A comparative metabologenomic approach reveals mechanistic insights into Streptomyces antibiotic crypticity

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Streptomyces genomes harbor numerous, biosynthetic gene clusters (BGCs) encoding for drug-like compounds. While some of these BGCs readily yield expected products, many do not. Biosynthetic crypticity represents a significant hurdle to drug discovery, and the biological mechanisms that underpin it remain poorly understood. Polycyclic tetramic acid macrolactam (PTM) antibiotic production is widespread within the Streptomyces genus, and examples of active and cryptic PTM BGCs are known. To reveal further insights into the causes of biosynthetic crypticity, we employed a PTM-targeted comparative metabologenomics approach to analyze a panel of S. griseus clade strains that included both poor and robust PTM producers. By comparing the genomes and PTM production profiles of these strains, we systematically mapped the PTM promoter architecture within the group, revealed that these promoters are directly activated via the global regulator AdpA, and discovered that small promoter insertion–deletion lesions (indels) differentiate weaker PTM producers from stronger ones. We also revealed an unexpected link between robust PTM expression and griseorhodin pigment coproduction, with weaker S. griseus–clade PTM producers being unable to produce the latter compound. This study highlights promoter indels and biosynthetic interactions as important, genetically encoded factors that impact BGC outputs, providing mechanistic insights that will undoubtedly extend to other Streptomyces BGCs. We highlight comparative metabologenomics as a powerful approach to expose genomic features that differentiate strong, antibiotic producers from weaker ones. This should prove useful for rational discovery efforts and is orthogonal to current engineering and molecular signaling approaches now standard in the field.

Significance

Streptomyces genomes harbor a trove of biosynthetic gene clusters (BGCs) that encode for drug-like molecules. However, only a fraction of these readily yield expected products. To investigate why this is, we used polycyclic tetramate macrolactam (PTM) antibiotic production as a model system. By comparing the genomes and PTM production profiles of several closely related Streptomyces griseus clade members, we uncovered two mechanistic mechanisms that differentiate more robust producers from weaker ones. The first involves small insertion–deletion lesions in PTM BGC promoters that significantly modulate production. The second mechanism involves biosynthetic pathway interactions, in which robust PTM producers unexpectedly benefit from griseorhodin coproduction, and weaker producers lack the pathway. We highlight comparative metabologenomics as a powerful approach to understand antibiotic crypticity.

Many therapeutics derive from natural products and their synthetic analogs (1). Historically, Streptomyces and related actinobacteria were heavily screened for these molecules, which resulted in numerous essential medicines. These include many clinical antibiotics (2), and there is an urgent need for new anti-infectives to counter increasing drug resistance (3). A massive reservoir of uncharacterized, biosynthetic gene clusters (BGCs) encoding drug-like molecules resides within Streptomyces genomes, which has triggered resurgent interest in these organisms (4). However, a large proportion of these BGCs fail to produce detectable levels of the expected compounds under laboratory conditions. This phenomenon of cryptic or silent metabolism thus poses a significant hurdle to genomics-driven drug discovery (5, 6). Silent BGCs are often thought to be transcriptionally deficient, and synthetic biology, cell signaling, and stress mechanisms are commonly used to activate silent BGCs for molecule discovery (7). Despite decades of research on the regulation of antibiotic production in Streptomyces (8, 9), a deeper mechanistic understanding of Streptomyces silent metabolism is still needed to access the full biosynthetic potential of these organisms to overcome the drug discovery gap (10).

Here, we employed a comparative metabologenomic approach to dissect why certain Streptomyces strains are ready antibiotic producers while others have apparently silent BGCs. Polycyclic tetramate macrolactam (PTM) antibiotics were specifically targeted in this study because they provide an opportune model system for multistrain comparative analyses. This is because PTM BGCs can contain as few as three genes (11), greatly simplifying regulatory studies. Another advantage of PTM BGCs is their relative commonality (12). A survey of bacterial genomes in GenBank, published in June 2016, reported that over 80 PTM BGCs were detected within 669 Streptomyces genomes available on GenBank at the time (13), and this commonality is leverageable for in-depth comparisons between PTM producers. The environmentally and biotechnologically important Streptomyces griseus clade (14) was identified as a particularly advantageous cohort for these comparisons because multiple strains within it had established active or cryptic PTM BGCs (12, 15), plus several additional family members with sequenced genomes and yet unstudied PTM clusters were available from public collections. Finally, PTM biosynthesis is increasingly understood, and this foundational knowledge was necessary to enact a targeted metabologenomics approach to document sensitive production differences. PTMs have experienced intense study toward understanding their unusual hybrid nonribosomal peptide/polyketide origins.

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investigating PTM therapeutic potential, and leveraging their ease of manipulation via synthetic biology (13).

This study was initiated by comparing the genomes and PTM production profiles among a cohort of PTM locus-bearing *S. griseus* clade strains, which included known examples of both strong and poor PTM producers. This led to the discovery of a subclade that has consistently higher PTM production and PTM BGC promoter strengths compared to the rest of the test strains. Despite the exceptional commonality of PTM biosynthetic loci in *Streptomyces* bacteria, PTM regulation remains poorly understood. To reveal how promoter sequence variations might underpin the observed PTM phenotype differences, the promoter driving production in the robust producer *Streptomyces* species (sp.) strain JV180 was thus mapped and compared against the rest of the clade. Overall, many promoter features appeared to be largely conserved within the group, regardless of host–strain PTM capability. Additionally, the well-characterized global regulator AdpA (16) was confirmed to play a direct positive role on PTM locus control through gene deletion, binding site mutation, and in vitro binding experiments. AdpA binding sites were detected in the PTM promoters of all tested clade members, in which they display a contextually unusual arrangement downstream of promoter –10 boxes. Critically, comparisons of strong and weak PTM promoters from several *S. griseus* clade members identified a 2 to 3 bp indel, located between the –10 box and AdpA operator site, that strongly influences PTM production strength. Yet another PTM control mechanism that differentiates weak from stronger PTM producers was discovered following the mutagenesis of strain JV180’s griseorhodin BGC. Weak *S. griseus* clade PTM producers natively lack griseorhodin BGCs, and loss of the JV180 cluster severely curtailed PTM production. Further dissection revealed that strain JV180’s PTM production likely benefits from PTM–griseorhodin coexpression via a yet uncharacterized transcriptional mechanism. In sum, this work revealed two atypical mechanisms by which stronger and weaker PTM producers are differentiated, and it highlights the application of targeted comparative metabologenomics to cohorts of related strains to successfully reveal otherwise difficult-to-detect genomic features that tune antibiotic production.

