative TSS of *kasO*p, which is 35 nucleotides (nt) upstream of the *kasO* translation start site, was determined previously by high-resolution S1 nuclease protection analysis (30) (Fig. 1A). Upstream of the *kasO*

TSS, two binding sites for GBL receptor ScbR, designated sites OA and OB, were identified. Site OA lies at −15 to −33 nt overlapping the core promoter region, whereas site OB lies at −222 nt to −242 nt (30) (Fig. 1A). The pseudo-GBL receptor ScbR2 binds only the OB site (32). To evaluate *kasO*p's activity without influence from ScbR and ScbR2, it was inserted into the Lux reporter plasmid and transformed into *E. coli* DH5α. The bioluminescence curve indicated that, in contrast to the expression pattern in *S. coelicolor* (see Fig. S1 in the supplemental material), *kasO*p exhibited continuous expression in heterologous host *E. coli* (Fig. 1B). To verify that *kasO*p was recognized by HrdB, a *hrdB* expression plasmid was constructed and transformed into *E. coli* BL21(DE3) harboring the *kasO*p reporter plasmid pK-Lux. As shown in Fig. 1C, the bioluminescence of the resulting transformants was significantly boosted after IPTG induction of HrdB expression. The results indicate that *kasO*p is indeed recognized by HrdB.

**Identification of engineered promoters not repressed by ScbR2.** To abolish the binding site of ScbR2 and determine the optimal length of *kasO*p, four 5′-truncated promoters of *kasO*p, designated *kasO*$p_1$, *kasO*$p_2$, *kasO*$p_3$, and *kasO*$p_4$, were amplified (Fig. 1A). The four truncated promoters were inserted into the Lux reporter plasmid and transformed into DH5α. The bioluminescence of those transformants was measured. As shown in Fig. 2A, four truncated promoters (without site OB) all displayed higher activities than the full-length promoter *kasO*p, especially the 97-bp *kasO*$p_3$, which showed activity that was nearly 40 times higher. To confirm that the binding of ScbR2 had been abolished *in vivo*, plasmid pScbR2 (31), which could constitutively express ScbR2, was transformed into the DH5α bearing the *kasO*$p_3$ reporter plasmid. The bioluminescence of the resulting strain was unaffected by the expression of ScbR2, thus verifying that *kasO*$p_3$ was no longer controlled by ScbR2 (Fig. 2B). In contrast, in DH5α harboring both the *kasO*$p_3$ reporter plasmid and ScbR expression plasmid pScbR, bioluminescence was obviously repressed (Fig. 2B), indicating that the *kasO*$p_3$ was still repressed by ScbR. In contrast to the *kasO*$p_3$ results, the bioluminescence of pK-Lux transformants was unaffected in the presence of the pACYC184 plasmid but was severely repressed by plasmid expressing ScbR or ScbR2 (Fig. 2B). The conclusions mentioned above were further supported by the results of an *in vitro* electrophoretic mobility shift assay (EMSA), which showed that ScbR2 could not bind *kasO*$p_3$ whereas ScbR could still bind *kasO*$p_3$ (see Fig. S2 in the supplemental material).

**Identification of *kasO*$p_3$ mutant promoters not repressed by ScbR.** The ScbR binding OA site overlaps the −10 and −35 regions of *kasO*p. Besides the core promoter region, the sequence between the −10 and −35 regions is also important for promoter activity. To abolish the binding of ScbR while preserving the strength of the promoter, a random-site OA mutant library of *kasO*$p_3$ was constructed. The mutants were created by using a pair of degenerate primers and then inserted into the Lux pCS26-Pac reporter plasmid to form a promoter library. The promoter library was transformed into DH5α bearing pScbR (Fig. 3A). Facilitated by the two-plasmid reporter system, the promoter library was conveniently screened. Four *kasO*$p_3$ mutants (designated *kasO*$p_{314}$, *kasO*$p_{361}$, *kasO*$p_{382}$, and *kasO*$p_{3154}$) showed strong luminescence unaffected by the presence of ScbR (Fig. 3B). The four mutants were subsequently sequenced, confirming that the OA sites were indeed altered (Fig. 3C). Furthermore, *in vitro*, EMSA was performed to verify that these mutants had lost the ability to bind ScbR or ScbR2 (see Fig. S3 in the supplemental material for the *kasO*$p_{361}$ EMSA result).

