Yeast homologous recombination-based promoter engineering for the activation of silent natural product biosynthetic gene clusters

Daniel Montiel¹, Hahk-Soo Kang¹, Fang-Yuan Chang, Zachary Charlop-Powers, and Sean F. Brady²

Laboratory of Genetically Encoded Small Molecules, The Rockefeller University, New York, NY 10065

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Large-scale sequencing of prokaryotic (meta)genomic DNA suggests that most bacterial natural product gene clusters are not expressed under common laboratory culture conditions. Silent gene clusters represent a promising resource for natural product discovery and the development of a new generation of therapeutics. Unfortunately, the characterization of molecules encoded by these clusters is hampered owing to our inability to express these gene clusters in the laboratory. To address this bottleneck, we have developed a promoter-engineering platform to transcriptionally activate silent gene clusters in a model heterologous host. Our approach uses yeast homologous recombination, an auxotrophy complementation-based yeast selection system and sequence orthogonal promoter cassettes to exchange all native promoters in silent gene clusters with constitutively active promoters. As part of this platform, we constructed and validated a set of bidirectional promoter cassettes consisting of orthogonal promoter sequences, Streptomyces ribosome binding sites, and yeast selectable marker genes. Using these tools we demonstrate the ability to simultaneously insert multiple promoter cassettes into a gene cluster, thereby expediting the reengineering process. We apply this method to model active and silent gene clusters (rebecamycin and tetaramycin) and to the silent, cryptic pseudogene-containing, environmental DNA-derived Lzr gene cluster. Complete promoter refactoring and targeted gene exchange in this “dead” cluster led to the discovery of potent indolotryptoline antiproliferative agents, lazarimides A and B. This potentially scalable and cost-effective promoter reengineering platform should streamline the discovery of natural products from silent natural product biosynthetic gene clusters.


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¹D.M. and H.-S.K. contributed equally to this work.

²To whom correspondence should be addressed. Email: sbrady@rockefeller.edu.

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Significance

A rapidly growing number of cryptic natural product biosynthetic gene clusters have been identified in bacterial DNA sequencing datasets. The metabolites encoded by most of these gene clusters remain uncharacterized because they are not readily activated using monoculture fermentation methods. The development of generic gene cluster activation strategies is needed to access molecules encoded by this rapidly growing collection of sequenced gene clusters. The promoter engineering platform outlined here provides a simple, cost-effective, and potentially scalable tool for the characterization of molecules encoded by gene clusters found in sequenced microbial (meta)genomes. We believe that this gene cluster activation platform will accelerate the discovery of biomedically relevant metabolites using (meta)genomics-driven natural products discovery methods.
auxotrophic yeast strain (i.e., “auxotrophic marker”), pairs of sequence orthogonal actinomycetes constitutive promoters, and ribosome binding sites (RBSs). These cassettes enable the promoter engineering of gene clusters in yeast, followed by molecule production in *Streptomyces*. This method retains native operon structures, allowing for transcriptional refactoring with minimal synthetic nucleic acid input, thereby streamlining the promoter engineering process and rendering the procedure technically and economically accessible. The utility of this gene cluster activation approach is shown using model gene clusters that encode for either the indolocarbazole beccamycin (Reb cluster) or the aromatic polyketide tetracyrism (Tam cluster) (6, 13, 15). We then apply the method to the refactoring of a silent, and we believe naturally dead, environmental DNA (eDNA)-derived gene cluster that we predicted would encode for a novel indolotryptoline-based metabolite. This work resulted in the isolation of lazaramides A and B, which are alkaloids belonging to a rare family of indolotryptoline vacuolar ATPase inhibitors (16). The application of this method to the targeted activation of gene clusters related to other biomedically relevant metabolites or new gene clusters representing new molecular families should provide a simple potentially scalable approach for either improving known bioactive natural products or discovering new families of bioactive natural products.

