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CRISPR–Cas9 strategy for activation of silent *Streptomyces* biosynthetic gene clusters

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Here we report an efficient CRISPR–Cas9 knock-in strategy to activate silent biosynthetic gene clusters (BGCs) in streptomycetes. We applied this one-step strategy to activate multiple BGCs of different classes in five *Streptomyces* species and triggered the production of unique metabolites, including a novel pentangular type II polyketide in *Streptomyces viridochromogenes*. This potentially scalable strategy complements existing activation approaches and facilitates discovery efforts to uncover new compounds with interesting bioactivities.

Microbial natural products are a rich source of pharmaceutical agents, and current advances in genomics have unveiled a vast source of unexplored BGCs. Because the majority of encoded metabolites of these BGCs are undetectable using current analytical methods, due to minimal or zero BGC expression under laboratory conditions (commonly defined as silent BGCs[1]), strategies to activate BGC expression and trigger metabolite production are critical to realize the full potential of Nature's chemical repertoire[1]. While heterologous expression bypasses native regulation networks and can be engineered rationally[2], entire biosynthetic pathways, often spanning large areas of genomes, will have to be cloned and refactored[3,4]. Additionally, heterologous hosts may lack regulatory, enzymatic, or metabolic requirements for product biosynthesis. Inducing cluster expression in native hosts circumvents these limitations but may be hindered by low homologous recombination efficiency. Technologies that improve genetic manipulation of streptomycetes will expedite discovery and characterization of BGCs in their native contexts, as well as guide improvement of heterologous systems[5].

CRISPR technology has enabled the genetic manipulation of many genetically recalcitrant organisms[6,7]. The *Streptococcus pyogenes* CRISPR–Cas9 system was recently reconstituted in model streptomycetes to delete genes and entire BGCs and to perform site-directed mutagenesis and gene replacement with significantly improved efficiencies[8–11]. Here, we extend this CRISPR–Cas9 technology to perform strategic knock-in for the activation of silent BGCs in native *Streptomyces* hosts (**Fig. 1a**).

To demonstrate that CRISPR–Cas9 can be used to efficiently and precisely introduce heterologous promoters into *Streptomyces* genomes for BGC activation, we selected well-characterized pigment BGCs, namely the indigoidine cluster in *Streptomyces albus*[12], as well as the actinorhodin (ACT) and undecylprodigiosin (RED) clusters in *Streptomyces lividans*[13,14]. Using CRISPR–Cas9-mediated knock-in, we replaced upstream promoter regions of main biosynthetic operons or pathway-specific activators with constitutive promoters that are stronger than the commonly used *ermE** promoter and work in multiple *Streptomyces* species (**Supplementary Results**, **Supplementary Fig. 1**)[15,16]. In *S. albus*, use of CRISPR–Cas9 increased the knock-in efficiency of the *kasO** promoter (*kasO**p) upstream of the *indC-like* indigoidine synthase gene (**Fig. 1b**). Higher knock-in efficiency observed when using 2-kb as compared to 1-kb homologous arms is consistent with homology-directed repair of Cas9-induced double-stranded breaks. Co-introduction of longer inserts, such as the ~1-kb thiostrepton-resistance cassette (*tsr*) with *kasO**p, was achieved at lower efficiencies, but was still more efficient than inserting *kasO**p alone without CRISPR–Cas9. Selected solely on apramycin, *tsr-kasO**p knock-in strains grew on thiostrepton plates while maintaining pigment production, as expected of an activated indigoidine synthase cluster (**Fig. 1c**, **Supplementary Fig. 2**). As for *S. albus*, recovery of desired *kasO**p knock-in strains in *S. lividans* was greatly enhanced with the use of CRISPR–Cas9 (**Fig. 1b**). Confirming activation of the RED and ACT clusters, the engineered strains produced red undecylprodigiosin and pH-responsive actinorhodin-related metabolites, respectively (**Fig. 1d**, **Supplementary Figs. 3–5**).

