Activation and characterization of a cryptic polycyclic tetramate macrolactam biosynthetic gene cluster

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Polycyclic tetramate macrolactams (PTMs) are a widely distributed class of natural products with important biological activities. However, many of these PTMs have not been characterized. Here we apply a plug-and-play synthetic biology strategy to activate a cryptic PTM biosynthetic gene cluster SGR810-815 from Streptomyces griseus and discover three new PTMs. This gene cluster is highly conserved in phylogenetically diverse bacterial strains and contains an unusual hybrid polyketide synthase-nonribosomal peptide synthetase, which resembles iterative polyketide synthases known in fungi. To further characterize this gene cluster, we use the same synthetic biology approach to create a series of gene deletion constructs and elucidate the biosynthetic steps for the formation of the polycyclic system. The strategy we employ bypasses the traditional laborious processes to elicit gene cluster expression and should be generally applicable to many other silent or cryptic gene clusters for discovery and characterization of new natural products.
**Results**

**Development of a gene cluster reconstruction system.** To discover and characterize new PTM biosynthetic gene clusters, we developed a simple synthetic biology strategy in which a plug-and-play scaffold is used to decouple gene cluster expression from native regulation (Fig. 1). The key step in this strategy is to use a set of well-characterized promoters and the DNA assembler method to reconstruct the target cryptic biosynthetic gene cluster in a genetically tractable heterologous host. The product(s) will be detected and characterized using analytical chemistry methods such as LC-MS and NMR or biological activity assays.

Because the DNA assembler method used for natural product gene cluster reconstruction relies on homologous recombination,

**Isolation and characterization of putative PTMs.** To determine the chemical structures of these compounds, large-scale cultivation on solid plates (equivalent to ~41 liquid culture) of the heterologous host harbouring the target reconstructed gene cluster was carried out to obtain sufficient amounts of these compounds. High-resolution electrospray ionization mass spectrometry in positive mode showed an $m/z$ of 511.2807 for compound a [M(C$_{29}$H$_{38}$O$_{6}$N$_{2}$)$_{+}$] $^+$ (calculated: 511.2808) and 511.2781 for compound b [M(C$_{29}$H$_{38}$O$_{6}$N$_{2}$)$_{+}$] $^+$ (calculated: 511.2808). Their corresponding chemical structures (Fig. 2b) were elucidated by extensive one- and two-dimensional NMR spectroscopy ($^1$H, $^{13}$C, Double-Quantum Filtered Correlation spectroscopy (COSY), Total correlation spectroscopy (TOCSY)),
Heteronuclear single-quantum correlation spectroscopy (gHSQC), Heteronuclear multiple-bond correlation spectroscopy (gHMBC) and Nuclear Overhauser Effect Spectroscopy (NOESY) and MS experiments (Supplementary Figs S2–S4, Supplementary Table S3). Key COSY and heteronuclear multiple bond correlation (HMBC) correlations were summarized in Supplementary Fig. S5. For compound a, the Double-Quantum Filtered COSY (Phase-sensitive) spectrum revealed the spin system, and the HMBC supported the coupling of H2 and H3 to C1, as well as H23 to C19. For compound b, the characteristic C-H coupling constants (C14, $J_{C-H}=172$ Hz and C15, $J_{C-H}=181$ Hz, measured using a high-resolution coupled gHSQC) suggested the presence of an epoxide ring. All compounds belong to the group of tetramic acid-containing macrolactam natural products. Compound a shares the same structure as previously reported alteramide A, whereas compound b has never been reported in literature. The NOESY correlations to confirm the stereochemistry of compound b were summarized in Supplementary Fig. S6. The production of these tetramic acid-containing macrolactams in a heterologous host clearly confirms the iterative use of the single PKS-NRPS module, which is rare in bacteria. Moreover, we have cultivated the previously described control strains and the native producer S. griseus under multiple different cultivation conditions (MYG liquid or solid media and YMS liquid or solid media), and the fermentation broths were extracted and analysed by HPLC. For the control strains, no distinct difference was observed compared with the wild-type S. lividans. For the native producer, neither compound a nor compound b could be detected.

**Elucidation of the biosynthetic steps.** Next, we attempted to elucidate the biosynthetic steps of this new gene cluster using the same synthetic biology strategy. We first determined the exact boundary of the gene cluster by bioinformatics analysis of upstream and downstream regions of this gene cluster and then constructed another seven-gene gene cluster with an upstream putative aminotransferase gene (Supplementary Fig. S7a and Supplementary Table S4). The expression of this putative aminotransferase gene was confirmed by quantitative PCR and the products were identified to be the same as the ones from the six-gene cluster (Supplementary Fig. S7b), indicating that the putative aminotransferase gene was not essential.
Figure 2 | The structures of PTM compounds. (a) Chemical structures of known PTMs. (b) Chemical structures of the products of the SGR810-815 gene cluster from S. griseus. Note: the stereochemistry assignments of compound b,c,d are relative.

