Native Promoter Strategy for High-Yielding Synthesis and Engineering of Fungal Secondary Metabolites

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Supporting Information

ABSTRACT: Strategies are needed for the robust production of cryptic, silenced, or engineered secondary metabolites in fungi. The filamentous fungus *Fusarium heterosporum* natively synthesizes the polyketide equisetin at >2 g L⁻¹ in a controllable manner. We hypothesized that this production level was achieved by regulatory elements in the equisetin pathway, leading to the prediction that the same regulatory elements would be useful in producing other secondary metabolites. This was tested by using the native *eqxS* promoter and *eqxR* regulator in *F. heterosporum*, synthesizing heterologous natural products in yields of ~1 g L⁻¹. As proof of concept for the practical application, we resurrected an extinct pathway from an endophytic fungus with an initial yield of >800 mg L⁻¹, leading to the practical synthesis of a selective antituberculosis agent. Finally, the method enabled new insights into the function of polyketide synthases in filamentous fungi. These results demonstrate a strategy for optimally employing native regulators for the robust synthesis of secondary metabolites.

Because of new technologies in sequencing and bioinformatics, it is now relatively trivial to identify novel biosynthetic pathways to secondary metabolites in cultivated organisms and in the environment.¹ Research in this area has led to a renewed appreciation that many secondary metabolites have yet to be described and that a vast resource awaits discovery.²,³ Although pathway identification is simple, discovering the compounds produced by these pathways remains much more challenging, especially when the pathways are silent or encoded in the genomes of uncultivated organisms. To obtain the new compound, in many cases, the identified genes must be transferred to a new host and successfully expressed.⁴ Despite many advances in technology, this is still nontrivial.

To date, many heterologous expression hosts have been developed for secondary metabolite production.⁴⁻⁸ Most of these focus on expression of bacterial biosynthetic pathways, for which numerous hosts exist. For eukaryotic pathways, such as those from filamentous fungi, genes have been heterologously expressed in *Escherichia coli*, *Saccharomyces cerevisiae*, *Aspergillus oryzae*, and several other yeasts and filamentous fungi.⁴⁻⁵,⁷⁻⁹,¹⁰ Practical scale production of fungal compounds in bacteria has proven to be challenging.⁴ *S. cerevisae* provides a robust platform but with relatively modest purified yields. Additionally, it does not handle introns well, requiring that introns be removed prior to expression. Similarly, heterologous expression platforms in various filamentous fungi have led to relatively modest yields, although in many cases fungal introns are tolerated, allowing genomic DNA to be employed directly.⁵,⁹ These expression systems generally use housekeeping or related primary metabolic promoters that induce robust transcription of the desired genes, indicating that perhaps seeking improvements in level of transcription alone is insufficient to provide high levels of secondary metabolites, and suggesting room for the development of other strategies.

Here, we sought to take advantage of high-titer production of equisetin in the filamentous fungus *Fusarium heterosporum* ATCC 74349.¹¹,¹² *F. heterosporum* produces equisetin at ~2 g L⁻¹ on corn grit agar (CGA), yet production is undetectable (10 ng L⁻¹ detection limit) in many other types of media. We thus hypothesized that if an exogenous biosynthetic gene was placed under the control of the equisetin biosynthetic regulon, the heterologous compound would be synthesized in a yield similar to that observed for equisetin in the wild-type strain. Moreover, the highly controlled regulation of the equisetin locus might enable the production of compounds that are natively toxic to *F. heterosporum*. This strategy bears similarity to that previously used in actinomycetes to synthesize...
polyketides\textsuperscript{13} but stands in contrast to a commonly used strategy in filamentous fungi, wherein promoters or regulators from primary metabolism are used to produce recombinant polyketides.