**Results and Discussion**

**Design of Promoter Cassettes for Yeast Homologous Recombination.** Actinomycetes produce the majority of characterized biomedically relevant natural products (17), and as a result we believe that they are likely to be the most appropriate hosts for most heterologous expression studies focused on the discovery of novel bioactive metabolites. A key feature of our promoter exchange strategy was therefore the construction of a set of constitutively active actinomycetes promoters that could be used to drive transcription without the risk of interpromoter recombination. To this end we designed a set of sequence orthogonal promoter cassettes that are based on promoters identified by Seghezzi et al. (18) in a screen for active promoters from a library of sequences containing a random 17-bp spacer between consensus −10 (TGGACN) and −35 (TASVDT) sequences recognized by the housekeeping sigma factor σ^{70}. The exact sequences from this study could not be used for promoter reengineering because each contains an identical RBS and spacer region, which would make them prone to interpromoter recombination if more than one were introduced into a gene cluster. To solve this problem, we added random 15-bp insulator sequences and unique natural *Streptomyces* RBSs (19) to 24 promoters identified by Seghezzi et al. (18). The spacer and RBS sequences were matched to give a general GC content of ~65% for each promoter element (SI Appendix). Pairs of promoters/spacer/RBS sequences were synthesized as primers (SI Appendix) containing 20-bp sequences homologous to the ends of yeast selectable marker genes. Five yeast auxotrophic genes (LEU2, MET15, TRP1, HIS3, and LYS2 encoding genes involved in leucine, methionine, tryptophan, histidine, and lysine biosynthesis, respectively) (20) and one antibiotic resistance gene (KanMX, aminoglycoside G418) (21) were chosen as selectable markers. Primer pairs were used to amplify each selectable marker to give a set of selectable promoter-exchange cassettes (Fig. 1B).

Each promoter cassette was tested in a promoter exchange experiment using the beccamycin (Reb) gene cluster (13). All gene clusters used in this study were cloned from soil metagenomes using Proteobacterial specific cosmids vectors. To permit promotor engineering in yeast and heterologous expression in actinomycetes hosts, clones carrying gene clusters were

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**Fig. 1.** Construction of bidirectional promoter cassettes and multiplexed promoter exchange strategy. (A) Overview of promoter exchange strategy. (B) The bidirectional promoter cassettes were generated by PCR amplification of six yeast selectable markers with primers containing promoter/insulator/RBS combinations (SI Appendix). The generated promoter cassettes were cloned into TOPO vector 2.1 for their future maintenance. These bidirectional promoter cassettes were tested on the Reb gene cluster, and those that produced beccamycin were used for promoter engineering (SI Appendix). (C) The method to generate the 500-bp homology arms is outlined. A promoter cassette is inserted into a gene cluster using 40-bp homology arms and the region around this insertion is PCR-amplified to generate 500-bp homology arms. (D) The number of colonies generated from the insertion of one, two, or three promoter cassettes with 40-bp or 500-bp homology arms were compared using the Reb gene cluster (cluster A) and an unrelated eDNA-derived type II polyketide synthase gene cluster (cluster B). A representative collection of yeast colonies (>10) from each experiment was examined by PCR to determine the frequency with which all promoter cassettes were correctly inserted. The solid portion of the bars represents the fraction of colonies with correctly inserted promoter cassettes.
retrieved with a yeast origin of replication (CEN/ARS), a yeast selectable marker (URA3), an origin of transfer (oriT) for intergeneric conjugation, the Streptomyces σC31 integrase and the Streptomyces apramycin resistance gene (22). This results in an Escherichia coli::yeast/Streptomyces shuttle vector that permits the rapid movement of gene clusters between all three organisms.

To test our selectable promoter-replacement cassettes using the Reb gene cluster, each bidirectional promoter exchange cassette was amplified with primers designed to contain 40-bp homology homologous to the sequences upstream of the Reb biosynthetic genes rebG and rebO, which flank the bidirectional promoter region in the Reb gene cluster (Fig. 1B). Each cassette was then cotransformed along with the Reb gene cluster containing cosmid into S. cerevisiae (BY4727 ΔΔnl4), and transformation, which is replicated on cassette-specific selective media to identify promoter exchange recombinants. Promoter-replaced Reb constructs isolated from yeast were transformed into E. coli S17 and conjugated into Streptomyces albus where they were examined for the ability to confer the production of rebeccamycin to S. albus (SI Appendix).