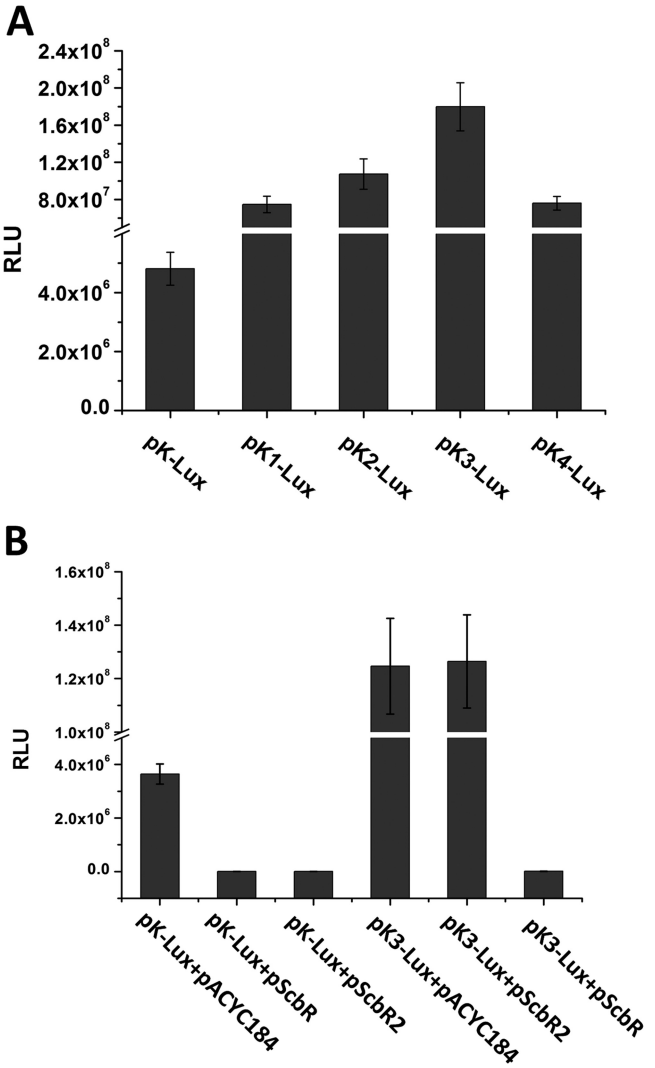


**FIG 2** Truncation of *kasO*p to remove the binding site OB and obtain the optimal upstream length. Bioluminescence levels were detected in *E. coli* DH5α containing various plasmid combinations. All values are in relative light units (RLU) and represent the averages of the results of at least three independent readings. Error bars indicate means ± SDs.

**Evaluation of the strength of different engineered promoters in *Streptomycetes*.** The engineering and evaluation of *kasO*p-based promoters mentioned above were carried out in *E. coli*. To evaluate their performance in streptomycetes, *kasO*$p_{314}$, *kasO*$p_{361}$, *kasO*$p_{382}$, and *kasO*$p_{3154}$ as well as the native *kasO*p were cloned in front of the *xylE-neo* double-reporter cassette and integrated into *S. coelicolor* M145 by conjugation. XylE activities of these transformants were monitored in SMM after 36 h of growth. The results revealed that the four engineered promoters show much higher activities than the native one (see Fig. S4 in the supplemental material). Similar to the results obtained in *E. coli*, *kasO*$p_{361}$ exhibited the highest activity in *S. coelicolor* M145 (Fig. S4). So we chose *kasO*$p_{361}$ for further evaluation and designated it *kasO*p*.