Together, these results demonstrated that CRISPR–Cas9 can be used to precisely introduce heterologous genetic elements into *Streptomyces* genomes, at relatively high efficiencies, to initiate secondary metabolite production from silent BGCs. The enhanced knock-in efficiencies allowed the use of donor DNA with shorter homology flanks as well as the introduction of larger genetic elements, both of which would have been challenging without CRISPR–Cas9. While homologous recombination occurs efficiently using conventional methods in model strains like *S. lividans* and *S. albus*, the efficiency increase afforded by CRISPR–Cas9 is critical in allowing genetic manipulation of otherwise challenging strains such as *Streptomyces roseosporus* (**Fig. 1b**).

Next, we employed this strategy to activate two unexplored, silent BGCs from *S. roseosporus* with relatively high homology to known BGCs. The *S. roseosporus* NRRL15998 genome contains 29 predicted BGCs[17], the majority of which have yet to be characterized (**Supplementary Table 2**)[18]. One of the predicted BGCs showed >90% sequence identity to the polycyclic tetramate macrolactam (PTM) cluster in *Streptomyces griseus* (**Supplementary Table 3**), which was refactored for expression in *S. lividans* by introducing individual promoters in front of each of the six cluster genes[19]. Notably, insertion of a single strong promoter failed to drive cluster expression in the heterologous system[19]. Here in the native *S. roseosporus* host, knock-in of *kasO**p upstream of the first open reading frame (ORF) was sufficient to drive expression of PTM biosynthetic genes (**Supplementary Fig. 6**) and yielded the production of photocyclized alteramide A (**1**, *m/z* 511.2808, $[M+H]^+$) and a second PTM **2** (*m/z* 513.2961, $[M+H]^+$) with the same planar structure as dihydromaltophilin (**Fig. 2a**, **Supplementary Note**). Since alteramide A photocyclization is spontaneous[20], we surmised that alteramide A was the original

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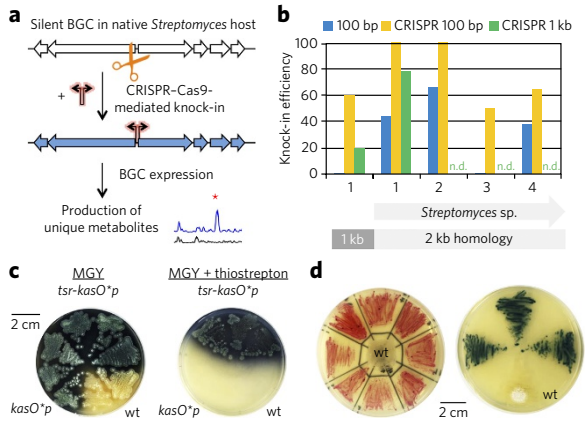


Figure 1 | CRISPR–Cas9-based promoter knock-in strategy to activate silent biosynthetic gene clusters in streptomycetes. (**a**) Using CRISPR–Cas9, efficient and precise introduction of promoter cassettes (red-shaded arrows) drives expression of biosynthetic genes (blue) and triggers the production of unique metabolites (*) that are not detected in the parent strain. (**b**) Knock-in efficiencies with and without use of CRISPR–Cas9 in different *Streptomyces* species: (1) *S. albus*, (2) *S. lividans*, (3) *S. roseosporus*, and (4) *S. venezuelae*. See **Supplementary Table 1** for actual values. For *S. albus*, the knock-in efficiencies for different-sized inserts (100-bp versus 1-kb) using editing templates with different homology lengths (1-kb versus 2-kb) were examined. No knock-in was observed for *S. roseosporus* without CRISPR–Cas9. n.d., not determined. (**c**) Wild-type (wt) or indicated engineered *S. albus* strains on MGY or MGY + thiostrepton plates. (**d**) Wild-type and engineered *S. lividans* strains with activated RED (left) or ACT (right) clusters on MGY plates. Ammonia fuming confirmed production of pH-sensitive actinorhodin-related pigments (**Supplementary Fig. 4**).

metabolite produced by *S. roseosporus*. Interestingly, **2** was not identified in the previous study involving the almost identical *S. griseus* cluster[19], suggesting that there may be host-dependent factors or differences between native and heterologous hosts.