Using the model described below, we produced several heterologous fungal polyketides in \textit{F. heterosporum}. In fungi, polyketides are biosynthesized by iterative decarboxylative condensation of malonyl units.\textsuperscript{5,15} The minimal domains that make up the polyketide synthase (PKS) include acyltransferase (AT) domain, which selects the substrate; acyl carrier protein (ACP), which tethers the growing chain during extension; and the ketosynthase (KS) domain, which catalyzes the condensation reaction.\textsuperscript{14–16} In addition, PKS enzymes may contain other modifying domains such as the methyltransferase (MT) domain, which introduces varying patterns of C-methylations along the polyketide backbone. For the reducing-type PKS, varying levels of reduction are achieved by ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains.\textsuperscript{14} In general, all domains required to produce a fungal polyketide are contained on a single polypeptide, but for several PKSs, the ER is trans-acting and is translated as a separate polypeptide from the other PKS domains.\textsuperscript{17–20} In the latter case, the trans-ER protein is required to produce the natural product. In many fungi, PKS modules exist as hybrids with nonribosomal peptide synthetases (NRPS), resulting in formation of polyketides fused to amino acids.\textsuperscript{14,15,21,22} Unlike standalone PKSs that rely on hydrolysis via a thioesterase domain (TE) for product release, several PKS-NRPSs use a terminal reductase (R) domain, which catalyzes a Dieckmann reaction in tandem with product release to form a tetramic acid ring.\textsuperscript{23,24}

Here, we describe a platform that enables production of heterologous fungal secondary metabolites in high titers (\~{}1 g L\textsuperscript{−1} unoptimized yield). The platform was applied to several problems in biosynthesis and drug discovery, including resurrection of a silenced pathway of potential use in tuberculosis.

\section*{RESULTS AND DISCUSSION}

\textbf{Design of Expression Strategy.} The strategy was based upon prior knowledge of the regulation of equisetin production. Equisetin biosynthesis requires the coordinated action of the PKS EqxS, the auxiliary ER EqxC, and the N-MT EqxD (Figure 1).\textsuperscript{11} The first two of these are synthesized from genes that are divergently transcribed from a promoter region, \textit{peqxS}. A regulatory transcription factor, EqxR, drives production of equisetin.\textsuperscript{11} On CGA media, a large amount of equisetin is produced, while on other media such as potato dextrose agar (PDA; Difco) or potato dextrose broth (PDB; Difco), equisetin is not produced.\textsuperscript{11} Equisetin is constitutively produced on CGA and slowly accumulates over a 21-day period. We thus selected \textit{peqxS} as a platform for producing metabolites, under control of EqxR. In this conception, the \textit{peqxS} region also contains a hypothetical gene, \textit{eqx3}, which is commonly found in equisetin-like pathways. For simplicity, this region was left in place in the current study. In some vectors, we used only one-half of \textit{peqxS} to express genes in one direction; in this series, \textit{eqx3} is disrupted, and the promoter region is referred to as \textit{peqxS′}. In others, the bidirectional promoter region \textit{peqxS} was used; in these cases \textit{eqx3} is not disrupted.

While the wild-type regulator \textit{eqxR} was well controlled to produce equisetin solely on CGA, we envisioned applications in which we might desire more rapid production in liquid media. In previous work, we fused the \textit{alcA} promoter (\textit{palca}) with \textit{eqxR} (Figure 1C).\textsuperscript{11} While \textit{palca} is inducible, it exhibits significant basal expression. Indeed, \textit{palca-eqxR} led to constitutive production of equisetin even in broth.\textsuperscript{11} However, production yields were lower in broth, and there was a concern that toxic metabolites may delay or halt fungal growth under control of \textit{palca-eqxR}. By contrast, an advantage of the PDB method was that production could be achieved in 5–7 days, rather than the 21+ days used with CGA. The strategy thus took advantage of this strong control of equisetin production. If toxicity was a concern or if high yields were required, the wild-type \textit{eqxR} construct was used. For a faster assessment of whether a recombinant metabolite could be produced, the \textit{palca-eqxR} construct could be employed. In this study, we employed two strategies to introduce \textit{palca-eqxR}: (1) we recently reported a modified \textit{F. heterosporum} strain, Palc-eqxR, which overexpresses the equisetin positive regulator \textit{eqxR} under control of the \textit{alcA} promoter to allow equisetin production in broth culture after 5 days, whereas normally no equisetin is produced in liquid broth;\textsuperscript{11} (2) we included the \textit{palca-eqxR}}
gene in an expression vector that could be transferred to *F. heterosporum*, along with desired secondary metabolic genes.