Then, to decipher the biosynthetic steps, a series of mutant gene clusters consisting of single or multiple gene deletions were constructed and analyzed (Fig. 3d and e, Supplementary Tables S5 and S6, Supplementary Figs S8 and S9). As expected, the SGR814 single-deletion construct lost the ability to produce any target compounds, indicating the essential role of the PKS-NRPS hybrid gene (Fig. 3d). The SGR815 single-deletion construct (Fig. 3d) abolished the production of compound a and instead produced compound c [M(C_{29}H_{38}O_5N_2) + H]^+ with an m/z of 495.2864 (Fig. 2b and Supplementary Figs S5, S6, S10 and S11, Supplementary Table S7; calculated: 495.2859). Compound c shares the similar structure as the previously reported alteramide A 

Compound a

\[ C_{29}H_{38}N_2O_5 \] 510.2808

Compound b

\[ C_{29}H_{38}N_2O_5 \] 510.2808

Compound c

\[ C_{29}H_{38}N_2O_5 \] 494.2848

Compound d

\[ C_{29}H_{38}N_2O_5 \] 476.2753

Dihydromaltophilin

Cylindramide

Maltophilin

Alteramide A

Ikarugamycin

Frontalamide A

Frontalamide B

These results suggest that both SGR812 and SGR813 are involved in the formation of the polycyclic system.

To further confirm this, a three-gene gene cluster consisting of SGR812, SGR813 and SGR814 was constructed and evaluated (Supplementary Fig. S9). Compound c was found to be the major product (Fig. 3e), indicating that this compound is a key biosynthetic intermediate. In addition, a four-gene gene cluster consisting of SGR812, SGR813, SGR814 and SGR815 was constructed (Supplementary Fig. S9) and its main product was compound a (Fig. 3e), which again confirmed the functional roles of these four genes. Although the SGR810 and SGR811 single-deletion constructs both maintained the production of compound a and compound b, the loss of compound b in these three-gene and four-gene constructs indicates that SGR810 and SGR811 are involved in the formation of the epoxide ring and their functions might be redundant (Fig. 3d and e). Taken together, a biosynthetic route for this group of PTMs has been proposed (Fig. 4).

Discussion

We have successfully activated a silent PTM gene cluster and discovered three novel PTM compounds by fully reconstructing the target gene cluster in a heterologous host. The production of these novel tetramic acid-containing macrolactams in a heterologous host directly confirms the iterative use of the single PKS-NRPS module, which is an intriguing feature of this type of enzymes in bacteria. Although this cluster shares sequence homology with other characterized PTM gene clusters and the PTMs characterized from these gene clusters all share similar backbones, there are notable differences in their chemical structures. There are basically two different groups of PTMs,
one with a 5,5,6-tricyclic system or 5,6,5-tricyclic system fused to the macro lactam and the other with a 5,5-bicyclic system (Fig. 2). It appears that the products we have discovered belong to the second group. It should be noted that these products may be shunt products from the gene cluster because of heterologous expression, but the different structures still reveal the diversity of PTMs.

In addition, we used the same synthetic biology strategy to investigate the biosynthetic route, especially for the formation of the polycyclic system. SGR815 was confirmed to be a hydroxylase, which is conserved in all known PTM gene clusters. SGR812 and SGR813 were annotated as oxidoreductases and can be found in most of the known PTM gene clusters. Based on phylogenetic analysis, these two enzymes are distinct and may catalyse different reactions even though they belong to the same family of oxidoreductases. Indeed, our results from the gene deletion constructs suggest that SGR812 is involved in the formation of the first ring, whereas SGR813 is responsible for the closure of the second ring. Interestingly, we noticed that two related PTM gene clusters from *Saccharophagus degradans* and *Salinospora arenicola* only contain homologs of SGR812, which supports the proposed role of SGR812 in ring formation. That is because the reaction catalysed by SGR812 takes place before the ones catalysed by other oxidoreductases. The last two enzymes, SGR811 and SGR810, are also conserved in most of the related PTM gene clusters, but with greater sequence diversity. Our results suggest that they are likely to perform oxidative reactions on the backbone.