S. cerevisiae strain BY4727 contains complete deletions of LEU2, MET15, HIS3, TRP1, LYS2, and URA3, making it useful for selection with all selectable markers used in our promoter cassettes (20). To avoid the nonhomologous end joining (NHEJ) of homologous integration, several overlapping DNA sequences (SI Appendix) were used to construct the bidirectional promoter cassette. The longer homology arm promoter cassette was amplified with primers designed to contain 500-bp homology arms. The longer homology arm promoter cassettes used in multiplexed promoter exchange experiments can be obtained by PCR using single promoter constructs created in parallel with 40-bp homology arms as a template (Fig. 1C). Although the production of longer homology arms using this two-step process requires an additional set of primers, the ability to multiplex the second recombination reaction should significantly simplify the reengineering of complex gene clusters.

Gene Cluster-Wide Promoter Exchange and Natural Product Production Using Model Systems. With the construction of a set of constitutive bidirectional promoter cassettes and the ability to efficiently introduce these cassettes into gene clusters we sought to evaluate their utility for activating gene clusters using a multiplexed promoter exchange strategy. We initially explored this using well-characterized natural product biosynthetic gene clusters known to encode for either the tryptophan dimer rebeccamycin (Reb) or the aromatic polypeptide tetarimycin (Tam). The Reb cluster is natively transcriptionally active (13), whereas the Tam cluster is known to be transcriptionally silent (6).

Reb cluster. The Reb gene cluster used in this study was originally isolated from an Arizona soil metagenome and found to natively produce rebeccamycin when introduced into S. albus (13). The Reb gene cluster is predicted to contain three promoters: two promoters oriented in opposite directions between the glycosyltransferase rebG and oxidase rebO genes that were used to test two-step promoter reengineering experiments and a third promoter upstream of the transcriptional regulator rebr gene (Fig. 2A). The bidirectional promoter site between the rebG and rebO genes was replaced with a TRP1 bidirectional promoter cassette and the unidirectional promoter in the upstream region of the rebh gene was replaced with a MET15 cassette in which only one promoter was incorporated into the ampiclon used for recombination (i.e., a unidirectional promoter cassette) (SI Appendix). This construct was then moved from yeast through E. coli into S. albus for heterologous expression studies. HPLC analysis of TAR extracts from cultures of S. albus transformed with the wild-type Reb gene cluster or with the promoter-exchanged Reb gene cluster showed no significant difference in the production of rebeccamycin, indicating that our promoter reengineering tools should be able to induce molecule production from transcriptionally silent natural product biosynthetic gene clusters.

Tam cluster. The Tam gene cluster is an eDNA-derived type II (aromatic) polypeptide synthase biosynthetic gene cluster that encodes for the antibiotics tetarimycin A (2) and B. In S. albus, this gene cluster is transcriptionally silent unless faml, the gene cluster-specific SARF family positive regulator, is artificially up-regulated (6). The Tam gene cluster is predicted to contain six biosynthetic operons driven by four promoter regions (SI Appendix). As a second proof-of-concept experiment, we replaced all four promoter regions with synthetic promoter cassettes using two rounds of TAR (SI Appendix). In the first yeast transformation, LEU2-, MET15-, TRP1-, and HIS3-based promoter cassettes were inserted in parallel into the Tam gene cluster using 40-bp homology arms (SI Appendix). Promoter cassettes with 500-bp homology arms were then amplified from each reengineered gene cluster. In the second round of TAR, LEU2-, MET15-, and TRP1-based promoter cassettes with 500-bp homology arms were simultaneously inserted into the Tam gene cluster harboring the HIS3 promoter cassette. The successful insertion of all four promoter cassettes into the Tam gene cluster was confirmed by genotyping refactored gene clusters using PCR.
Refactoring of the Reb (rebeccamycin, GenBank accession no. SARP gene (cultures con- www.pnas.org/cgi/doi/10.1073/pnas.1507606112 – transformed with either the S. albus for heterologous expression studies, but unfortu- ii of one of the indole rings to form an gene. gene (i.e., pseudogene).