S. roseosporus also possesses a phosphonate BGC with genes showing high homology and synteny to the *Streptomyces rubellomurinus* FR-900098 BGC (**Supplementary Table 4**)[21,22]. Intriguingly, a BLASTP search within ~2,000 NCBI-deposited actinobacteria assemblies for FR-900098 biosynthetic enzymes did not uncover similar BGCs (accessed in May 2016), suggesting that *S. roseosporus* has the uncommon biosynthetic potential to synthesize the antimalarial compound, which to date has only been attributed to *S. rubellomurinus* and *Streptomyces lavendulae*[21]. To determine whether *S. roseosporus* can produce FR-900098, we introduced a bidirectional *P8-kasO*p* promoter cassette to drive expression of the putative *frbD* operon and *frbC* homolog (**Supplementary Figs. 8** and **9**). The engineered strain produced **3** with ^{31}P-NMR, heteronuclear multiple bond correlation (HMBC), and mass values consistent with FR-900098 (**Fig. 2b**, **Supplementary Note**), validating the inherent ability of *S. roseosporus* to make FR-900098. The estimated FR-900098 titer of 6–10 mg/L in the engineered strain, while lower than the 22.5 mg/L reported for *S. rubellomurinus*[21], is ~1,000-fold higher than the minimum inhibitory concentration against the malarial parasite[23], suggesting that this activation strategy may facilitate bioactivity-guided discovery.

We next asked whether this activation strategy can be generally applied to uncharacterized BGCs of different classes in multiple *Streptomyces* species, namely *S. roseosporus* (**Supplementary Table 2**), *Streptomyces venezuelae* (**Supplementary Table 5**), and *Streptomyces viridochromogenes* (**Supplementary Table 6**). For pathway-specific activation, we targeted single and bidirectional promoter cassettes to the first ORF(s) of the main biosynthetic operon(s) predicted on the basis of either gene directionality alone or, if available, predicted transcriptional activators (**Supplementary Figs. 10** and **11**). Introduction of single- or bidirectional promoter cassettes into additional clusters in *S. roseosporus* and *S. venezuelae* led to the production of unique compounds that were not observed in the parent strains (**Fig. 2c–e**). For example, cluster 3 in *S. roseosporus* was predicted to encode a nucleoside type I polyketide synthase (PKS) with biosynthetic enzymes for incorporation of a 3-amino-5-hydroxybenzoic acid starter unit and formation of a naphthalene ring. Insertion of *kasO*p* upstream of the main synthase gene, encoding a loading domain and three PKS modules, triggered the production of a major metabolite with *m/z* 405 (**Fig. 2c**). A distinct compound with *m/z* 780 was observed for another engineered *S. roseosporus* strain in which *kasO*p* was introduced upstream of a predicted LuxR-type regulator within a type I PKS cluster (**Fig. 2d**). In *S. venezuelae*, insertion of a bidirectional promoter cassette between a type III PKS gene encoding an RppA synthase and a cytochrome P450 gene resulted in production of pigmented products (**Fig. 2e**). Production of these