To systematically compare the strength of *kasO*p* with that of *ermE*p* and SF14p in streptomycetes, their activities were evalu-

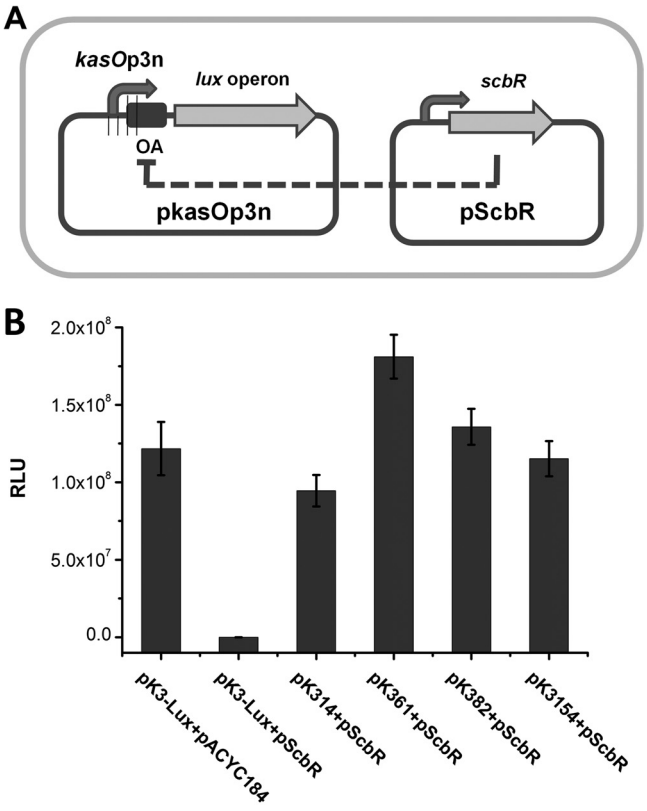


**FIG 3** Reporter system for the screening of randomized *kasO*p$_3$ mutants not repressed by ScbR. (A) Schematic representation of the reporter system, which bears two plasmids: a ScbR expression plasmid and a reporter plasmid. The promoter library was constructed by inserting the mutant promoters into the reporter plasmid. The bioluminescence of the reporter indicates whether the mutant promoters were regulated by ScbR. (B) Bioluminescence levels of the strains in which ScbR repression was abolished screened by the two-plasmid reporter system at the time of the stationary phase. (C) Sequences of the site OA region of native *kasO*p and four mutants not repressed by ScbR. Promoter *kasO*p$_{361}$ is renamed *kasO*p*. RBR is the abbreviation of Random mutational Binding Region. The gray letters represent the sequence of the partial −35 and −10 region. The dashed arrows represent the palindromic sequence of the OA site.

ated in three *Streptomyces* strains. These promoters were inserted into a *xylE-neo* reporter plasmid (pDR4) and integrated into the genomes of *S. coelicolor*, *S. venezuelae*, and *S. avermitilis*. First, the kanamycin resistance levels of these strains were evaluated. As shown in Table 2, *kasO*p* conferred much higher kanamycin resistance than *ermE*p* and SF14p, especially in *S. venezuelae*. Also, XylE (the second reporter) activity assay showed a similar trend of higher expression levels under the control of *kasO*p* (see Fig. S5 in the supplemental material). Furthermore, at the transcription level, real-time qPCR was performed and showed that the mRNA levels of *neo* were markedly higher under the control of *kasO*p* in all three different *Streptomyces* hosts during both the exponential (Fig. 4A) and stationary growth phases (Fig. 4B).

To compare the expression dynamics of *kasO*p* with those of the known strong promoter SF14p and *ermE*p*, the relative expression levels of reporter genes under the control of *kasO*p*, SF14p, *ermE*p*, and native *kasO*p were profiled by qPCR during growth of *S. coelicolor* M145 containing the corresponding plasmids in liquid medium (see Fig. S1 in the supplemental material). As expected, the activity of native *kasO*p rapidly declined below the expression level of *ermE*p* after 24 h, whereas *kasO*p* constantly exhibited a higher level of expression than *ermE*p* and SF14p at all time points. The increases of *kasO*p* activity relative to *kasO*p activity were significant at all time points: a 1.8-fold increase at the peak level (24 h), an 85-fold increase at 12 h, a 69-fold increase at 36 h, a 112-fold increase at 48 h, and an 82-fold increase at 60 h. In addition, it is worth noting that the reporter expression level under the control of all promoters did not remain constant.