It should be noted that the strategy we employed in this study is simple, generally applicable and potentially scalable in studying other cryptic natural product gene clusters. For example, we recently used the same strategy to successfully reconstruct a putative phosphonic acid gene cluster from a *Streptomyces* sp. soil isolate. Analysis of the *S. lividans* strain containing the completely reconstructed 13-gene pathway revealed multiple new putative phosphonate peaks by $^{31}$P-NMR, which were not observed in the native host or the *S. lividans* background (Supplementary Fig. S13). The key innovative aspect of this strategy is the use of the DNA assembler method to achieve full gene cluster reconstruction for cryptic gene clusters in a single-step manner. This strategy is distinct from other related strategies such as using promoter replacement to activate cryptic single genes$^{28}$, which focuses on single genes rather than the entire gene cluster, gene-by-gene reassembly in a heterologous host$^{29-31}$, which use

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**Figure 3** Activation and characterization of a cryptic PTM gene cluster. (a) The reconstructed cryptic SGR810-815 gene cluster from *S. griseus*. P1: gapdh (*Kocuria rhizophila*), P2: gapdh (*S. griseus*), P3: ermE*, P4: rpsL (*Tsukamurella paurometabola*), P5: rpsL (*S. griseus*), P6: rpsL (*Cellulomonas flavigena*). (b) Real-time PCR analysis of SGR genes in the reconstructed gene cluster and the control gene clusters. Ep, original cluster with ermE* in front of the whole gene cluster; OC, original cluster; SG, native producer *S. griseus*; RC, reconstructed cluster. Error bars represent the standard errors and triplicates were performed for each reaction. (c) HPLC analysis of the extract from the *S. lividans* strain carrying the reconstructed gene cluster SGR810-815. *Major products, #minor products. Ultraviolet chromatographs were recorded at 260 nm. (d) HPLC analysis of single-deletion constructs. (e) HPLC analysis of three-gene and four-gene constructs (# indicates a small production of compound c).
continually decreasing cost of DNA synthesis, the entire gene clusters per day with minimal human intervention. With the and LC-MS (compound detection), this versatile platform will be separator (DNA purification), shake incubators (cell cultivation) (synthesis of DNA fragments by PCR reactions), magnetic bead transformation in blocks (setting up PCR reactions, isolating plasmid DNAs, DNA integrated robotic system featuring liquid handler with Peltier throughput platform for natural product discovery. Utilizing an long-term goal is to build an automated, scalable and high-
reconstructed and the entire process may be fully automated. Our
interest in streptomycetes and other actinomycetes. Moreover, this strategy may be further modified for activation of cryptic gene clusters from other organisms, such as myxobacteria, cyanobacteria and fungi, as long as appropriate heterologous hosts and functional promoters can be obtained. For the promoters, strong constitutive promoters used to drive the expression of each gene in a target gene cluster can be readily identified from the heterologous host by real-time PCR or RNA-seq. It is noteworthy that because of the plug-and-play nature of the system, a cryptic gene cluster of interest can be readily reconstructed and the entire process may be fully automated. Our long-term goal is to build an automated, scalable and high-throughput platform for natural product discovery. Utilizing an integrated robotic system featuring liquid handler with Peltier blocks (setting up PCR reactions, isolating plasmid DNAs, DNA transformation in S. cerevisiae and S. lividans), thermal cyclers (synthesis of DNA fragments by PCR reactions), magnetic bead separator (DNA purification), shake incubators (cell cultivation) and LC-MS (compound detection), this versatile platform will be capable of assembling and reconstruction hundreds of gene clusters per day with minimal human intervention. With the continually decreasing cost of DNA synthesis, the entire gene cluster may be reconstructed by using synthetic genes and/or promoters. Finally, this strategy is much more flexible and versatile for generating gene deletions or additions for mechanistic studies or creation of novel compounds than traditional combinatorial biosynthesis approaches. Overall, the synthetic biology approach we developed represents a new technology platform for discovery and characterization of novel natural products from sequenced genomes and metagenomes for pharmaceutical and biotechnological applications.

**Methods**

Materials and reagents. *S. lividans* 66 and *S. griseus* were obtained from the Agricultural Research Service Culture Collection (Peoria, IL, USA). The vector map and complete sequences of *Streptomyces* integration parts of plasmid pAE4 (ref. 33) is provided in Supplementary Fig. S14. *E. coli* strain WM1788 is the equivalent of strain BW25141 (ref. 34) [lacP rrmB14 ΔlacZΔM15 ΔapbR R580 hsdR514 ΔaraBAD ΔaraBADΔ279 galU95 endA4333 uvdA1(M61Δ::psr⁻ recA1, derived from *E. coli* K-12 strain BD792) and was provided by Dr William Metcalf (University of Illinois, Urbana, IL, USA). *E. coli* strain WM6026 (refs 35,36) [lacP rrmB3 ΔlacZΔM15 ΔapbR R580 ΔaraBADΔ279 ΔaraBADΔ967 ΔaraBADΔ568 rph-1 attP:ΔAE12 (ΔoriR6K:cat::Frt5) ΔendA:ΔFrt uvdA1(M61Δ::psr⁻ attHck::pik1006Δ(oriR6K- cat::Frt5::Frt5)]; was provided by Dr William Metcalf (University of Illinois, Urbana, IL, USA). The plasmid pRS416 was purchased from New England Biolabs (Beverly, MA). Nalidixic acid and isopropyl-β-D-thiogalactopyranoside were obtained from Sigma-Aldrich (St Louis, MO). ISPC, agar, beef extract, yeast extract, malt extract and other reagents required for cell culture were obtained from Difco (Franklin Lakes, NJ). All restriction endonucleases, as well as T4 DNA ligase and antarctic phosphatase, were purchased from New England Biolabs (Beverly, MA). Phusion DNA polymerase was from Finnzymes (Waltham, MA). FailSafe PCR Master Mix was purchased from Applied Biosystems (San Francisco, CA). The QiAprep Spin Plasmid Mini-prep Kit, QiAquick PCR Purification Kit, QiAquick Gel Extraction Kit and RNasey MinElute Cleanup Kit were purchased from Qiagen.
Streptomycete cultivation and expression analysis