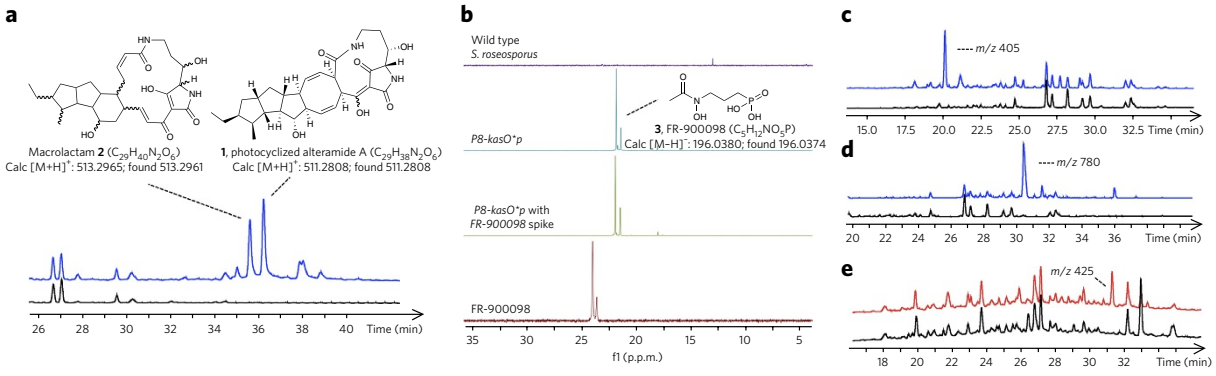


Figure 2 | Activation of biosynthetic gene clusters in multiple streptomycetes. (**a**) HPLC analysis of ethyl acetate extracts from wild-type *S. roseosporus* and an engineered strain in which *kasO*p* was introduced into cluster 24. Indicated are the two major PTM compounds isolated from the engineered strain. Stereochemistry was not assigned for **2**. See **Supplementary Figure 7** and **Supplementary Note** for full chromatograms and chemical characterization data. (**b**) ^{31}P-NMR spectra of methanol extracts from wild-type *S. roseosporus* and an engineered strain in which a bidirectional *P8-kasO*p* cassette was introduced into the phosphonate cluster 10. Also shown are the ^{31}P-NMR spectra of authentic FR-900098 sample and FR-900098 spiked into the extract of the *P8-kasO*p* strain. HRMS and HMBC analyses confirmed FR-900098 production (**Supplementary Note**). Difference in chemical shift of authentic and spiked FR-900098 is due to difference in sample pH[22]. (**c–e**) LC-MS analyses of culture extracts from wild-type (black) and, engineered *S. roseosporus* (blue) and from *S. venezuelae* (red) strains in which *kasO*p* was introduced into the respective clusters. Major metabolites uniquely produced by the engineered strains and not observed in wild-type strains are highlighted with their indicated *m/z* values. We note that there are additional differences between the metabolic profiles of the engineered and the respective wild-type strains. For details, full chromatograms and mass spectra, see **Supplementary Figures 12–14**.

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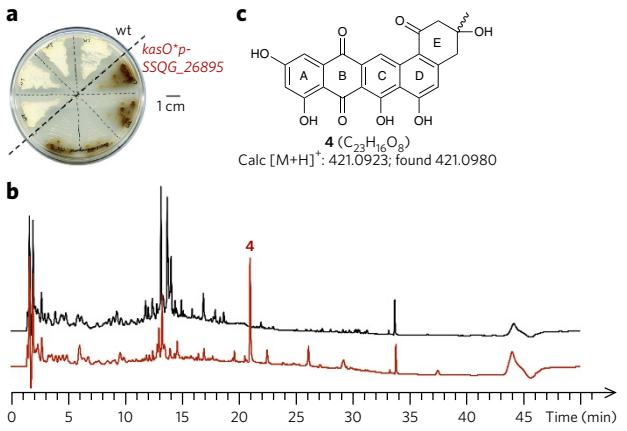


Figure 3 | Activation of type II PKS biosynthetic gene cluster in *S. viridochromogenes* yields a novel pigmented compound. (**a**) Production of brown pigment by the engineered strain but not wild-type (wt) *S. viridochromogenes* on MGY medium. (**b**) HPLC analysis of extracts from an engineered *S. viridochromogenes* strain harboring a *kasO*p* knock-in in front of SSQG_RS26895 (red) and the parent wt strain (black). Indicated is the major metabolite **4** that is uniquely produced by the engineered strain. Here, we focus on the major distinct metabolite produced by the engineered strain, but we note that there are additional differences between the engineered and wt strains (**Supplementary Fig. 15**). (**c**) Structure of **4**. The five rings are labeled A–E.

newly observed metabolites was independently validated at least three times in solid and liquid MGY media as being unique to the respective knock-in strains and not detected in the wild-type strains.