We had two major concerns in terms of practical genetics: could we repetitively use a single promoter element, and could we process different classes of introns? *F. heterosporum* largely undergoes ectopic recombination in our hands, and it is quite difficult to obtain homologous recombinants. For this reason, we believed that repetitive use of *peqS* would be tolerated by the strain, enabling production of more than two gene products without recombination. Indeed, in this study, we show that multiple copies of this element are stable in recombination, meaning that the strategy is scalable to recapitulate multigene biosynthetic pathways. We were also concerned about the ability to splice different types of introns. While this will presumably always be a problem in some cases, here, we show that a quite divergent set of introns can be processed. However, our overall strategy mainly uses artificially spliced, intron-free DNA.

Our construction strategy involved using shuttle vectors, in which we could employ yeast recombination in *S. cerevisiae* to build the desired vectors, *E. coli* to amplify those vectors, and *F. heterosporum* for production of compounds. Each vector thus required selection and replication elements for each strain (see Methods). We constructed and tested multiple types of selection markers so that multiple different vectors could be inserted into *F. heterosporum*. Previously, we used hygromycin (*hph*) and phleomycin (*ble*) resistance effectively. Here, we also employed uracil auxotrophy as an additional selection marker. By combining these elements, it becomes possible to use stepwise engineering to insert multiple copies of genes under control of *peqS*. In addition, it is always possible to insert multiple copies of the promoter into a single vector prior to transformation into *F. heterosporum*, making the strategy highly scalable.

Finally, we used an 8-cutter restriction endonuclease site, either AscI or PacI, to linearize the vectors before fungal transformation. These sites were selected so that the linearized vectors would contain the complete promoter-heterologous gene construct in the correct order. Otherwise, there was a danger of integration with a disrupted reading frame. In addition, we put these sites in some cases between selectable markers and synthetic green fluorescent protein (sGFP) to ensure that the vector was inserted in the correct manner. However, in the event, we never found a rearrangement that necessitated using sGFP. In *F. heterosporum*, linearized vectors integrated ectopically and intact.

Below, we describe a stepwise application of these principles to construct a heterologous expression platform. (1) The basics of transcription using both wild-type *eqxR* and *palCA-eqxR* were examined using sGFP. Although simple chemical analysis of equisetin production previously revealed fundamental aspects of pathway regulation, employing sGFP enabled a direct translational readout that would complement our understanding of *eqxR* and *peqS*. (2) We desired to test this strategy by adding a single, discrete, heterologous PKS that leads to a known and well characterized product. We selected the *cpaS* gene from the *Aspergillus flaus* cyclopiazonic acid pathway for this purpose. Not only is *cpaS* exceptionally well characterized, but *A. flaus* is in a different class (Eurotiomycetes) from *Fusarium* (Sordariomycetes). (3) We next tested the applicability to systems requiring two genes for production of compounds. In the event, we used both the homologous equisetin pathway and the heterologous lovastatin pathway, again from an *Aspergillus*. By examining equisetin production, we hoped to determine whether chromosomal location impacted production; in the event, it did not. The lovastatin PKS genes are well characterized and biomedically of great importance, making this an interesting target for production. Moreover, only two proteins are required to synthesize the complex core of the molecule. (4) We aimed to produce more than two genes and to test the practical application of the platform to a real-world problem. This involved production of the “extinct” metabolite, pyrrolocin. Taken together, these approaches fully define the application of equisetin regulatory elements to produce diverse secondary metabolites in high yield.

**Knockout of the eqx Locus.** We desired to compare expression in the wild-type *F. heterosporum* with expression in an *eqx* knockout strain. Although *eqxC* has been previously deleted, *eqxS* was difficult to delete. Here, we created a knockout vector in which *eqxS*, *eqxC*, *eqxD*, and the promoter region *peqS* were deleted (Figure 2). The resulting strain, Fus*ΔeqxS* was confirmed to be an *eqx* cluster knockout by PCR (Supporting Information Figure S4). Subsequent genome sequencing of Fus*ΔeqxS* revealed that the *eqxS* gene was knocked out in the S′ end and that the deletion vector was inserted into a total of 3 locations in the genome (Supporting Information Figure S5).

**eqxS Promoter Drives Expression of sGFP in Recombinant Strain.** To test whether the *peqS* promoter sequence could be used to express exogenous genes, and to determine timing and control of heterologous expression, the *peqS*′ was fused to sGFP and transferred to wild-type *F. heterosporum* in vector FH-1 (Figure 3A; Supporting Information Figure S1). After 21 days on CGA, sGFP was observed in fungal filaments when examined by confocal fluorescence microscopy. Moreover, the fungus was fluorescent over the full expression period on CGA, but it lacked any observable fluorescence on PDA. This was a clear indication that *peqS*′ was sufficient for heterologous expression. It also further supported previous observations that production was constitutive on CGA but completely shut off on PDA and other media. By contrast, when sGFP was added to strains containing the leaky *palCA-eqxR* gene (Figure 3B), the resulting *F. heterosporum* strain was constitutively fluorescent on all media.