**Evaluation of *kasO*p* performance in overexpressing Act.** Act is an aromatic polyketide produced by *S. coelicolor* during the stationary phase (41). The pathway-specific activator of the Act biosynthesis gene cluster, *actII*-ORF4, controls the onset of Act biosynthesis (42). Overexpression of *actII*-ORF4 on a multicopy plasmid resulted in increased Act production (42). Manipulation of regulatory genes that govern secondary metabolite production is an effective strategy to improve antibiotic production levels (43). Here, the performance of *kasO*p* in increasing Act production by overexpressing *actII*-ORF4 was tested and compared with that of SF14p and *ermE*p*. As shown in Fig. 5, in two different fermentation media, the highest Act production levels was detected in strain actII-K*OE, which contains the *kasO*p*-based overexpression plasmid. The Act production in R2YE with strain actII-K*OE was significantly higher than the levels seen with the *ermE*p* and SF14p overexpression strains in SMM.

## DISCUSSION

In *S. coelicolor*, *kasO*p is a relatively well-characterized promoter with a core promoter region highly similar to the consensus sequence recognized by the housekeeping sigma factor HrdB (30). Previously, *in vitro* reconstitution of *E. coli* RNA polymerase core

**TABLE 2** Kanamycin resistance levels conferred by different promoters in different *Streptomyces* strains

| Strain | Promoter | Growth result at indicated kanamycin concn (μg/ml)[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 50 | 100 | 200 | 400 | 600 | 800 |
| *S. coelicolor* | *kasO*p* | + | + | + | + | + | + |
| | SF14p | + | + | + | + | − | − |
| | *ermE*p* | + | + | + | + | − | − |
| *S. venezuelae* | *kasO*p* | + | + | + | + | + | + |
| | SF14p | + | + | − | − | − | − |
| | *ermE*p* | + | + | − | − | − | − |
| *S. avermitilis* | *kasO*p* | + | + | + | + | + | + |
| | SF14p | + | + | + | + | + | − |
| | *ermE*p* | + | + | − | − | − | − |

[a] The symbols + and − indicate growth and no growth, respectively.

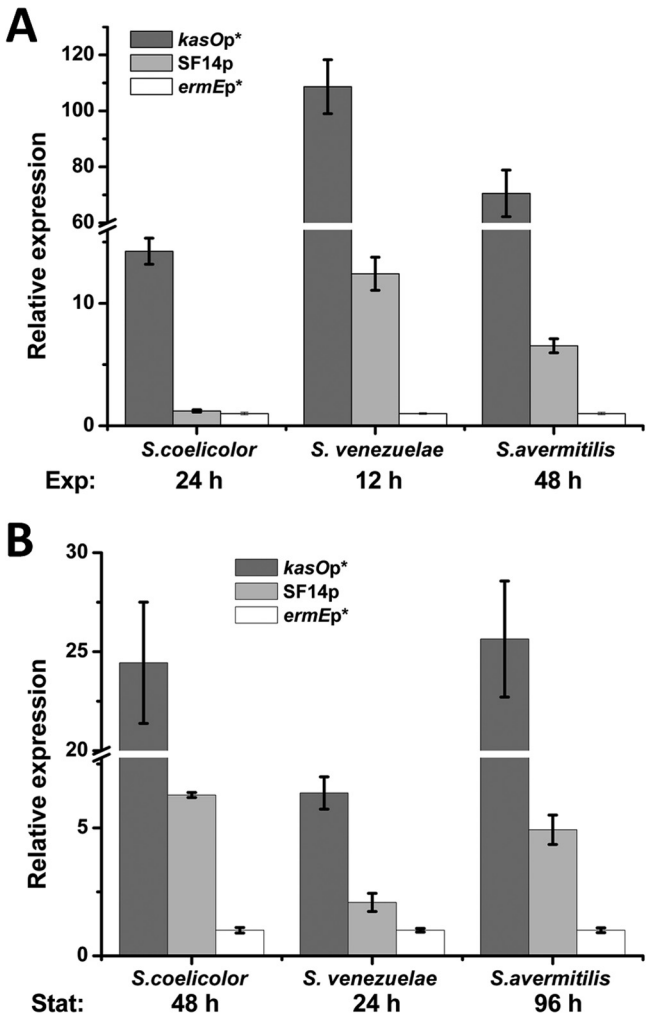


**FIG 4** Quantitative analysis of the transcriptional profiles of *xylE-neo* double reporter genes in *S. coelicolor* M145, *S. venezuelae* ISP5230, and *S. avermitilis* NRRL8165 by real-time qPCR. Panels A and B show the relative expression levels of the *neo* gene in the exponential phase and stationary phase, respectively. Relative values were obtained using *hrdB* as the internal reference. The relative values of *ermE*p*-directed expression were arbitrarily assigned as 1 in the respective three strains. Error bars indicate means ± SDs.