Identification of a novel compound using CRISPR–Cas9-based promoter knock-in further demonstrates the potential of this strategy for natural product discovery. We isolated and characterized the major product selectively produced by an engineered *S. viridochromogenes* strain in which *kasO**p was inserted in front of the main biosynthetic operon, SSQG_RS26895–RS26920, of an uncharacterized type II PKS gene cluster, NZ_GG657757 (**Supplementary Fig. 15**). Except for an additional cytochrome P450, NZ_GG657757 has high homology and similar gene arrangement to a gene encoding a spore pigment BGC in *Streptomyces avermitilis* (AB070937.1). The engineered *S. viridochromogenes* strain produced an obvious brown pigment in liquid and solid media before sporulation with a major unique metabolite **4** observed by HPLC (**Fig. 3a**,**b**). HRMS of **4** predicted a molecular formula $C_{23}H_{16}O_8$. ^{1}H-NMR, ^{13}C-NMR, COSY/TOCSY, HSQC, and HMBC analyses revealed a novel polyketide with a dihydrobenzo[α] naphthacenequinone core that is shared by a family of polyketides including frankiamycin, benastatin, and pradimicin (**Fig. 3c**, **Supplementary Note**)[24]. The cyclohexanone (ring E) in **4** is atypical and has not been observed for pentangular aromatic polyketides[25]. Further mechanistic studies will elucidate the contributions of each enzyme in the biosynthesis of this pentangular polyketide.

In this study, we showed that relatively small genome perturbations in the form of promoters strategically introduced using CRISPR–Cas9 technology are sufficient to activate BGCs of different classes in multiple *Streptomyces* species including type I, II, and III PKSs, non-ribosomal peptide synthetase (NRPS), hybrid PKS–NRPS, and phosphonate clusters. Our current efforts focus on BGCs with one or two major predicted biosynthetic operons. We do not expect this strategy to work for all BGCs, especially if global transcriptional changes are required or if there are multiple major operons within the target BGC. For activation of these BGCs, more complex engineering is required involving knock-in of multiple promoters, manipulation of regulators, domain swaps and mutations—all of which may also be performed using CRISPR–Cas9. An obvious limitation of this strategy is that it requires introduction of recombinant DNA, which may be challenging for some strains, especially natural isolates. Nonetheless, the activation of multiple clusters in different *Streptomyces* species highlights the potential of this approach to complement existing strategies, including heterologous expression, to discover, characterize, and re-engineer BGCs[1]. This strategy should be generally applicable and potentially scalable to better explore the biosynthetic potential of streptomycetes.

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Methods

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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Author contributions

M.M.Z., F.T.W., and H.Z. conceived and designed the research. M.M.Z., F.T.W., Y.W., E.H., W.L.Y., R.E.C., and B.E. performed the molecular biology, conjugation and fermentation experiments. S.L. and Y.H.L. performed structure elucidation of compounds. M.M.Z., F.T.W., E.L.A., and H.Z. analyzed the data. M.M.Z., F.T.W., and H.Z. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Any supplementary information, chemical compound information and source data are available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to H.Z.

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ONLINE METHODS

Reagents and media. Unless otherwise indicated, all reagents are obtained from Sigma. 1 L of MGY medium contains 10 g malt extract broth, 4 g Bacto yeast extract (BD Biosciences), 4 g glucose (1st Base, Axil Scientific) and for MGY agar plates, an additional 20 g of Bacto agar (BD Biosciences). Conjugation experiments involving WM6026 and WM3780 *E. coli* strains were performed on R2 agar without sucrose: 0.25 g K_2SO_4, 10.12 g $MgCl_2{\cdot}6H_2O$, 10 g glucose, 0.1 g Bacto casamino acids (BD Biosciences), 5.73 g TES, 20 g agar in 1 L water, autoclaved, after which 1 mL filter-sterilized 50 mg/mL KH_2PO_4 solution and filter-sterilized 2.94 g $CaCl_2{\cdot}2H_2O$ and 3 g L-proline in 5 mL 1 N NaOH were added to the medium.