promoter refactoring was able to replicate native levels of me- The two naturally occurring indolotryptolines that have been to date rarely encountered, and therefore underexplored, family of genes surrounding the core indolotryptoline biosynthesis genes are a structurally and functionally diverse class of natural prod-ucts (29). The best studied of these are the indolocarbazoles staurosporine and rebeccamycin, which are kinase and top-

metabolite with potent human cell cytotoxicity. The biosynthesis of indolotryptolines is well-characterized, making it possible to predict the function of most genes in the Lzr gene cluster (32). The four key stages of indolotryptoline biosynthesis involve dimerization of oxo-tryptophan to form a chromopyrrolic acid, oxidative aroyl–aryl coupling to form an indolocarbazole, “flipping” of one of the indole rings to form an indolotryptoline, and tailoring to generate the final product. The Lzr gene cluster is predicted to contain seven transcriptional units controlled by three bidirectional (P1, P2, and P3) and one unidirectional (P4) promoter regions (Fig. 3 B, i). This cluster is conveniently organized such that successive activation of the three bidirectional promoter regions (P1, P2, and P3) is predicted to drive the expression of genes required to achieve the first, second, and third stages in indolotryptoline biosynthesis, respectively (Fig. 3C).

In a series of single cassette insertions we replaced each bi- directional Lzr promoter region with a synthetic promoter cas-
sette. As expected, P1 and P1+P2 replacement constructs produced chromopyrrolic and indolocarbazole intermediates, respectively (SI Appendix). The P1+P2+P3-replaced gene cluster, however, produced an indolocarbazole intermediate instead of the expected indolotryptoline intermediate (Fig. 3 B, ii). A close examination of lzx1, the gene predicted to encode the oxidative enzyme that installs the C4c/C7a diol (32, 40), sug-
gested the presence of a single base deletion that leads to a truncated and likely nonfunctional lzx1 gene (i.e., pseudogene). Hence, the Lzr gene cluster seems to be not only silent but also dead owing to the disruption of the lzx1 gene. Although natural mutation rates for most environmental bacteria are not known, even if these rates are relatively low it would not be surprising if many secondary metabolite gene clusters, which are expected to be required only during specific potentially rare envi-
ronmental events, accumulate mutations in key biosynthetic genes. Ideally, a gene cluster activation tool should therefore not only be able to awaken silent gene clusters through the replacement of promoters but also have the flexibility to “resuscitate” dead gene

The promoter reengineered Tam cluster was transformed into E. coli S17.1 and conjugated into S. albus for heterologous expres-
sion studies. Liquid chromatography–mass spectrometry analysis of culture broth extracts from S. albus cultures containing either the refactored (i) or wild-type cluster (ii) indicates that these cultures produce comparable levels of rebeccamycin. (B) The Tam gene cluster encodes tetarimycin A (2) but is silent in S. albus (iii). The three bi-
directional (P1–P3) and one unidirectional (P4) promoter region in the Tam gene cluster was exchanged with synthetic bidirectional promoter casettes. HPLC analysis of extracts from S. albus cultures either transformed with the fully promoter refactored gene cluster (i) or the Tam gene clusters activated through expression of the tamI SARP gene (ii) indicates that these cultures produce comparable levels of tetarimycin A (2).

Resuscitation of a Dead Indolotryptoline Gene Cluster. Silent gene clusters in need of activation increasingly appear in (meta)geno-
ic DNA sequencing datasets. Here we use our promoter engi-
neering method to activate a previously uncharacterized silent, eDNA-derived indolotryptoline gene cluster as a third model system for testing our promoter replacement tools.

Recovery of the SARP gene cluster. The Lzr gene cluster was recovered from a previously archived Arizona desert soil eDNA library (39) on two overlapping eDNA cosmids clones (AZ22-292 and AZ25-153). The full-length Lzr gene cluster was reassembled from these two cosmids using TAR and a pTARa-based pathway-specific E. coli:yeast:Streptomyces shuttle vector system to aid in the identification of the Lzr gene cluster. We used our promoter replacement tools.

Recovery of the silenced SARP-derived Lzr gene cluster. The outer edges of the Lzr gene cluster were defined based on comparisons to the BE-54017 and cladonamide gene clusters and a BLAST analysis of genes surrounding the SARP biosynthesis genes (Fig. 3A). The biosynthesis of indolotryptolines is well-characterized, indicating that the Lzr gene cluster is silent in S. albus. We used this silent cryptic gene cluster as a third model system for testing our promoter replacement tools.