**Results**

The *S. griseus* Clade Is an Ideal Model Group to Compare and Reveal Genetic Underpinnings of PTM Crypticity. Several features of the *S. griseus* clade make the group attractive for comparison-based approaches to reveal BGC-silencing mechanisms. Clade member *Streptomyces* sp. strain JV180 readily produces PTM compounds (12, 17), while *S. griseus* subspecies *griseus* strain IFO13350 harbors a silent PTM BGC (15, 18). Nevertheless, promoter refactoring of the IFO13350 PTM BGC and its expression in a heterologous host successfully yielded PTMs with 5/5-carbocyclic ring systems, proving the functionality of its encoded enzymes. The PTM production status of most other clade members remained unknown and, to benefit from the increased analytical depth afforded by expanded cohort sizes, several additional clade members having PTM loci in their sequenced genomes (19) were obtained from public strain repositories (JV251 to JV258). To complete the cohort, the environmental clade member *Streptomyces* sp. strain SP18CM02, whose genome was recently reported by our group (17), (*SI Appendix, Table S1*) was also included. The phylogenetic relationships of these strains were inferred through multilocus phylogeny (14) (*SI Appendix, Fig. S1*). Importantly, the PTM BGCs of these strains appeared to be orthologous. This was based on several observations, including that their PTM BGCs share identically ordered biosynthetic genes (*ftdA, ftdB, ftdC, ftdD, ftdE,* and *ftdF*), and the chromosomal regions that immediately flank their respective PTM BGCs also have identical gene content (see Fig. 1A for a PTM BGC diagram representative of all strains studied here and the figure legend for additional detail). Finally, the PTM enzymes encoded within each studied BGC also shared high pairwise identities to those of strain JV180 (*SI Appendix, Table S4*).

To assess and compare PTM production in these strains, the robust PTM-producing strain JV180 served as an archetype. Strain JV180’s PTM production was extensively analyzed using approaches similar to those reported for clifednamide-type PTM analyses in other *Streptomyces* by our group (20). Putative PTM peaks were identified using 13C-labeled ornithine precursor incorporation and diagnostic daughter ion production following collision-induced dissociation in liquid chromatography–coupled tandem mass spectrometry (LC-MS/MS). These target the conserved ornithine–derived tetramate region, which has two possible hydroxylation states (*SI Appendix, Figs. S2 B–E and S3*). As expected, all PTM peaks identified through these methods were absent when the JV180 PTM BGC was deleted (*ftdABCDEF*, Fig. 1A and *SI Appendix, S2A*), yielding a high-confidence set of *S. griseus* clade PTM congensers for quantitative production comparisons.

The remaining *S. griseus* clade strains were then grown under several conditions, and their extracts were analyzed via LC-MS/MS and ultraviolet (UV) spectrometry for PTM production (see *Materials and Methods*). As expected from orthologous PTM BGCs, all producing strains gave PTM signals that largely overlapped with those established in JV180, but strain-to-strain differences in quantity and relative PTM congener ratios were observed. From these comparative PTM production data, an interesting trend emerged: Strains JV180, SP18CM02, and JV251 to JV253 displayed robust PTM production, while the remaining strains generally showed little production (Fig. 1B and *SI Appendix, Figs. S4–S6*). We noted that these strains belong to a distinct subclade on the *S. griseus* clade phylogenetic tree (*SI Appendix, Fig. S1*, group VI in green), suggesting they might share a conserved genetic basis for increased PTM production versus the other studied clade members. Interestingly, these comparative production analyses revealed a clear bias for higher PTM production on solid media over shake flask cultures for all tested strains (Fig. 1B). The strongest PTM producers continued to have the highest production in liquid media as well. Throughout, to obtain consistent transcriptional information, liquid media was used. Furthermore, in agreement with prior publications, PTM production could be detected by UV absorbance in JV180 (12), while strain IFO13350 produced insufficient PTMs for detection using this method (*SI Appendix, Fig. S7A*).

In contrast, our PTM-targeted LC-MS/MS analyses revealed detectable production in all tested clade members, but the amounts varied widely by strain. This suggests that prior efforts to characterize weak PTM producers may have overlooked scant actual production due to inherent UV limitations, overcome here by MS/MS methodology (*SI Appendix, Fig. S7 B and C*).

**PTM Promoter Sequence Heterogeneity Contributes to Differences in Antibiotic Production.** Because the most robust PTM production was found in strains of the JV180 subclade, we hypothesized that comparing these genomes against other *S. griseus* clade strains could reveal specific differences that underpin the observed production disparities. PTM BGC transcriptional differences were immediately targeted as a potential mechanism. While weak transcription is often implicated in the cryptic biosynthesis literature, this assertion is often speculative or left without mechanistic investigation (21). The possibility of PTM biosynthetic enzyme defects in some strains was ruled out as a potential cause. This is because all tested strains have high–PTM BGC protein sequence identity (*SI Appendix, Table S4*), and previous transcriptional refactoring experiments were able to successfully activate the cryptic PTM BGC of strain IFO13350 in heterologous hosts, as a demonstration of BGC functionality (15, 18).

Possible strain-linked differences in PTM BGC transcription were initially explored by comparing the presumed PTM promoter regions of each strain tested here. *S. griseus* clade PTM BGCs have a simple and conserved gene arrangement with minimal intergenic
gaps, suggesting a single operon ([SI Appendix](https://doi.org/10.1073/pnas.2103515118), Fig. S8A). In JV180, PCR amplification of complementary DNA (cDNA) intergenic junctions confirmed this supposition ([SI Appendix](https://doi.org/10.1073/pnas.2103515118), Fig. S8B).