Strains and growth conditions. Strains and plasmids used in this study are listed in **Supplementary Table 7**. Unless otherwise indicated, strains are propagated in MGY medium at 30 °C. Spore preparations and conjugation protocols were similar to those described by Keiser and Bibb[26]. For spore preparations, 1:1000 of a spore preparation or 1:100 dilution of a saturated seed culture is plated on MGY plates and incubated at 30 °C until thick spores are observed. Spores were removed from the plate using 5-mm glass beads (Sigma) and resuspended in sterile TX buffer (50 mM Tris, pH 7.4, 0.001% (*v*/*v*) Triton X) by vigorous vortexing for 30 s. The eluant containing free spores was pelleted by spinning at maximum speed in an Eppendorf 5810R centrifuge for 10 min, resuspended in 1 mL sterile water and repelleted. The spores were then resuspended in water and stored at −80 °C. A typical spore prep contains ~10^7–10^9 spores per milliliter as determined by serial dilution plating.

Construction of genome editing plasmids. All DNA manipulations were carried out in *Escherichia coli* DH5α or OmniMAX™ (Thermo Fisher). Primers used in this study are listed in **Supplementary Table 8**. Restriction enzymes were obtained from New England Biolabs. Helper pCRISPomyces-2 plasmids for making promoter knock-in constructs were made by ligating adaptor sequences, containing restriction sites flanking the promoter of choice (**Supplementary Fig. 16**) to facilitate insertion of homology arms at the *Xba*I site of pCRISPomyces-2 (ref. 8). The protospacer of a target cluster was first inserted via *Bbs*I-mediated Golden Gate Assembly as previously described[8]. The helper plasmid (pCRISPomyces-2-*kasO*p*, pCRISPomyces-2-*P8-kasO*p*) was linearized using *Spe*I and assembled with the downstream homology arm (2-kb unless otherwise indicated) by Gibson assembly (New England Biolabs). The second upstream homology arm (2-kb unless otherwise indicated) was subsequently inserted by Gibson assembly using *Hin*dIII or *Nhe*I linearized construct containing the first homology arm. See **Supplementary Figure 17** for workflow to construct genome editing plasmids.

Interspecies conjugation. Promoter knock-in constructs were used to transform conjugating *E. coli* strains and colonies with the appropriate antibiotic resistance (for example, 50 mg/L apramycin) were picked into LB with antibiotics. WM6026 requires diaminopimelic acid in LB for growth, which was added to LB for subsequent wash and resuspension steps. Overnight cultures were diluted 1:100 into fresh LB with antibiotics and grown to an OD_{600} of 0.4–0.6. 400 µL of the culture was pelleted, washed twice and resuspended in LB without antibiotics. The washed *E. coli* cells were then mixed with spores at 1:5 volume ratio and spotted on R2 without sucrose plates. After incubation for 16–20 h at 30 °C, the plates were flooded with nalidixic acid and apramycin and incubated until exconjugants appear. Exconjugants were streaked onto MGY plates containing apramycin at 30 °C followed by restreaking to MGY plates at 37 °C to cure the CRISPR–Cas9 plasmid containing a temperature-sensitive origin of replication. Apramycin-sensitive clones growing at 37 °C were then subjected to validation of promoter knock-in and genome editing as described below.

Validation of promoter knock-in and genome editing. Genomic DNA from wild type and exconjugants from the indicated strains were isolated from liquid cultures using the Blood and Tissue DNeasy kit (Qiagen) after pretreating the cells with 20 mg/mL lysozyme for 0.5–1 h at 30 °C. PCR was performed using control primers beyond the homology regions or knock-in specific primers (**Supplementary Table 8**) with KODXtreme Taq polymerase (Millipore). Where indicated, PCR products were subjected to digestion with specific restriction enzymes to differentiate between PCR products of wild type genomic sequences and successful genome editing by knock-ins. Positive samples were purified using Qiaquick PCR purification kit (Qiagen) and validated by Sanger sequencing.