**Expression of the cpaS Gene from Aspergillus flaus Produces Expected Metabolite.** To determine whether the *peqS*′ could lead to biosynthesis of new compounds, the well-characterized *cpaS* gene from *Aspergillus flaus* was cloned into FH-1 to make pHgB-Cpas. The resulting *F. heterosporum*
mutant, Peqx:Cpas, was then cultured in potato dextrose broth (PDB) for 7 days. The predicted product, cAATrp \( \text{I} \) was identified by high-pressure liquid chromatography (HPLC) (Figure 4A) and isolated. Comparison of the \(^1\)H NMR spectrum and the molecular formula of \( \text{I} \) with those of the previously reported cAATrp showed that they were identical (Supporting Information Figure S13).\(^{23,27}\) This confirmed the value of \( \text{Peqx} \) in producing heterologous compounds.

However, high levels of equisetin \( \text{II} \) were coproduced (Figure 4A).

In other systems, it has been shown that eliminating the production of undesired metabolites increases substrate flux to the target pathway resulting in higher yields of the desired compounds.\(^5\) In our case, we reasoned that deleting the equisetin biosynthetic genes would not only increase flux of the building block, malonyl CoA, to polyketide-type heterologous pathways, but also ease downstream target compound purification and analysis. The \( A. \text{flavus} \) \( \text{cpaS} \) gene was cloned into vector FH-2 (Supporting Information Figure S2), which contained a copy of \( \text{palcA}-\text{eqxR} \) for constitutive expression in broth. An sGFP tag was fused to the C-terminus of \( \text{cpaS} \). After transformation of this construct into the \( \text{eqx} \) knockout strain \( \text{Fus} \Delta\text{eqxS} \), we isolated a mutant \( \Delta\text{eqxS}:\text{Peqx:CpaSgfp} \) that glowed bright yellow on a dark reader (Figure 4B). Production of \( \text{I} \) was measured to be 100 mg \( \text{L}^{-1} \) by HPLC-DAD in comparison with a standard curve. This is an order of magnitude greater than the previously reported production of the same compound when the \( \text{cpaS} \) was expressed in \( A. \text{oryzae} \) under control of a housekeeping promoter.\(^{27} \) In addition, this experiment provided some support for our hypothesis, since the yield in the deletion mutant was increased by approximately 4-fold in comparison to production in the wild-type equisetin producer.

When the \( \Delta\text{eqxS}:\text{Peqx:CpaSgfp} \) mutant was cultured for 21 days on CGA, production of \( \text{I} \) rose to over 1 g \( \text{kg}^{-1} \) without any optimization of production conditions. \( A. \text{flavus} \) is quite phylogenetically distant to \( F. \text{heterosporum} \), with the former in Class Eurotiomycetes and the latter in Class Sordariomycetes, indicating that the strategy may be widely applicable to fungal metabolites from different groups.

**Use of the Divergent Promoter for Simultaneous Dual Gene Introduction.** In initial experiments, only one side of \( \text{Peqx} \) was used, comprising 1.5 kbp of gene sequence. To express two genes, \( \text{Peqx} \) was synthesized, containing the entire \( \sim 2.5 \) kbp of the divergent promoter, to generate vector FH-3 (Figure 5A). Previously, we showed by knockout mutagenesis that \( \text{eqxC} \) was critical for the production of equisetin \( \text{II} \) and trichosetin \( \text{III} \), and we proposed that it was the trans-acting ER regulator. Shown here is a photograph of constitutively fluorescent fungal colonies grown on PDA, visualized on a Dark Reader.

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gCOSY, gHSQC, and gHMBC, to be the ring-opened derivative of trichosetin that had not undergone the terminal Dieckmann reaction. This result implied that eqxS is indeed the equisetin synthetase, but the gfp tag interferes with the proper functioning of the reductase domain, preventing Dieckmann cyclization and instead promoting water-mediated hydrolysis of the intermediate. This was, in fact, found to be the case because, when eqxS was cloned without a C-terminal tag, trichosetin was robustly synthesized at wild-type levels (Figure SB).