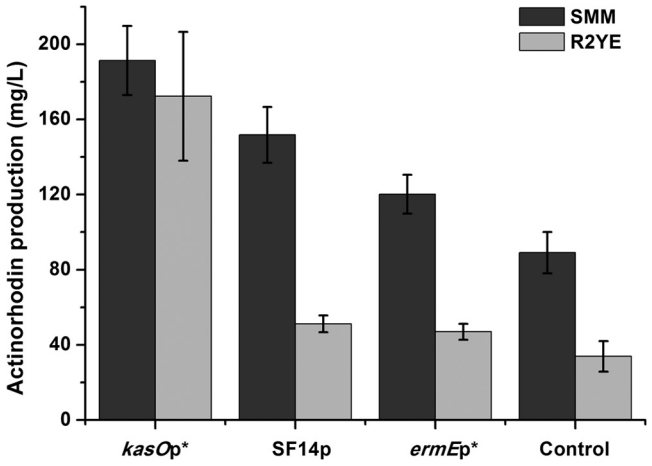


**FIG 5** Act production levels in the *S. coelicolor* strains overexpressing *actII*-ORF4 under the control of different promoters. The dark gray bars display the production of Act in SMM at 60 h, and the light gray bars show the production of Act in R2YE medium at 48 h. Data are expressed as average values and standard deviations (SD) of the results of three parallel studies. The results are all statistically significant ($P < 0.05$).

enzyme activity with *Streptomyces* sigma factors (including a HrdB homologue) was reported (44, 45), indicating that *E. coli* RNA polymerase could recognize *Streptomyces* sigma factors. Here, the increased bioluminescence of *E. coli* containing pK-Lux in response to HrdB expression also suggests that the *E. coli* RNA polymerase core enzyme can recognize HrdB. Two rational steps were taken to remove or abolish ScbR and ScbR2 binding sites in *kasO*p. The first step was removing the ScbR/ScbR2 binding site at −222 nt to −242 nt by truncating *kasO*p. As shown in Fig. 2A, the activity of the shortened promoters exhibited an continuous increase until the optimal length was reached. The second step was to abolish the ScbR binding site overlapping the −35-to-−10 region while still preserving promoter strength. This could not be easily accomplished because the promoter strength was also dependent on this region. Therefore, a *kasO*p$_3$ mutant library with random sequences in the ScbR binding site was constructed and screened to obtain the best promoter. Several ScbR-deregulated promoters were identified; among them, *kasO*p* (*kasO*p$_{361}$) showed the highest activity (Fig. 3B).

A good reporter system is extremely useful for promoter evaluation, particularly for screening a promoter library. As shown in Fig. 3A, our reporter system contains a Lux reporter plasmid and a protein expression plasmid, which has greatly facilitated the screening and identification of mutant promoters no longer repressed by ScbR. Moreover, it is applicable in the verification of other promoter-regulator interactions as long as the promoter is recognized by the *E. coli* RNA polymerase holoenzyme. *E. coli* worked well as a heterologous host for the evaluation of *Streptomyces* promoters recognized by the essential sigma factor HrdB in this instance: it provided a relatively clean background without the noise presented by *Streptomyces*. The promoter activity detected in *E. coli* showed good agreement with the results obtained in *Streptomyces* (see Fig. S4 in the supplemental material), which is probably due to the fact that the core promoter recognized by the essential sigma factor HrdB of *S. coelicolor* is very similar to those recognized by the housekeeping sigma factor ($\sigma^{70}$) of *E. coli*. However, promoters recognized by other *Streptomyces* sigma factors may not be recognized in *E. coli*.