Phylogenetic analysis of the ∼500 nt located upstream of the first gene in each studied PTM BGC, ftdA, formed two groups: one by JV180-like strains and one by the remaining strains (these sequences are hereafter referred to as JV180- or IFO13350-like P<sub>ftdA</sub>s, respectively; Fig. 1C and [SI Appendix](https://doi.org/10.1073/pnas.2103515118), Fig. S9). To test if the sequence differences in P<sub>ftdA</sub> regions are transcriptionally relevant, we leveraged the strong PTM producer JV180. JV180 represents a uniform background to eliminate complicating, metabolic, and genetic variables inherent to strain-to-strain comparisons. After
replacing the native promoter region of JV180 with the corre-
spanding region of the other 10 *S. griseus* clade strains in this
study, we generally observed higher PTM production and *ftdB*
transcription from JV180-like *P* _ftdB_ than IFO13350-like *P* _ftdB_
(Fig. 1 D and E). This suggests the idea that stronger PTM pro-
ducers might result from inherently stronger promoters.

Surmising that the basic transcriptional machinery of all *S. gri-
seus* clade members should be highly similar, we also hypothesized
that if JV180-like *P* _ftdB_ are stronger than IFO13350-like *P* _ftdB_,
then introducing the former promoter type into strain IFO13350’s PTM BGC should increase PTM production and transcription.
However, exhaustive attempts to introduce the necessary plasmids to
engineer increased production in IFO13350 (and other group
member JV257; *SI Appendix*, Fig. S1), failed by both conjugation
and protoplast transformation. Instead, the effects of *P* _ftdA_ sequence
variation were assayed in strain IFO13350 using an *xylE* (22) re-
porter gene chromosomally integrated into the *F. coli* cell. Controls
testing the efficacy of colormetric *xylE* assays in both strains JV180
and IFO13350 were successful when using the strong, constitutive
promoter *P* _ermC*+, but both *P* _ftdA_ _JV180_ and *P* _ftdA_ _IFO13350_
failed to drive observable *xylE* in both strains. However, qRT-PCR assays to
detect *xylE* transcripts were successful in making the desired com-
parison, confirming *P* _ftdA_ _JV180_ to be stronger than *P* _ftdA_ _IFO13350_
in both JV180 and IFO13350 hosts (*SI Appendix*, Fig. S10). Together,
the above data supported a model in which *P* _ftdA_ sequence varia-
tions might significantly influence PTM production or silence. While BGC promoter heterogeneity is a concept that remains
underexplored in the biosynthetic literature, the idea of tuning
BGC outputs through promoter strength is grounded in numerous
studies in which silent BGCs can be activated by replacing their
native promoters with stronger ones (see recent review in ref. 23).

**Mapping Promoter Regions of the JV180 PTM BGC Enables Structure–Function Comparisons.** Existing PTM regulatory knowledge is sparse
(24, 25), including within the *S. griseus* clade. Our above results
suggested that *S. griseus* clade PTM regulation involves cis-regu-
latory elements located directly upstream of *ftdA* (∼500 nt),
although we could not rule out additional influences from trans-
regulatory elements. To define the PTM promoter architecture of
these strains, the transcriptional start site (TSS) of strain JV180
was mapped via circular rapid amplification of cDNA ends. The
putative TSS was a cytosine residue 195-nt upstream of the pre-
dicted start codon of *ftdA* (*SI Appendix*, Fig. S11). Likely −10
and −35 boxes were assigned based upon established spacing (26,
27) (Fig. 2.4). The high-sequence conservation between the −35 box
and the TSS (82.4 to 100% pairwise sequence identity) sug-
gested that all examined *S. griseus* clade strains share the same
core promoter (−35, −10, and TSS).

To better resolve *P* _ftdA_ promoter architecture and probe for
the presence of cis-regulatory signatures upstream of the pre-
dicted −35 boxes, a series of nested deletions in this region were
created in JV180 (Fig. 2.4). PTM transcription and production in
these deletions were largely unaffected, except where the puta-
tive −35 box was disrupted (Fig. 2 B and C, ∆−528 to −31). This
confirmed the position of the JV180 −35 box and suggested this
region lacks any critical, regulatory residues. Furthermore, we
observed highly variable sequence conservation within this region
upstream for all studied *S. griseus* clade strains (38.2 to 97.8% pairwise sequence identity). This contrasts with the stricter sequence
conservation seen in the core promoter region (−35, −10, and TSS).

The −10 boxes and TSS residues of all examined strains are
perfectly conserved, but several single-nucleotide polymorphisms
(SNPs) differentiate the −35 boxes of the IFO13350-like *P*_ftdA_*8*
(Fig. 2.4, red box) from the strictly conserved −35 boxes of the
JV180 group (Fig. 2.4, green box). It is known that changes in
bacterial −35 boxes can greatly affect promoter strength (28, 29)
and could thus affect metabolite production. Therefore, a panel of
JV180 mutants carrying each observed −35 box SNP in the
IFO13350 group was created and tested for PTM production and transcription (*SI Appendix*, Fig. S124). Overall, the IFO13350
group −35 box SNPs failed to significantly change PTM pro-
duction and only slightly decreased transcription in the chimeric
JV180 hosts (*SI Appendix*, Figs. S12 B and C). These differences
were subtle compared to the far more substantial ones seen in
our −500-nt replacements upstream of the *ftdA* gene (Fig. 1 D and E), indicating that *P* _ftdA_ strength differences must originate
through mechanisms other than −35 box differences.

**AdpA Positively Regulates *S. griseus* Clade PTM BGCs.** Many Streptomyces messenger RNAs (mRNAs) contain long 5′ untranslated
regions (5′ UTRs) (26, 27), and these can contribute to regulatory
tuning. The DNA regions encoding 5′ UTRs can modulate gene
expression through direct regulator binding (30), and their corre-
spounding mRNAs can further modulate expression via ribos
witches (31) or other RNA secondary structures (32). A series of
−20 nt deletions were constructed (Fig. 2D) across the 195-nt 5′
UTR region of JV180 to probe contributions to PTM regulation.
This revealed multiple lesions with strongly decreased PTM pro-
duction and transcript levels, while others had little effect (Fig. 2 E
and F). mFold (33) modeling to reveal possible 5′ UTR mRNA
secondary structures across *S. griseus* clade strains yielded several
energetically favored outputs, and we surmise some of the dele-
terious, mutational effects seen in JV180 might stem from the
disruption of these types of structures (Fig. 2D, inset and *SI
Appendix*, Fig. S13–17).