**Production of Multiple Gene Products Leads to Synthesis of Lovastatin Precursors in F. heterosporum.** To establish the utility of this new expression strategy for coexpression of heterologous genes, we cloned the well-studied lovastatin nonaketide synthase (lovB) from *Aspergillus terreus* together with its cognate trans-ER (lovC) to make the hphlovC+lovBgfp plasmid. We also cloned only lovB into FH-3 to make the plasmid hpheqcX+lovBgfp. Transformation of these constructs independently into FusΔeqx resulted in production of the expected products. Without its cognate trans-ER, the expressed LovB synthesized the previously reported truncated intermediates, 5 and 6 (Figure 6A). Coexpression of lovB with lovC produced the expected reduced metabolites 7 (Figure 6A), the dihydromonacolin L acid 8, and the lovastatin precursor lactone 9 (Figure 6B). These metabolites were characterized by comparing their liquid chromatography/mass spectroscopy (LC/MS), ultraviolet (UV), and 1H NMR data to previous reports (Supporting Information Figures S8–S10, S12–S14). This confirmed that, indeed, the intergenic sequence between eqxS and eqxC could guide transcription divergently to heterologously coexpress two genes. The purified yield of 9 was 130 mg kg−1, and 8 was produced in about equal proportions.

Figure 5. Trichosetin synthesis requires EqxC and unmodified EqxS. (A) Expression vector FH-3 designed with complete intergenic sequence peqxS, for dual expression of genes. Also shown are elements that permit cloning by recombination in *S. cerevisiae* and selection in *E. coli.* (B) Analytical HPLC of crude extracts of PDB cultures of FusAleqs5 transformed with eqxSgfp together with either eqxC or noncognate trans-ER lovC. Also shown is nontagged eqxS coexpressed with eqxC and the trichosetin standard. Trichosetin is only produced in the presence of eqxC and unmodified eqxS. (C) LC/MS analysis of crude extracts shows that a gfp tag on the C-terminus of EqxS interferes with formation of trichosetin and instead results in production of only the ring-open form 4.

Figure 6. Dual expression with peqxS promoter reconstitutes pathway to lovastatin precursor. (A) Analytical HPLC of crude extracts of PDB cultures of FusAleqs5 transformed with lovBgfp together with lovC or a noncognate trans-ER eqxC. In the presence of eqxC, lovB produces the polyene pyrone 5 and ketone 6; and more reduced pyrone 7 with lovC coexpression. (B) LC/MS analysis of crude extracts shows formation of the lovastatin precursor, dihydromonacolin L acid 8 and the lactone 9 when lovBgfp is coexpressed with lovC. EqxC is not able to complement LovB to form 8 or 9.
EqsX Requires Cognate trans-ER. In the course of cloning and analyzing locV, we coexpressed locV and eqsX under control of the divergent promoter. No trichosetin was produced under these conditions (Figure 5B). LocV could not complement EqsC, indicating that EqsS could not interact with the noncognate ER. Further, the complementary experiment in which EqsC was coexpressed with LovB failed to yield reduced intermediates (Figure 6A). By contrast, the EqsS were fully functional when coexpressed with their cognate PKS proteins.

Resurrection of Antituberculosis Agent from a Silenced Biosynthetic Pathway. In collaboration with scientists at Wyeth (now Pfizer), researchers in the Barrows lab cultivated an endophytic fungus, designated strain NRRL 50135, obtained in Papua New Guinea as part of their International Cooperative Drug Discovery Group (ICBG) project. The crude extract was potently active against Mycobacterium tuberculosis, and assay-guided purification led to the identification of a novel compound, pyrrolocin A (10), as the active principle. In 2007, a tentative structure was proposed for this compound, which contained a tetramate ring. The structure of 10, at 458 Da (M+H)+ initially isolated from endophytic fungus NRRL 50135. (B) The identified candidate gene cluster for the biosynthesis of 10 after genome sequencing contains a PKS-NRPS hybrid gene prlD, an enoyl reductase prlC, two transcription factors prlF and prlR, and two export genes prlG and prlL. (C) Analytical HPLC of crude extract of FusAeqsXΔpyrG10 transformed with both prlS and prlC driven by the eqsX promoter shows synthesis of two new products 11 and 12 with corresponding mass of 444 Da (M+H)+. Further introduction of methyltransferases prlD and eqxD led to synthesis of new minor products 13 and 10, respectively. (D) Compounds 11 and 12 were found to have antituberculosis activity and to have the core structure of 10.33