medium lacking uracil (SC-Ura) was used to select transformants containing the
100), which was used as the host for DNA assembly. Synthetic complete drop-out
medium (5 g l

1gl

2-1, 112 and

1 yeast extract, 20 g l

250 mm2), on the Agilent 1100 series LC/MSD XCT with an isocratic solvent
26.8 min (compound b). 28.5 min (compound c) and

346 min (compound d). The MS system was operated using a drying temperature of
350 °C, a nebulizer pressure of 35 psi, a drying gas flow of 8.5 min

and

a viapolyamide sigma factor, was used as the internal control for
promoter screening. The expression levels of other candidate genes were normal-
ized by the expression of the control. Data were analysed using SDS2.4 software
(Applied Biosystems).

Gene cluster reconstruction and yeast transformation

Gene cluster fragments were amplified from the genomic DNA of S. griesei. The primer sequences are listed in Supplementary Tables S2–S5. The S. cerevisiae helper fragment was amplified from the plasmid pRS456, whereas the E. coli helper fragment and the S. lividans helper fragment were amplified from pA4E. The PCR products were individually gel-purified from 0.7% agarose. For gpdA(SG) and rpsL(SG), the genomic DNA of S. griesei was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) and then used as the template for PCR amplification of the two target promoters. Promoters from other hosts were obtained through primer splicing in which six to ten overlapping oligonucleotides designed on the sequence of each promoter were joined together by overlap extension PCR. Genes with corresponding promoters were also ligated together by overlap extension PCR. To ensure high efficiency of yeast homologous recombi-
nation, the promoter upstream-gene-promoter downstream’ cassette was built to generate a ~200-bp overlap region with adjacent fragments. Individual cassettes (200–300 ng of each) were mixed and precipitated with ethanol. The resulting DNA pellet was air-dried and re-suspended in 4 μl of Milli-Q double deionized water. The concentrated mixture of DNA was electroporated into S. cerevisiae using the protocol reported elsewhere [16,17].

Restriction digestion analysis

Colonies were randomly picked to SC-Ura liquid plates and grown for 1 day, after which the plasmids from yeast were isolated using the NucleoBond AX960 kit (Macherey-Nagel). The plasmids were then sequenced by using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed using an Applied Biosystems 377 sequencer.

Heterologous expression in S. lividans

The verified clones were transformed to E. coli WM6026 (ref. 35) and selected on LB agar plates supplemented with 15 μg ml

1 bacterial colonies were inoculated into 20 ml of ATCC72 liquid medium (5 g l

2-1, 1 µl). The strains were cultured at 30 °C for 24 h, after which the frozen cultures were thawed completely and the release of actinomycin D was measured. The liquids were extracted with ethyl acetate with a ratio of 1:1 twice, concentrated 1,000-fold and subjected to HPLC analysis. HPLC was performed on the Agilent 1100 series LC/MSD XCT plus ion trap mass spectrometer with a Phenomenex Luna C18 reverse-phase column (3.0 × 15 mm2, 3.5-μm). HPLC parameters were as follows: solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in water (with a flow rate of 3 ml min

and a wavelength of 260 nm). The high-resolution MS was performed on a Waters Q-ToF Ultima system (Waters, Milford, MA). All NMR experiments were carried out on a Varian UNITY INOVA 600 MHz spectrometer, except the 13C spectra, which were recorded on a Bruker Avance III 500 with a 5 mm 1H/13C cryogenic probe at the National Magnetic Resonance Facility at the University of Wisconsin at Madison. The structures were determined based on 1D and 2D NMR data. The relative configurations were determined using 2D NOESY experiments.

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Author contributions
Y.L. and H.Z. designed the experiments and wrote the manuscript. Y.L. performed the experiments to activate and characterize the cryptic gene cluster. H.H., J.L., M.W. and L.L. contributed to the characterization of the compounds from the cryptic gene cluster. Z.S. and Y.L. contributed to promoter identification and characterization. R.E.C. contributed to the study of the cryptic phosphonic acid cluster.

Additional information
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