While *S. griseus* clade *P* _ftdA_ 5′ UTR folding remains to be
further explored, the above deletions were particularly useful for
identifying a critical region that we subsequently characterized as
an AdpA binding site (Fig. 2 D–F, Δ29_48 and *SI Appendix*, Fig.
S9). AdpA is a global regulator that is well studied in strain
IFO13350, such that it is known to bind >500 chromosomal sites
via its weak consensus sequence TGGCSNGWWY (34). *S. griseus*
AdpA is involved in the hierarchical control of morphological
differentiation, the production of streptomycin and other antibio-
tics, and several other important processes (see review in ref. 16).
Prior chromatin immunoprecipitation sequencing and RNA se-
quencing data indicated that AdpA might bind upstream of
IFO13350’s PTM BGC (35), but because prior efforts to elicit PTM
production from wild-type (WT) IFO13350 were unsuccessful (15,
18), the biological significance of AdpA’s interaction with the gene
cluster was tenuous. Our searches for potential AdpA consensus
motifs within the *P* _ftdA_ regions of the studied *S. griseus* clade strains
revealed an imperfect inverted repeat 29- to 48-nt downstream of
the TSS in the JV180 *P* _ftdA_ (within the 5′ UTR region) that was
conserved in all *S. griseus* clade strains examined (*SI Appendix*, Fig.
S9). Because our nested deletions revealed that this sequence is
essential for JV180 PTM production, it renewed the notion that
AdpA might positively regulate PTM expression.

Several experiments were carried out to investigate whether
AdpA regulates *P* _ftdA_, including *adpA* deletion and complement-
analysis, mutating the putative AdpA binding site in *P* _ftdA_,
and in vitro binding assays. As expected from prior *adpA* studies
in strain IFO13350 (36), deletion of the JV180 ortholog (*SadpA*)
led to the loss of morphological differentiation and pigmentation
(*SI Appendix*, Fig. S18). PTM production and BGC transcription
were also abrogated in JV180, and these defects were rescued by
ectopically expressing either native *adpA*, its IFO13350 ortholog
(97% amino acid identity versus JV180 AdpA), or by replacing
*P* _ftdA_ with the strong, constitutive promoter *P* _ermC*+ to drive PTM
BGC expression independent of AdpA (Fig. 3 A and B and *SI
Appendix*, Fig. S19). These results were consistent with AdpA being
a transcriptional activator for *P* _ftdA_. However, in bacterial regulation,
regulators binding downstream of the RNA–polymerase complex
typically cause transcriptional downshifts (37, 38). Thus, the loca-
tion of the putative AdpA operator site downstream of the conserved
−10 box is unusual for a transcriptional activator. Importantly, another
AdpA-activated promoter with a downstream operator site controls *S. griseus* IFO13350 *adca*, encoding an extracytoplasmic function sigma factor (30). This precedent thus supports the idea that AdpA could positively regulate PTM expression, despite its atypical, putative binding arrangement in P	extsubscript{adca}.

AdpA–DNA cococrystallization studies indicate that the protein binds target operators as a homoduplex that recognizes a highly conserved, putative binding arrangement in P	extsubscript{adca} that AdpA could positively regulate PTM expression, despite its atypical, putative binding arrangement in P	extsubscript{adca}. Exchanging the JV180 AdpA operator site with that from IFO13350 was PTM proficient, although it showed transcriptional AdpA operator. Evidence further suggests that the identified region acts as a functional AdpA operator. Exchanging the JV180 AdpA operator site with that from IFO13350 was PTM proficient, although it showed a slight decrease in PTM titer and ftdB transcription (Fig. 3 C–E). This additional evidence further suggests that the identified region acts as a functional AdpA operator. Exchanging the JV180 AdpA operator site with that from IFO13350 was PTM proficient, although it showed a slight decrease in PTM titer and ftdB transcription (Fig. 3 C–E). AdpA–P	extsubscript{adca} interactions were further examined via electrophoretic mobility shift assays using operator sequences from JV180 and IFO13350, plus the critical point mutants that disrupt essential DNA–AdpA interactions. As expected, recombinant histidine-tagged AdpA bound the JV180 and IFO13350 P	extsubscript{adca} AdpA binding sites (Fig. 3 F and G) but failed to shift operators with the in vivo tested point mutations (Fig. 3H and SI Appendix, S20). Together, our in vivo and in vitro data strongly suggest that AdpA directly binds P	extsubscript{adca} in both JV180 and IFO13350 but also indicate that the native SNPs (Fig. 3C, red residues) in the nonessential residues of the AdpA operator sites are not the main cause of PTM expression differences seen between P	extsubscript{adca,JV180} and P	extsubscript{adca,IFO13350} (Fig. 3 D and E).

**Comparative Promoter Analyses Reveal an Indel “Switch” that Tunes P	extsubscript{adca} Strength and PTM Production.** Aside from the AdpA operator, the JV180 P	extsubscript{adca}′ S′ UTR truncation experiments revealed that the 28 nucleotides between the TSS and the AdpA binding site are also critical for PTM expression (Fig. 2 B–F, Δ2–Δ28). Nucleotide alignments between promoters in this region revealed generally high conservation, except for two nucleotides (AG) that are present in all JV180-like P	extsubscript{adca} but are missing from IFO13350-like P	extsubscript{adca} (Fig. 4A and SI Appendix, Fig. S9). The effects of this indel on PTM regulation were tested by deleting this AG dinucleotide from JV180 and by introducing the dinucleotide at the corresponding position in P	extsubscript{adca,IFO13350} (P	extsubscript{adca,IFO13350+AG}). P	extsubscript{adca,IFO13350+AG} was tested heterologously in strain JV180. Strikingly, the dinucleotide deletion led to strongly reduced PTM production and transcription, while the amended P	extsubscript{adca,IFO13350+AG} insertion variant led to a substantial increase in JV180 PTM production and transcription, compared to the WT P	extsubscript{adca,IFO13350} sequence (Fig. 4 B and C). To test if the indel’s effect was sequence specific, a JV180 transversion (ΔAG→CT) mutation was created, which exhibited reduced PTM production and transcription compared to WT but was much less deleterious than ΔAG (Fig. 4 B and C). This region is seemingly prone to sequence plasticity within the *S. griseus* clade; the recently isolated strain SP18CM02 contains an additional guanosine in this region compared to all other JV180-like P	extsubscript{adca}s (Fig. 4A). Despite having otherwise high, overall identity to all other JV180-like P	extsubscript{adca}s,
P\textsubscript{ftdA}_SP18CM02 drove slightly less transcription and resultant PTM production when heterologously introduced into JV180 (Fig. 1D and E). Together, these data suggest that, of the natural sequence variants in this region, having the AG dinucleotide is important for PTM production. These data clearly reveal this indel region as a key factor in modulating natural \textit{S. griseus} clade \textit{P\textsubscript{ftdA}} strength. Further work is necessary to discern how this indel region modulates promoter strength, possibly via mechanisms such as perturbed AdpA–RNA polymerase interactions or recruitment of another yet unknown, regulatory component.