Figure 7. (A) Preliminary structure of antituberculosis agent 10 with mass 458 Da (M+H)+ initially isolated from endophytic fungus NRRL 50135. (B) The identified candidate gene cluster for the biosynthesis of 10 after genome sequencing contains a PKS-NRPS hybrid gene prlD, an enoyl reductase prlC, two transcription factors prlF and prlR, and two export genes prlG and prlL. (C) Analytical HPLC of crude extract of FusAeqsXΔpyrG10 transformed with both prlS and prlC driven by the eqsX promoter shows synthesis of two new products 11 and 12 with corresponding mass of 444 Da (M+H)+. Further introduction of methyltransferases prlD and eqxD led to synthesis of new minor products 13 and 10, respectively. (D) Compounds 11 and 12 were found to have antituberculosis activity and to have the core structure of 10.33

Days. The crude extract contained pyrrolocins B (11) and C (12), the desmethyl derivatives of 10 (444 Da [M + H]+).33 Compounds 11 and 12 (Figure 7C) were produced in a 2:1 ratio, totaling >800 mg kg−1 on CGA, which is greatly in excess of what was initially found for 10 in the native producer before production was lost (∼50 mg L−1).

Two MTs were cloned in attempts to methylate the tetramate ring. The first, PrlD, was the top hit resulting from BLAST analysis of NRRL 50135 when EqsD was used as the query (36% identity). prlD was from a contig that was not linked with prl, meaning that it might result from a separate biosynthetic pathway. EqsD itself was also coexpressed with PrlS and PrlC. Each MT was cloned into complementary vector FH-1 and separately transformed into F. heterosporum ΔpyrG::Peqs::prlS+prlC. The mutants were cultivated for 7 days. Analysis of the crude extracts by HPLC-DAD and LC/MS showed that both PrlD and EqsD led to synthesis of methylated products. Interestingly, the EqsD-expressing strain
produced a minor amount of authentic pyrrolocin A 10 (<10 mg kg\(^{-1}\)) in a background of ~800 mg kg\(^{-1}\) of nonmethylated 11 and 12. The methylated derivative 13 produced by the PrlD-expressing strain did not match the NMR data for 10 and is therefore not the correct MT. No obvious MT exists in the sequenced genome that would be predicted to perform that transformation.

**Conclusion.** Heterologous expression is potentially the most universal solution to produce natural products from cryptic pathways and unculturable organisms.\(^4\),\(^5\) We set out to develop such a production platform based on the equisetin biosynthetic reguloan, which would enable production of important metabolites with sufficient yield to ease purification and downstream assays. This platform was designed based upon the hypothesis that high-level transcription alone was insufficient to produce high levels of secondary metabolites. Instead, exploitation of a natively high-producing biosynthetic pathway would provide the ideal environment for secondary metabolite biosynthesis. In developing and testing this platform, we designed derivative strains of *F. heterosporum* and a suite of expression vectors into which various heterologous genes could be cloned. These new tools were validated by producing a range of fungal polyketides from widely different fungi, in unoptimized yields ranging from several hundred mg to >1 g per liter. In fact, we found that cultures on just 50 g of CGA provided enough material for complete characterization of novel compounds. We provided proof of concept by resurrecting an important silenced pathway.

Another advantage of this platform is that it seems to process introns from a broad phylogenetic diversity of fungi. prlS and prlC contained introns that were properly processed (otherwise interrupting the reading frame of the proteins), demonstrating the breadth of the platform in handling introns from diverse strains. As has been found in other heterologous expression strategies, a disadvantage is that pathway shunt products are often produced along with the major natural product. However, in this case, the abundant amount of desired products compensated for this problem.

We have demonstrated practical application of this platform in reviving antituberculosis activity previously observed from pyrrolocin A, a product of the NRRL S0135 strain. Direct cloning of prlS and prlC from genomic DNA of strain NRRL S0135 into our expression vectors and subsequent high-level production of related active compounds 11 and 12 demonstrated the ease with which such eukaryotic genes riddien with introns could be characterized. This work enabled the structures of the pyrrolocins to be fully elucidated and the biological activity to be characterized.