RNA isolation and real-time quantitative PCR (RT-qPCR). RNA from wild type and engineered *S. roseosporus* were isolated using RNeasy Midi Kit (Qiagen) 72 h after seed cultures were diluted 1:100 into 50 mL of MGY broth in 250-mL baffled flasks containing ~30–40 5-mm glass beads and growth at 30 °C. Isolated RNA was treated with DNase (Qiagen) before being reverse transcribed with random hexamers using SuperScript III (Invitrogen). RT-qPCR was performed on a Roche LightCycler 480 using SYBR FAST qPCR master mix (KAPA). The housekeeping *rpsL* gene of *S. roseosporus* was used as constitutive reference to normalize gene expression of each target gene such that *rpsL* expression = 1 (ref. 27). Technical triplicates of three biological repeats were performed per condition. Gene-specific primers are listed in **Supplementary Table 8**.

Fermentation, ethyl acetate extraction, and liquid chromatography–mass spectrometry analysis of metabolites from wild type and engineered Streptomyces strains. Liquid seed cultures (2 mL MGY) of wild type and engineered *S. roseosporus* and *S. venezuelae* strains were inoculated from a plate or spore stock in 14-mL culture tubes. Seed cultures were incubated at 30 °C with 250-rpm shaking until achieving turbidity or high particle density (typically 2–3 days). Seed cultures were diluted 1:100 into 50 mL of MGY broth in 250-mL baffled flasks containing ~30–40 5-mm glass beads and incubated at 30 °C with 250-rpm shaking (10–14 days for *S. roseosporus*, 5–7 days for *S. venezuelae*). The cultures were harvested by pelleting at maximum speed in an Eppendorf 5810R centrifuge for 10 min. The cell pellet was stored at −80 °C while the supernatants were split into two 50-mL Falcon tubes. Culture supernatants were extracted three times with equal volume ethyl acetate. For solid-state cultures, the strains were grown on MGY plates at 30 °C for 10 days. The plates were chopped into small pieces and extracted twice with ethyl acetate. Extracts were dried and resuspended in methanol and analyzed by LC-MS using ESI source in positive ion mode (Bruker, Amazon SL Ion Trap) equipped with a Kinetex 2.6 µm XB-C18 100 Å (Phenomenex). HPLC parameters were as follows: solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile; gradient at a constant flow rate of 0.2 mL/min, 10% B for 5 min, 10% to 100% B in 35 min, maintain at 100% B for 10 min, return to 10% B in 1 min, and finally, maintain at 10% B for 10 min; detection by UV spectroscopy at 210, 254, 280, and 320 nm.

Extraction and NMR analysis of phosphonate compounds. Liquid seed cultures (2 mL MGY) of wild type and engineered *S. roseosporus* strains were inoculated from a plate or spore stock into 14-mL culture tubes. Seed cultures were incubated at 30 °C with 250-rpm shaking until achieving turbidity or high particle density (typically 2–3 days). Seed cultures were diluted 1:100 into 50 mL of MGY broth in 250-mL baffled flasks containing ~30–40 5-mm glass beads and incubated at 30 °C with 250-rpm shaking for 10–14 days. The cultures were harvested by pelleting at maximum speed in an Eppendorf 5810R centrifuge for 10 min. The cell pellet was stored at −80 °C while the supernatants were split into two 50-mL Falcon tubes, flash frozen liquid nitrogen, and lyophilized to dryness. 25 and 10 mL of methanol was added to each tube containing dried supernatant and frozen cell pellets, respectively. The methanol mixtures were vortexed for 1 min each and incubated on a platform shaker at 4 °C for 2 h. Samples were clarified by spinning at maximum speed in an Eppendorf 5810R centrifuge for 10 min twice and pooling the methanol extracts from the respective pellets and lyophilized culture supernatants. A generous amount of anhydrous sodium sulfate was added to the extracts and stirred. The extracts were decanted, concentrated to dryness, and resuspended in 700 µL deuterium oxide added in two 350-µL aliquots. A spatula-full of Chelex-100 resin (Bio-Rad) was added to each sample in a 1.7-mL centrifuge tube, which was incubated for 30 min at room temperature with agitation on a Thermo microplate shaker. The samples were clarified twice by centrifuging at maximum speed in an Eppendorf benchtop centrifuge for 1 min each time.