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clusters through the exchange of pseudogenes with functional homologs found in closely related gene clusters. In an effort to reconstitute the Lzr cluster, we extended our promoter exchange method to allow for simultaneous insertion of both synthetic promoters and new genes into the gene cluster of interest (SI Appendix). In this case the abeX1 gene from the BE-54017 gene cluster (32), a full-length homolog of the \( \text{lzx} \) pseudogene, and a promoter selection cassette were independently PCR-amplified to produce amplicons with 20-bp overlaps. A second round of PCR was then carried out to link the resulting amplicons into a single cassette containing 40-bp Lzr cluster-specific homology arms, two promoters, the full-length abeX1 oxidative gene, and the LYS2 marker gene (Fig. 3B and SI Appendix). This cassette was then used in a standard TAR promoter exchange reaction to replace both the disrupted \( \text{lzx} \) gene and the P3 promoter region. Upon introduction of this cassette into the P3 site, the new P1+P2+P3 reengineered gene cluster was found to confer to \( S. \) \( \text{albus} \) the ability to produce new indolocarbazole [lazarimide C (5)]- and indolotryptoline [lazarimide B (6)]-based metabolites (Fig. 3B, iii).

To complete the refactoring of the Lzr gene cluster, we replaced P4 with a unidirectional synthetic promoter cassette. Heterologous expression studies with this fully reengineered Lzr gene cluster showed the presence of one additional major metabolite (7) not seen in cultures of \( S. \) \( \text{albus} \) transformed with any previous reengineered constructs (Fig. 3B, iv). Compound 7, which we have given the trivial name lazaramide A, was purified from large-scale cultures of \( S. \) \( \text{albus} \) transformed with the completely reengineered Lzr gene cluster, and its structure was solved using high-resolution electrospray ionization mass spectrometry and 1D and 2D NMR (SI Appendix). The general structure of the lazaramide series of metabolites was further confirmed with a crystal structure of the lazaramide intermediate lazaramide C (5) (Fig. 3C).

Lazarimide A (7) differs from cladonamide and BE-54017 but both its halogenation pattern and the oxidation of the flipped indole moiety. The biosynthesis of lazaramides can be rationalized based on the predicted function of each gene in the Lzr gene cluster (Fig. 3C). Key novel features of the proposed biosynthetic scheme include the action of the predicted oxygenase \( \text{lzxG} \) and two predicted halogenases \( \text{lzxH1} \) and \( \text{lzxH2} \). The cytochrome P450, \( \text{lzxG} \), is predicted to carry out the unique indolotryptoline core hydroxylation seen on Lazaramide A (7), \( \text{lzxH1} \) and \( \text{lzxH2} \) are predicted to be tryptophan-5 and tryptophan-6 halogenases, respectively. This difference in regiospecificity affords the unique halogenation pattern seen in lazaramides A (7), B (6), and C (5).

Because known indolotryptolines are potent human cell line toxins, previously unknown compounds 5-7, as well as cladonamides A (3) as a control, were tested for cytotoxicity against HCT-116 human colon carcinoma cells. The \( IC_{50} \)s observed for compounds 3 and 5-7 were 0.6 nM, 25.8 \( \mu \)M, 11.6 nM, and 8.4 nM, respectively (Fig. 3D). In this series of closely related natural products, lazaramide A (7) exhibits the most potent cytotoxicity against human cells. Activation of the Lzr gene cluster demonstrates the utility of our platform toward characterizing new biomedically relevant metabolites through the activation not only of silent but also of naturally dead biosynthetic gene clusters.

Conclusions

The functional characterization of cryptic biosynthetic gene clusters identified in (meta)genome sequencing efforts remains a significant challenge because most of these clusters are silent under common laboratory culture conditions. Here, we demonstrate a potentially general and scalable yeast homologous recombination-based promoter reengineering platform for activating silent gene clusters through replacement of native promoters with constitutively active synthetic promoters. We initially use our promoter-reengineering platform to refactor known clusters resulting in molecule production efficiencies that are comparable to those seen for the naturally active clusters. We then demonstrate the successful application of this method to the expression of the dead eDNA-derived Lzr gene cluster in a heterologous host, leading to the characterization of a potent, previously unknown, indolotryptoline-based natural product.