This strategy is widely applicable to many types of natural product synthesis in different organisms. The key factors that led us to select *F. heterosporum* as the production host were as follows: (1) the robust synthesis of the native compound, which meant that even if the recombinant yield were greatly reduced it should still be sufficient for chemical analysis; (2) the strict controllability of compound production, where essentially no natural product is produced on most media types, leading to a reduction in problems related to toxicity; (3) the apparent constitutive expression of the natural pathway, where compounds are slowly produced, exported, and accumulated in the medium over the course of weeks on CGA. It is likely that other systems with similar features would be amenable to the same approach.

A few biochemical observations were also enabled by these studies. During the complementation of the eqx knockout strain with eqxC and eqxS, we directly show that both these genes are required for equisetin production. However, we found that R domain function is altered when fused to an sGFP tag. Instead of the expected tetramic acid 3, we obtained the ring-open 4. We speculate that sGFP alters the structure of the R domain to prevent either transfer from the T domain or to allow water to enter the R domain active site. The tetramic acid derivative is nearly instantaneously formed from thioesters under neutral buffer conditions in water,\(^4\),\(^5\) making it especially remarkable that the linear form can be obtained from thioenamdated synthesis. Potentially, if desired, this problem might be circumvented by experimenting with different types of linkers that do not disrupt the R domain. A cleavable linker strategy may also be feasible. However, in our hands, we found that sGFP is optional; for example, the complete vector is usually integrated intact in *F. heterosporum*. We also found that the ER proteins cannot be crossed between these pathways but that the wild-type ERs are required at least in these cases. The interaction of these ER proteins is also of interest, since having the correct set of protein partners is essential in the synthesis of the desired natural products.

In the wild-type fungus NRRL S013S, 10 was the major compound produced, with a small amount (<1% estimated from HPLC-MS) of 12 as a side-product.\(^3\) Based upon the sequenced gene cluster and the recombinant expression performed here, it is clear that the combination of PKS-NRPS and auxiliary ER lead to the formation of 12. Unlike our findings here, the wild-type fungus did not produce any cis-decalin product, such as 11. It is possible that one of the other hypothetical genes in the gene cluster could be responsible for this discrepancy and may act as the pyrrolocin Diels–Alderase, but there are also other interesting possibilities. Of note, no obvious MT was present in the prl cluster that might produce 10. The closest homologue of EqxD from the genome did not produce 10. By contrast, EqxD itself produced ~10 mg kg\(^{-1}\) of 10. This minor production, using a combination of heterologous and homologous proteins, would likely not have been observable if starting with a less efficient expression system. This emphasizes the value of starting with a high-yielding platform. The yield was still sufficient for biological and chemical characterization from a single 1 kg scale experiment. The reaction with EqxD is also remarkable in that 12 contains d-Ser, while 3 contains l-Ser, and the decalin ring is also enantiomeric between 3 and 12. This indicates that, perhaps, EqxD has fairly relaxed substrate selectivity.

### METHODS

**Cloning of Vectors and Expression Plasmids.** Standard PCR techniques were employed and plasmid construction carried out as previously described.\(^1\) Details of vector construction can be found in the Supporting Information. Vector images were generated with Vector NTI software (Invitrogen).

Transformation of *Fusarium heterosporum* was done as previously described except that protoplasts were prepared from 8 to 10-h germinating spores.\(^1\) When antibiotic selection was required, hygromycin or phleomycin was added to media at 150 \(\mu\)g mL\(^{-1}\). For uracil auxotrophy selection, protoplast regeneration agar lacking uracil was prepared containing 1 M sucrose, 0.02% yeast extract without amino acids, 0.02% BSM supplement, and 1% agar.
Fungal Mutagenesis. Knockout of eqxS FusΔeqxS was constructed by transforming FusΔeqxC with knockout vector ClusterPhleoKO (Supporting Information) and transformants selected on phleomycin. The knockout cassette was made of the phleomycin resistance marker flanked by sequences homologous to regions within eqxS and eqxD (Figure 2). Isolated transformants were counter-screened for hygromycin sensitivity and verified by colony PCR. The genome of the identified knockout FusΔeqxS was extracted and sequenced. The reads were aligned to the FusWT reference genome with Novoalign and output visualized with the integrative genomics browser (Supporting Information Figure S5).