**Discovery of an Unexpected Griseorhodin–Biosynthetic Interaction that Strengthens PTM Production in Strain JV180.** Our data thus far illustrated how leveraging within-clade comparative metabologenomics can assist regulatory region mapping and how small easily overlooked nucleotide changes in these regions can tune antibiotic production. The use of comparative genomics to understand \textit{Streptomyces} antibiotic production, particularly at the species level, is a relatively recent development in natural products functional genomics. Studies in this area tend to focus on BGC conservation and differentiation (e.g., comparison of the \textit{Streptomyces} albus clade; see ref. 39). From these comparisons, we anticipated that our \textit{S. griseus} clade strains would share several BGCs (beyond PTMs) and that some antibiotic BGCs in these strains would not be conserved clade wide. We found that our \textit{S. griseus} clade strains share a core set of 13 conserved BGCs. Some BGCs were found in only a few strains, and several BGCs were unique by strain (SI Appendix, Fig. S21). Strikingly, strain IFO13350 is one of the oldest known producers of streptomycin (40), but all members of the JV180 group were found to lack this BGC. Likewise, we noted that the JV180 group strains produced red pigments that are absent from the other studied \textit{S. griseus} clade strains (SI Appendix, Fig. S21). Through comparative BGC analysis and subsequent cluster deletion in strain JV180, we attributed this pigment to the production of griseorhodin polyketide congeners (41) (Fig. 5A, \textit{ΔgrhR2-V} and SI Appendix, S23A–C). Unexpectedly, this griseorhodin BGC...
Synthase (PKS) assembly-line enzymes were deleted (test this, four adjacent genes encoding griseorhodin polyketide outside of (Fig. 5

Abundance from JV180 PΔ in red. (Fig. 4.

To further probe how the griseorhodin BGC exerts its influence, we this mutant was PTM proficient, arguing against the signaling idea. This deletion mutant exhibited several additional phenotypes, including abrogated PTM production, down-regulated PTM BGC transcription, and reduced sporulation (Fig. 5 B and C and SI Appendix, S24).

The loss of PTM production in the JV180 ΔgrhI-R2-V mutant was wholly unexpected, and the mutant’s pleiotropic phenotypes suggested that griseorhodin might act as a signaling molecule. To test this, four adjacent genes encoding griseorhodin polyketide synthase (PKS) assembly-line enzymes were deleted (ΔgrhQSAB) (Fig. 5/4). This was done to specifically eliminate griseorhodin production, while leaving all other grh BGC genes intact. Interestingly, this mutant was PTM proficient, arguing against the signaling idea. To further probe how the griseorhodin BGC exerts its influence, we tested a griseorhodin-enriched JV180 growth extract (see Materials and Methods and SI Appendix, Fig. S25/) and authentic γ-rubromycin (42) (a griseorhodin analog) for their ability to chemically complement the ΔgrhR2-V strain. Neither sample could restore PTM production in the ΔgrhR2-V mutant in flask cultures (SI Appendix, Fig. S25B), and disk diffusion tests on agar plates failed to restore sporulation in the diffusion zone (SI Appendix, Fig. 25C). These experiments together ruled out the griseorhodin signaling hypothesis.

To continue probing the grh locus for key PTM-influencing genes outside of grhQSAB, multiple groups of genes were deleted from the BGC (grhR1-E, grhFGH, and grhL-P, Fig. S5A). All these mutations caused a complete loss of red griseorhodin pigmentation and showed reductions in PTM production and transcription (Fig. 5 B and C and SI Appendix, Figs. S23D and S26B). Homology-based annotations of the genes in these regions (41) led us to focus on a subset, which might affect PTM biosynthesis through transcriptional or metabolic mechanisms. Genes grhR2 and grhR3 encode transcriptional regulators, which could influence regulatory crosstalk, grFH encodes a phosphopantetheinyl transferase (essential for posttranslational modification of NRPS and PKS enzymes), and grhGH encodes β and ε subunits of acetyl-CoA carboxylase (ACC). ACC enzyme complexes are essential for malonyl-CoA production, a common precursor for fatty acid, griseorhodin, and PTM biosynthesis. PTM production in JV180 ΔgrhR2-V was not complemented by grhR2, grhR3, or grhF, but grhGH was able to restore some PTM production and transcription (Fig. 5 B and C and SI Appendix, Fig. S26A). Interestingly, expressing either grhG or grhH alone was sufficient to restore PTM production, similar to grhGH when expressed together (SI Appendix, Fig. S26A). A ΔgrhGH mutant was thus created, leading to partial griseorhodin pigmentation and reduced PTM production comparable to the initial ΔgrhFH mutant (SI Appendix, Figs. S23D and S26B). How the ΔgrhR1-E and ΔgrhL-P mutations caused decreased PTM production remains unclear. Because these lesions lead to the loss of putative regulatory genes (grhR2 and grhR3, see ref. 41), their phenotypes could be entangled with concomitant grhGH down-regulation.