This methodology can be expanded to gene clusters that require replacements of more than the 12 promoters we provide through either (i) the use of additional promoter selection cassettes containing other auxotrophic markers and yeast strains with additional auxotrophies or (ii) the use of multiple integration sites in the Streptomyces host that would allow introduction of partially refactored pathways and permit reuse of existing promoter cassettes. The simple and potentially scalable gene cluster activation method we have developed should greatly facilitate the isolation of bioactive natural products from silent gene clusters identified in both strain-based and metagenome-based next-generation sequencing campaigns.

Materials and Methods

Construction of Promoter Exchange Cassettes. The forward and reverse primers used to amplify yeast selectable markers were designed to contain unique promoter (44 bp)/spacer (15 bp)/RBS (12-18 bp) combinations (SI Appendix) and 20-bp sequences identical to the distal end of yeast marker genes, LEU2, MET15, TRP1, HIS3, LYS2, and KanMX selectable markers were amplified from plasmids pRS405, pRS401, pRS404, pRS403, pRS403, pRS403, and pFA6, respectively, using the primer sets listed in SI Appendix. PCR products were TA-cloned into
TOPO vector pCR2.1 (Life Technologies) for stable maintenance of each selection cassette. Cloned promoter cassettes were sequenced using M13F(-60) and M13R primers to confirm each sequence. Primer sets used for the promoter test experiment with the Reb gene cluster are listed in SI Appendix.

Promoter Exchanges. For each promoter engineering experiment, amplicons were generated from the TOPO-cloned promoter cassettes using primers containing 40-bp sequences matching the targeted insertion site. Specific primers used in the reengineering of different gene clusters are listed in SI Appendix. Promoter cassettes were amplified using AccuPrime Taq High Fidelity DNA polymerase (Invitrogen). A standard 50-µL PCR contained 1 µL of template (10 ng/µL), 2.5 µL of each primer (5 µM), 25 µL of buffer G (Epicentre), 18.5 µL of water, and 0.5 µL of polymerase. The following gradient amplification protocol was used for LEU2 and LYS2 promoter cassettes: initial denaturation (95 °C, 5 min), 36 cycles of denaturation (95 °C, 30 s), annealing (40-60 °C across 12 wells, 30 s) and extension (72 °C, 5 min), and final extension (72 °C, 5 min). MET15, TRP1, HIS3, and KanMX promoter cassettes were amplified using the following single annealing temperature protocol: initial denaturation (95 °C, 5 min), 36 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 40 s) and extension (72 °C, 3 min), and final extension (72 °C, 5 min). The resulting PCR products were column-purified (QIAprep spin columns, Qiagen) and either the cosmID or BAC vector harboring the target gene clusters were cotransformed into S. cerevisiae BY4742 (admH) using the LiAcS carrier DNA/PEG yeast transformation protocol published by Gietz and Schiestl (25). Briefly, yeast was grown overnight in 50 mL of YPD media containing G418 (200 µg/mL) at 30 °C. In the morning, 2 mL of the overnight culture was reincultured into 50 mL of fresh YPD media containing G418 (200 µg/mL) and grown for 4 h (OD600nm = 2.0). This culture was harvested by centrifugation (10 min, 3,200 × g), washed twice with sterile 4 °C water, and resuspended in 1 mL of sterile 4 °C water. For each transformation 100 µL of washed cells was transferred to a microfuge tube. The cells were collected by centrifugation (30 s, 18,000 × g), resuspended in 100 µL of water, and plated on the appropriate synthetic complete dropout media agar plates. Agar plates were incubated at 30 °C until colonies appeared. Colonies were checked by PCR for correct promoter insertion using primer pairs where one primer targeted the cassette and the second primer targeted the gene cluster (SI Appendix). DNA was isolated from PCR-positive yeast clones, transferred into E. coli S17.1, and then moved to S. albus by intergeneric conjugation for expression studies (SI Appendix).

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