Characterization of the strength and host range of promoters *in vivo* is a critical step before application in metabolic engineering and synthetic biology (10, 11). To our knowledge, although many promoters have been well defined by S1 nuclease mapping over a time course, none has been dynamically evaluated by real-time PCR in a whole growth phase in different *Streptomyces*. In this work, the temporal expression profiles of three different promoters were compared in three streptomycetes strains: *S. coelicolor*, *S. venezuelae*, and *S. avermitilis* (Fig. 4; see also Fig. S5 in the supplemental material). The evaluations at both the transcription and protein levels demonstrated that *kasO*p* is the strongest promoter followed by SF14p and *ermE*p*. This is most likely due to the differences in promoter sequences and structures. As mentioned earlier, both *ermE*p* and SF14p are heterogenous promoters; thus, their core promoter sequences may not be recognized by HrdB efficiently, or they are recognized by totally different sigma

factors. Additionally, both *ermE*p* and SF14p contain two overlapping core promoter regions (5, 6), which could result in sterical hindrance for the binding of RNA polymerase holoenzymes and hence affect transcription efficiency depending on the spacing between the two regions (46). In contrast, *kasO*p is an innate promoter of *Streptomyces*, whose core promoter with upstream and downstream regions is relatively optimized to allow efficient interaction with HrdB and RNA polymerase. It had been shown that these regions have a significant influence on transcription efficiency (47, 48). Preservation of the regions upstream of −35 and downstream of −10 in *kasO*p may help *kasO*p* perform consistently in various genetic contexts of streptomycetes. Although the optimal upstream sequence was selected in *E. coli*, the resulting 97-bp $kasOp_3$ also displayed the strongest activity in *S. coelicolor* (see Fig. S4 in the supplemental material). The fact that $kasOp_3$ showed higher activity than the 77-bp $kasOp_4$ in *E. coli* clearly demonstrates that the 20-bp region 5′ of the −35 site is important for transcription efficiency. This region is reported to be involved in the interaction with the carboxy-terminal domain of the RNA polymerase holoenzyme α subunit (49). Another critical factor determining promoter strength is the spacer length between the −35 and −10 sites. For *ermE*p*, the lengths of the two spacers between two putative −35 and −10 sites are 14 and 17 bp, whereas for SF14p, the lengths are 17 and 19 bp, respectively (5, 6). They are different from the 18-bp spacer of *kasO*p*. So 18 bp may be the optimal spacer length for HrdB-recognized promoters, but this needs further investigation.

Moreover, the transcription of *xylE-neo* reporter genes driven by the three promoters *kasO*p*, SF14p, and *ermE*p* was dynamically evaluated in *S. coelicolor* (see Fig. S1 in the supplemental material). To our surprise, the generally accepted constitutive promoter, *ermE*p*, was not constantly expressed during growth according to the temporal profile of reporter gene *neo*. Similarly, *kasO*p* and SF14p also peaked at the exponential phase and declined thereafter, showing obvious growth-dependent activity (see Fig. S1 in the supplemental material). If we take an open view of the concept of constitutive expression, these results are not surprising at all. Recent work revealed that the expression of essential sigma factor HrdB in *Streptomyces* is also growth phase dependent (44, 50); thus, the activities of promoters recognized by HrdB should follow a similar pattern. To our knowledge, a real constitutive promoter should drive gene expression constantly throughout a growth phase; this may demand a multiplexer promoter that could be continuously transcribed by different sigma factors. The rRNA promoter(s) in *S. coelicolor* may be such a promoter, having four transcriptional start sites probably initiated by different sigma factor and core RNA polymerase combinations (51). Similarly, in *Bacillus subtilis*, the widely used constitutive promoter $P_{43}$ contains two tandem core promoters recognized by $\sigma^{55}$ during growth and by $\sigma^{37}$ during the stationary phase, respectively (52).

Act production was greatly increased in *S. coelicolor* when *actII-ORF4* was overexpressed by *kasO*p* compared to that seen with overexpression by SF14p and *ermE*p*, again indicating that *kasO*p* was a stronger promoter. However, the increase in Act production did not correlate with the increase in expression levels reflected by qPCR results, where *kasO*p* shows remarkable superiority relative to the other two promoters (Fig. 4 and 5). This could be due to an imbalance between metabolic flux and gene expression levels. This explanation is supported by the Act fermentation results in different media, which may supply different levels of biosynthetic precursors for Act.

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