Generating the Uracl Auxotroph. The pyrG knockout cassette in the TOPO-pyrGKO plasmid was made by cloning a randomly selected sequence (first exon of lovC) into the F. heterosporum pyrG sequence. Translation of this sequence results in a truncated, nonfunctional PyrG. FusΔeqxS was transformed with TOPO-pyrGKO, and the protoplasts were regenerated for 72 h at 30 °C before plating on selection medium made of Czapek Dox Broth, 5-FOA (4 g L−1), uracil (1.12 g L−1), uridine (140 mg L−1), and 1.5% agar. Transformants were cross-streaked on Czapek–Dox agar with and without uracil to identify auxotrophs. Diagnostic PCR for homologous integration was done to confirm FusΔeqxSΔpyrG10 as a true knockout (Supporting Information Figure S6).

Fluorescence Microscopy. Visualization of gfp expressing mutants on CGA was done on an Olympus FV1000 spectral confocal microscope. Visualization of gfp expressing mutants on PDA was done on a Dark Reader (Clare Chemical).

Genome Sequencing and Analysis. All fungal genomes were extracted with the DNeasy Plant Minikit (Qiagen) and sequenced at the University of Utah Huntsman Cancer Institute sequencing facility on an Illumina HiSeq 2000. The raw reads from NRRL 50135 genome sequencing were assembled using the SPAdes de novo genome assembler with Novoalign and output visualized with the integrative genomics browser (Supporting Information Figure S5).

Chemical Analysis. Selected transformants were screened for compound production by culture in potato dextrose broth (PDB) for 7 d at 30 °C with shaking at 180 rpm. Speros of compound expressing mutants were then inoculated on to corn grit agar and incubated at room temperature for 21 d. Extraction of filtered PDB broth was done with ethyl acetate containing 1% acetic acid, and CGA was wholly extracted with acetone. Solvents were removed under vacuum, and the crude extracts analyzed by HPLC-DAD and LC/MS using C18 chromatography.

Purification of cAAATrp 1. The crude extract from a PDB culture (250 mL) of ΔeqxS::Peqx:CpaSgfp was purified by preparative HPLC (4 mL min−1; 5–70% acetonitrile/water-0.05% TFA in 35 min). The pooled fractions were dried under vacuum to a total of 0.05% TFA in 35 min). The pooled fractions were dried under vacuum to afford pure 1 (9 mg), which was then analyzed by 1H NMR and LC/MS (Supporting Information Figures S7 and S13), in comparison to previously published reference data. A standard curve was generated using pure 1 by HPLC-DAD and production was quantified from crude extracts of 100 mL PDB cultures and 50 g CGA cultures of ΔeqxS::Peqx:Cpasgfp to average 100 mg L−1 and 1.25 g/kg (±0.24 g kg−1, n = 3), respectively.

Purification of 4. CGA culture of ΔeqxS::Peqx:eqxC +eqx5gfp (50 g) was extracted with acetone. The crude residue was then fractionated by flash chromatography on end-capped C18 with a methanol/water gradient and fractions screened by LC/MS. The fraction containing 4 was further purified by preparative HPLC to afford previously undescribed compound 4 (4.7 mg), which was characterized spectroscopically (see Supporting Information).

Purification of Dihydromonacolin L 9. The crude extract from 50 g CGA culture of ΔeqxS::Peqx:lovC+lovBgfp was fractionated by flash chromatography on end-capped C18 with a methanol/water gradient. Fractions were screened by LC/MS and was found to be contained in one fraction. This fraction was dried, and the residue was separated on silica column with 2:1 ethyl acetate/hexanes mobile phase. Further preparative HPLC purification afforded 9 as a white solid (6.6 mg). MS and 1H NMR data were compared with those for the previously reported material.

Purification of 10–13. To purify 11, the crude extract from 200 g CGA culture was fractionated by flash chromatography on end-capped C18 using a methanol/water gradient. The fractions were analyzed by HPLC-DAD, and a portion (12.5%) of the fraction containing 11 was purified by several rounds of preparative HPLC to obtain pure 11 (8.6 mg; isolated yield 344 mg kg−1). A similar strategy was used to obtain other derivatives.

ASSOCIATED CONTENT

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