In addition to the griseorhodin BGC’s grhGH, several other Streptomyces PKS clusters are known to harbor additional, non-housekeeping copies of ACC genes. These include cpkKL in the coelimycin BGC of Streptomyces coelicolor (43) and jadN in the jadomycin BGC of Streptomyces venezuelae (44), and it can be reasoned that these ACC copies likely assist polyketide biosynthesis through increased malonyl-CoA. Because JV180 PTM production decreased in the ΔgrhR2-V and ΔgrhGH mutants but not the ΔgrhQSAR mutant (Fig. 5B), we likewise hypothesized that PTM downshifts in these mutants might be caused, at least partially, by reduced, intracellular malonyl-CoA concentration. We thus tested several additional S. griseus clade ACC subunit genes for their ability to rescue PTM production in the JV180 ΔgrhR2-V strain to discern if these effects were specific to grhGH or are more broadly attributable across ACC subunit homologs. The tested ACC genes included those housekeeping ACC subunits cloned from JV180 and IFO13350 [function assigned by sequence homology and gene neighborhood synteny to the accBE genes in S. coelicolor (45)] plus the previously uncharacterized, PKS-associated ACC genes SGR3280 to SGR3281 of strain IFO13350, all of which encode ACC β and ε subunits like grhGH. All of these ACC homologs partially restored PTM production in the ΔgrhR2-V strain (SI Appendix, Fig. S26A), further supporting a role for malonyl-CoA in the PTM production defects of the ΔgrhR2-V and ΔgrhGH mutants.

To test if JV180 could still produce PTMs independent of the griseorhodin BGC, we constitutively expressed the strain’s PTM BGC by replacing PftdA with PpmrE. This PftdA–PpmrE–promoter replacement in both of the ΔgrhR2-V or ΔgrhGH backgrounds led to increased PTM production and PTM BGC transcription, similar to a JV180 ΔgrhR2-V;ermE+ control (Fig. 5 B and C). Our data show that PTM transcription and its production is affected by the lack of grhR2-V and grhGH. It is possible that the griseorhodin BGC may affect the PTM BGC through biosynthetic malonyl-CoA availability. It is also likely that an as of yet undefined transcriptional regulatory interaction also connects the two BGCs. Malonyl-CoA–responsive regulators are well-characterized in other model organisms such as Bacillus subtilis (46), but no such regulators are known in Streptomyces. Further inquiry is underway to characterize this unusual cross-cluster interaction more fully. Intrigued by the finding that ectopic expression of just grhG or grhH could rescue PTM production in the JV180 ΔgrhR2-V mutant, we tested if the PTM BGCs of weak PTM-producing S. griseus clade
strains might be similarly stimulated for production. This was done by heterologously expressing \( \text{grhG} \) in strains IFO13350, JV254, and JV258 (the \( \text{grhGH} \) construct had low-conjugation efficiency). This resulted in increased PTM production from these natively low-producing strains on agar media and also increased PTM BGC expression when tested in strain IFO13350, showing that ACC subunit overexpression can stimulate PTM production in natively poor producers (SI Appendix, Fig. S27 A and B). Combined with the above data, these observations support a model in which \( S. \) griseus clade PTM production differences have complex origins. In the case of poor PTM producers, promoter indels and insufficient positive BGC interactions likely dampen potential production via low transcription. In contrast, better PTM producers appear to benefit from cross-BGC interactions that increase transcription from a more active P\( \text{ftdA} \) variant (+AG), resulting in PTM production. Strain JV180, and possibly other members of its subclade, do not seem to be bottlenecked for PTM production at the transcriptional level based on several findings throughout this study. This includes the failure of \( \text{grhG} \) merodiploids to boost WT JV180 PTM production (SI Appendix, Fig. S27 A and B) and evidence from several recombinant strains in which P\( \text{ftdA} \) expression was increased by only up to 1.4-fold (\( \Delta \text{grhQSAB} \), \( \text{adpA} \) complementation, and \( \Delta \text{P}_{\text{ftdA}}::\text{P}_{\text{ermE}}^* \) strains; Figs. 3 A and B and 5 B and C). The above findings also highlight ACC enzyme overexpression as a way to potentially up-regulate certain silent BGCs, adding to their current use for increasing fatty acid and polyketide titers via malonyl-CoA overproduction (47, 48).

Conclusions

\( S. \) griseus genomes contain many BGCs encoding for drug-like compounds, and to harness their full biosynthetic capacity for discovery, it is crucial to understand what underpins the differences between active and silent BGCs (10). Cryptic metabolism is a well-recognized problem in the field, and poor BGC transcription is often implicated as the predominant mechanism behind biosynthetic silence. However, this supposition is increasingly challenged by a growing body of research that suggests all cryptic clusters aren’t necessarily transcriptionally silent (49). Furthermore, it only addresses how biosynthetic silence could originate in a given organism but does little toward explaining why affected BGCs show little activity to begin with. A common but difficult-to-prove hypothesis is that axenic laboratory growth deprives microorganisms of signals needed to up-regulate quiescent BGCs (21). This idea is based on the complex lifestyles of \( S. \) griseus bacteria, which are known to be heavily influenced by molecular and environmental cues (50). Indeed, chemical elicitor screening, coculturing, and other regulatory manipulation strategies continue to yield new molecules from apparently silent BGCs (7, 50, 51). However, the number and types of molecular signals these organisms can sense and respond to must have a finite limit, constrained by the characteristics and capacities of their genetically encoded signal...
transduction pathways (10). If true, then extracellular signals can only explain one part of antibiotic crypticity, highlighting the need to understand how basic strain-to-strain genetic differences also contribute to the phenomenon.

Toward this, we leveraged the unusual commonality of PTM BGCs in *Streptomyces* to compare several highly related *S. griseus* clades to discover genomic features that differentiate strong and weak producers. These efforts led to a cohort of sequence-defined PTM promoters, confirmation that the global regulator AdpA acts directly on *S. griseus* clade PTM production by binding Pₜₙₙₜ, promoter regions in an atypical way, and that 2- to 3-bp lesions between −10 promoter regions and AdpA operator sites can cause substantial differences in transcription strength and biosynthetic output. To our knowledge, this type of inquiry is largely absent from the biosynthetic-regulatory literature, but similar promoter heterogeneity, with resultant-tuned transcription, has been documented to control phase variation in bacterial pathogenicity (52). Furthermore, because the AG indel discovered here resides in a polyguanosine-rich region, poly-N strand slippage (52) or a similar mechanism might plausibly explain how these promoter variants arise in *S. griseus* clade member populations. As more *Streptomyces* are sequenced and characterized, it is likely that additional promoter region sequence variations will emerge as drivers of silent metabolism in other biosynthetic pathways. In proof, scientists working at Warp Drive Bio, Inc. patented a method as drivers of silent metabolism in other biosynthetic pathways. In that additional promoter region sequence variations will emerge to activate select silent BGCs.