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The supernatants were then filtered using a 10-kDa Vivaspin column (GE Healthcare) and the filtrates were transferred to a 5-mm NMR tube for NMR analysis. ^{31}P-NMR has been acquired using a Bruker DRX-600 spectrometer equipped with a 5-mm BBFO cryoprobe. Proton decoupled ^{31}P-NMR spectra are referenced to an external H_3PO_4 (aq) standard (δ 0.0 ppm). All samples have been acquired for 6000 scans. Identity of FR-900098 was confirmed by (1) spiking the sample with authentic FR-900098, (2) ^{31}P-HMBC data comparison, (3) HRMS data. Production titers were estimated by spiking in known amounts of FR-900098.

Isolation and NMR analysis of PTM compounds. The crude extract was fractionated using silica gel flash chromatography and generated 11 fractions: F1 (5% ethyl acetate with 95% hexanes), F2 (15% ethyl acetate with 85% hexanes), F3 (20% ethyl acetate with 80% hexanes), F4 (30% ethyl acetate with 70% hexanes), F5 (40% ethyl acetate with 60% hexanes), F6 (50% ethyl acetate with 50% hexanes), F7 (60% ethyl acetate with 40% hexanes), F8 (80% ethyl acetate with 20% hexanes), F9 (100% ethyl acetate), F10 (100% acetone), and F11 (100% methanol). PTM was eluted in F11 according to LC-MS analysis. F11 was subjected to semi-prep HPLC using a C18 column (Phenomenex, 250 × 10 mm) with the following gradient: 5~40 min 5%~20% acetonitrile in water with 0.1% formic acid; 40~60 min 20%~50% acetonitrile in water with 0.1% formic acid; 60~70 min 50%~60% acetonitrile in water with 0.1% formic acid. **2** was eluted at 62 min. **1** was eluted at 61 min. NMR analysis was performed on an Agilent 600-MHz NMR spectrometer.

Isolation and nuclear magnetic resonance spectroscopy analysis of type II polyketide from *S. viridochromogenes*. Large-scale cultivation on solid plates (equivalent to 5 L liquid culture) of the knock-in strain was carried out to obtain sufficient amounts of potential new compound. 10-day-growth, solid plates were soaked in equal volume ethyl acetate overnight. The extract was fractionated using C18 flash column chromatography and the fraction containing the target compound was further subjected to silica gel flash column chromatography. The column elution was monitored by TLC and the fractions containing the target compound were further confirmed by HPLC. NMR analysis was performed on an Agilent 600-MHz NMR spectrometer.

Fermentation, extraction and liquid chromatography mass spectrometry analysis of RED, ACT and indigoidine from wild type and engineered *Streptomyces* strains. Liquid seed cultures (2 mL MGY) of wild type and engineered *S. lividans* and *S. albus* strains were inoculated from a plate or spore stock in 14-mL culture tubes. Seed cultures were incubated at 30 °C with 250-rpm shaking until achieving turbidity or high particle density (typically 1–2 days). For *S. lividans*, seed cultures were diluted 1:100, plated onto MGY plates, and grown at 30 °C for 3–4 days. The plates were chopped into small pieces and extracted with methanol (RED) or acidified methanol (ACT). For *S. albus*, seed cultures were diluted 1:100 into 50 mL of MGY broth in 250-mL baffled flasks and grown at 25 °C with 250-rpm shaking for 2–3 days. Culture supernatants of wild type and engineered *S. albus* strains were extracted twice with equal volume ethyl acetate containing 1% (*v*/*v*) formic acid. Extracts were dried and resuspended in methanol before analysis by LC-MS using ESI source in positive ion mode (Bruker, Amazon SL Ion Trap) equipped with a Kinetex 2.6 µm XB-C18 100 Å (Phenomenex). HPLC parameters were as follows: solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile; gradient at a constant flow rate of 1 mL/min, 5% B for 2 min, 5% to 100% B in 15 min, maintain at 100% B for 2 min, return to 5% B and maintain for 2 min; detection by UV spectroscopy at 500 nm (RED, ACT) or 600 nm (indigoidine). MS/MS was performed in positive auto MS(n) mode with scan range *m/z* 100-1000.

Data availability. The authors declare that the data supporting the findings of this study are available within the article and its supplementary information.

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