A comparative metabologenomic approach reveals mechanistic insights into *Streptomyces* antibiotic crypticity. Strains, plasmids, and primers are described in *SI Appendix, Tables S1–S3*. Strains IFO13350 and JV251 to JV258 were obtained from the Agricultural Research Service Culture Collection (NRRL). All primers were purchased from Integrated DNA Technologies. All restriction enzymes, Taq polymerase, and T4 ligase were purchased from New England BioLabs. PCR was generally carried out using KOD Hot Start DNA Polymerase (EMD Millipore) in FailSafe PCR 2× PreMix G (Epicentre). Taq polymerase was used for colony PCR to verify cloning and genome editing. Streptomyces genomic DNA was prepared for PCR by grinding a colony in DMSO, as described by Van Dessel et al. (59).

Most media and chemicals were purchased from Sigma-Aldrich or Thermo Fisher Scientific. Unless specified, γ-tubulin was purchased from Abcam. 13C₂-L-ornithine was purchased from Cambridge Isotope Laboratories. Standard protocols for manipulating *Escherichia coli* were based on those of Sambrook et al. (60). Streptomyces cultures were routinely propagated on International *Streptomyces* Project-2 medium (ISP2) agar and Trypticase Soy Broth (Difco) at 28 °C. Glass beads were added to liquid cultures to disrupt mycelial clumps.

**Streptomyces Conjugations.** Conjugations were performed using JV36 as the general *E. coli* donor, as previously described (61). Streptomyces sp. strain JV180 spore cultures were collected from lawns plated on 8,340 agar [1% Profilo cottonseed meal (AMD); 2% o-mannitol; 0.1% yeast extract; 0.01% KH₂PO₄; 0.01% MgSO₄·7H₂O; 0.002% CaCl₂·2H₂O; 0.2% (volume/volume) R2 trace elements solution (62); 2.067% MES hemisodium salt; 2% agar; and pH 6.5] in a 12.5 cm petri dish. Cotton swabs were injected into TX Buffer (63). Strain JV254 and JV258 spore cultures were collected from lawns plated on Soya Flour Mannitol (62) and ISP-5 (61), respectively. Exconjugants were selected with 50 μg/mL colistin and 50 μg/mL apramycin. Successful integrations by φC31 integrase–based vectors were verified by colony PCR, as previously described (20).

**Marker-Less Gene Deletion/Promoter Replacement.** All gene deletions and PTₚₙₙₜ mutants were grown on ISP2 plates and incubated at 28 °C for 2 to 3 d. A plug was cut from the plate and used to inoculate 3 mL Trypticase Soy Broth in 24-well deep well plates, which were shaken at 300 rpm at 28 °C. One 4-mm glass bead was added per well to disrupt mycelial clumps. After 2 d of growth, 200 μL cultures were either inoculated into 20 mL ATCC-MOPS (adapted from ATCC172: 1% dextrose; 2% soluble starch; 0.5% yeast extract; 0.2% saline; 0.63% MOPS; and pH 7.2) in a 125-mL flask with 6-mm glass beads for disrupting clumps or plated on 8,340 agar overlaid with cellophane and incubated at 28 °C. Flask cultures were shaken at 250 rpm. After 4 d of growth, 1 mL was collected in RILater for protein and RNA analyses (see qRT-PCR), and the rest of the cultures were extracted twice with equal volumes of ethyl acetate. Solid media (plate) cultures were incubated for 6 d, and the mycelia and fractions were collected from the cellophane combination, as previously described (20). To avoid undesired recombination between the WT and the mutant PTₚₙₙₜ sequences, an intermediate ΔPTₚₙₙₜ::tetR mutant was constructed, with specifics provided in *SI Appendix, Supplementary Methods*. This ΔPTₚₙₙₜ::tetR mutant was used as the parent strain for the construction of most PTₚₙₙₜ mutants. Some plasmids for genome editing were cloned using overlap extension PCR instead of Gibson assembly, as described previously (64) (*SI Appendix, Table S2*).

**PTM Production and Analysis.** Strains were streaked from ~80 °C glycerol stocks onto ISP2 plates and incubated at 28 °C for 2 to 3 d. A plug was cut from the plate and used to inoculate 3 mL Trypticase Soy Broth in 24-well deep well plates, which were shaken at 300 rpm at 28 °C. One 4-mm glass bead was added per well to disrupt mycelial clumps. After 2 d of growth, 200 μL cultures were either inoculated into 20 mL ATCC-MOPS (adapted from ATCC172: 1% dextrose; 2% soluble starch; 0.5% yeast extract; 0.2% saline; 0.63% MOPS; and pH 7.2) in a 125-mL flask with 6-mm glass beads for disrupting clumps or plated on 8,340 agar overlaid with cellophane and incubated at 28 °C. Flask cultures were shaken at 250 rpm. After 4 d of growth, 1 mL was collected in RILater for protein and RNA analyses (see qRT-PCR), and the rest of the cultures were extracted twice with equal volumes of ethyl acetate. Solid media (plate) cultures were incubated for 6 d, and the mycelia and fractions were collected from the cellophane combination, as previously described (20). The ethyl acetate extracts were dried at low pressure and resuspended in 500 μL LC-MS grade methanol and syringe filtered before LC-MS analysis. PTM analysis was performed using a Phenomenex Luna C18 column (75 × 3 mm, 3 μm pore size) installed on an Agilent 1260 Infinity HPLC connected to an Agilent 6420 Triple-Quad mass spectrometer. For each run, 10 μL sample was injected and the chromatography conditions were as follows: T = 30 °C; B = 45%; T = 4, 45%; B = 12, 53%; B = 16, 100%; B = 20, 100%; A: water + 0.1% formic acid, B: acetonitrile + 0.1% formic acid, and 0.8 μL/min. The diode array detector was set to measure absorbance at 320 nm. The mass spectrometer was set to precursor ion scan mode with the precursor ions m/z: 450 to 550, collision energy = 30 V, fragmentor = 70 V, and daughter ions m/z: 139.2 or 154.2. The resulting data were analyzed offline with Agilent MassHunter Qualitative Analysis software. Chromatograms were extracted for each parent–daughter ion mass transition, and the integrated areas for the major PTM congeners (*SI Appendix, Fig. S28*) were used to compare PTM production. One PTM peak had identical retention times and fragmentation spectra as an authentic standard of maltophilin (sourced from EMC Microcollections, GmbH; *SI Appendix, Fig. S29*), a stereoisomer of 10-epi-maltophilin produced by *Streptomyces griseus* strain SCSIO 40010, which has a similar BGC to the major PTM BGC to strain JV180 (66). The sum of PTM peak areas was normalized by total protein, and the relative PTM production was calculated relative to the appropriate control strain, typically JV180 rpsl. PTM production experiments were generally carried out in triplicates, unless specified. The statistical significance in the differences observed was calculated by two-tailed Student’s *t* test, typically relative to JV180 or its rpsl mutant, JV307, or otherwise indicated. *P < 0.05, *P < 0.01, and *P < 0.001. Error bars represent SD.

Generally, the PTM production data reported were based on liquid media cultures that were used to collect the corresponding qRT-PCR data. Relative PTM production trends were consistent between solid and liquid media. Some figures in the *SI Appendix* show relative PTM production on solid media, where flask culture/qRT-PCR data were not collected.
qRT-PCR. Strains were cultivated in flasks, as described in PTM Production and Analysis. After 4 d, 1 mL culture was added to 2 mL RNAlater and vortexed to stabilize the RNA. The transfer was centrifugated at 3,214 × g for 10 min, and the supernatant was discarded. The RNA-stabilized pellet was resuspended in 250 μL 10-mg/mL lysosome (Sigma-Aldrich) and incubated at 37 °C for 30 min. To the lysate, 750 μL TRIzol reagent (Thermo Fisher Scientific) was added, and protein and RNA were extracted following the manufacturer’s protocol from this point. Protein concentration was measured by Bradford assay. The RNA was resuspended in 84 μL nucleic-acid-free water. DNase treatment was carried out by adding 10 μL 10x Turbo DNase buffer, 4 μL Turbo DNase with 2 μL RNAsin (Promega), for ~6 h at 37 °C. Removal of leftover DNA was confirmed by PCR and gel electrophoresis before the DNase inactivation reagent from the Turbo DNase kit (Thermo Fisher Scientific) was added. RNA concentration was measured with a NanoDrop, and 5 μg RNA was used for reverse transcription with SuperScript II (Thermo Fisher Scientific) following the manufacturer’s protocol.

Primers for qPCR were designed using the Integrated DNA Technologies (IDT) PrimerQuest Tool. Real-time PCR was performed on a CFX Connect Real-Time PCR Detection System (BioRad) with the following program: 1 cycle at 95 °C for 3 min, 40 cycles of 95 °C for 10 s, and 65 °C for 30 s. Each reaction contained 5 μL Taq Universal SYBR Green Supermix (BioRad), 2 μL nucleic-acid-free water, 1 μL 10-mM forward primer, 1 μL 10-mM reverse primer, and 1 μL template cDNA. The relative transcript abundance was calculated using the ΔΔCt method, and hrdB was used as the housekeeping gene (67). The primer efficiency was determined as described by the qPCR instrument manufacturer for several pairs of PTM BGCs, and the ftdB primers YQ376-180fddt11153 and YQ377-180fddt12178 were chosen for subsequent experiments, as they had the highest efficiency and produced the most consistent results. Data shown represent at least 95% success rate and are each derived from at least two biological replicates. The statistical significance in the differences observed was calculated by two-tailed Student t test, typically relative to JV180 or its rpsL mutant, JV307, or otherwise indicated. *P < 0.05, **P < 0.01, and ***P < 0.001. Error bars represent SD.

Expression and Purification of His-Tagged AdpA. The full-length adpA gene of Streptomyces sp. strain JV180 was amplified by PCR using primers pET11α-AdeP-AdpA-F and pET11α-AdeP-AdpA-R. The PCR product was cloned into the expression plasmid pET11α. The expression recombinant plasmid, pKG052, contained the sequence adpA-CTC-GAG-(TGAC)-TAG-Y (under the control of the T7 promoter, similar to the construct reported by Yamazaki et al. (30). E. coli BL21 (DE3) Rosetta-harboring pKG052 was cultivated in lysogeny broth (LB) medium with 100 μg/mL ampicillin at 37 °C overnight. A total of 1 mL seed culture was transferred to 150 mL LB medium with 100 μg/mL ampicillin and incubated by shaking at 37 °C at 250 rpm. When OD600 reached 0.6 to 0.8, the cells were chilled on ice for 1 h. After adding IPTG to 1 mM, the culture was continued for shaking at 250 rpm for 22 h at 30 °C. The cells were harvested by centrifugation at 5,000 rpm for 30 min, resuspended in Tris HCl buffer (20 mM Tris-HCl, 200 mM NaCl, and 10% glycerol: pH 8.0), and stored at ~80 °C. To purify the protein, cells were disrupted by sonication (3 x 10 s, 0 to 10% 1534–1344 (1996). 15. R. Rigali, S. Andersson, A. Naeye, G. P. van Brakel, Coupling the regulatory code of biosynthetic gene clusters as a strategy for natural product discovery. Biochem. Pharmacol. 153, 24–34 (2016). 16. J. Antosch, F. Schaefer, T. A. M. Gulder, Heterologous reconstitution of ikarugamycin biosynthesis in E. coli. Angew. Chem. Int. Ed. Engl. 53, 3011–3014 (2014). 17. J. A. V. Blodgett et al., Common biosynthetic origins for polycyclic tetracenomacrolides from phylogenetically diverse bacteria. Proc. Natl. Acad. Sci. U.S.A. 107, 11692–11697 (2010). 18. S. Zhang, W. Zhang, S. Saha, C. Zhang, Recent advances in discovery, biosynthesis, and genome mining of medicinally relevant polycyclic tetracenomacrolides. Curr. Top. Med. Chem. 16, 1727–1739 (2016).

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