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Toward Awakening Cryptic Secondary Metabolite Gene Clusters in Filamentous Fungi

Fang Yun Lim^{*}, James F. Sanchez[†], Clay C.C. Wang^{†,‡}, and Nancy P. Keller^{*,1}

^{*}Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, Wisconsin, USA

[†]Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, School of Pharmacy, Los Angeles, California, USA

[‡]Department of Chemistry, University of Southern California, College of Letters, Arts, and Sciences, Los Angeles, California, USA

Abstract

Mining for novel natural compounds is of eminent importance owing to the continuous need for new pharmaceuticals. Filamentous fungi are historically known to harbor the genetic capacity for an arsenal of natural compounds, both beneficial and detrimental to humans. The majority of these metabolites are still cryptic or silent under standard laboratory culture conditions. Mining for these cryptic natural products can be an excellent source for identifying new compound classes. Capitalizing on the current knowledge on how secondary metabolite gene clusters are regulated has allowed the research community to unlock many hidden fungal treasures, as described in this chapter.

1. INTRODUCTION

Despite their obscure or sometimes unknown functions in the producing species, fungal natural compounds have certainly impacted humankind tremendously owing to their diverse and potent bioactivities. Compounds such as statins, cyclosporines, and penicillin are of clinical importance, whereas mycotoxins like aflatoxin and gliotoxin have adversely affected the food industry and human health. These low-molecular weight natural compounds are also termed secondary metabolites (SMs), in agreement with their “dispensable” role in the survival of an organism. Ongoing research in this field has not only increased our appreciation for SMs as an asset but has also shed light on their pivotal role in fungal biology, defense, and stress response and their potential contribution to human mycoses (Ben-Ami, Lewis, Leventakos, & Kontoyiannis, 2009; Bok et al., 2006; Losada, Ajayi, Frisvad, Yu, & Nierman, 2009; Rohlf, Albert, Keller, & Kempken, 2007; Stanzani et al., 2005; Yin et al., 2012). As more genomes are sequenced, it is found that, as in SM producing bacteria, the genomic capacity for natural product diversity far exceeds known isolated compounds, because many of these clusters are either dormant under standard laboratory culture conditions or expressed at extremely low levels (Hertweck, 2009).

Our current understanding of fungal SM gene clusters regulation allows us to use this knowledge to artificially induce these silent clusters under normally noninducing conditions. Most of these approaches have been reviewed recently (Brakhage et al., 2008; Brakhage &

Schroeckh, 2011; Chiang, Chang, Oakley, & Wang, 2011; Yin et al., 2012) and is briefly discussed in Section 2. Although various methods have been employed to mine novel compounds from cryptic clusters, including coculture with bacterial/fungal species that cohabit the same niche, heterologous expression in a different host (e.g., *Aspergillus oryzae*), and using chemical means to modify the chromatin landscape (e.g., 5-azacytidine, trichostatin A), this chapter focuses on *in silico*- and proteomic-based approaches to mine new compounds and their biosynthetic genes, molecular techniques used to force expression of cryptic SM gene clusters, and advances in isolating and identifying the natural products themselves.

2. ACTIVATION HANDLES OF FUNGAL SECONDARY METABOLITE GENE CLUSTERS

SM gene clusters are regulated in a multilevel fashion. The production of specific natural compounds is intimately linked to specific environmental cues. This is not surprising, given that these natural compounds have evolved to secure niches, avoid predation (Rohlf et al., 2007), and defend against hostile environmental confrontations. Expression of these clusters is therefore cryptic under standard laboratory growth conditions. Several groups have shown that simulating these natural triggers serves to activate dormant gene clusters (Brakhage et al., 2008; Losada et al., 2009). In addition, both global and SM gene cluster-specific regulators have been used for activating many SM gene clusters (Bergmann et al., 2010; Yin et al., 2012). Altering the chromatin landscape by either genetic or chemical manipulation of chromatin modifiers has also been shown to activate many cryptic SM gene clusters (Bok et al., 2009; Cichewicz, 2010; Lee et al., 2009; Palmer & Keller, 2010; Reyes-Dominguez et al., 2010; Shwab et al., 2007; Wang et al., 2011).

2.1. Biosynthetic genes

The SM biosynthetic genes for any one metabolite in filamentous fungi, as in bacteria, are typically clustered together (Keller & Hohn, 1997; Walton, 2000). SM clusters usually consist of one or more backbone gene(s) such as polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs), dimethylallyl tryptophan synthases (DMATs), and terpene cyclases (TCs), surrounded by genes for modifying enzymes including, but not limited to, oxidoreductases, oxygenases, dehydrogenases, reductases, and transferases. The backbone enzyme from a given cluster usually catalyzes the first step(s) of metabolite biosynthesis, forming a scaffold, which can then be modified, usually in multiple biosynthetic steps by cluster-specific modifying enzymes to produce the final compound. Altering the expression of these backbone genes affects the expression of other biosynthetic genes in the cluster, as seen by the deletion of the NRPS gene, *gliP*, leading to reduced expression of the *gli* genes (Cramer et al., 2006). The backbone gene can be artificially induced to high levels via promoter replacement at its native locus to yield high levels of the first intermediate (Seshime, Juvvadi, Kitamoto, Ebizuka, & Fujii, 2010).

2.2. Global and cluster-specific regulators

2.2.1 Global regulators—A hallmark discovery concerning fungal SM gene cluster regulation is the characterization of a nuclear methyltransferase-domain protein, LaeA, first characterized in *Aspergillus nidulans* (Bok & Keller, 2004). Loss of this protein decreased or eliminated production of many SMs. Since then, LaeA has been characterized in various *Aspergillus*, *Penicillium*, and *Fusarium* spp. and shown to both positively and negatively regulate production of natural compounds (Baba, Kinoshita, & Nihira, 2011; Bok et al., 2005; Kale et al., 2008; Oda, Kobayashi, Ohashi, & Sano, 2011; Perrin et al., 2007; Wiemann et al., 2010; Xing, Deng, & Hu, 2010). Altogether, these findings branded LaeA as a global regulator of secondary metabolism. Thus, manipulating the level of this protein

will inevitably alter the SM profile. Many groups have since used LaeA regulation of SM gene clusters to link orphan clusters to their respective metabolites or used this protein to enhance heterologously expressed clusters (Sakai, Kinoshita, & Nihira, 2012). Other global regulators that have been identified to affect SM gene cluster expression include development-related transcription factors such as StuA (Twumasi-Boateng et al., 2009) and a bZIP transcription factor in *A. nidulans* (Yin et al., 2012).

2.2.2 Pathway-specific regulators—Many SM clusters contain a gene encoding a transcription factor specific for activating the enzymatic genes in the cluster. The hallmark transcription factor is AflR, encoded by a gene in the aflatoxin and sterigmatocystin (ST) clusters in various *Aspergillus* spp. Overexpression of *aflR* leads to enhanced expression of SM cluster genes and concomitant metabolite production (Flaherty & Payne, 1997). This method can be used to enhance production of SMs (reviewed in Yin & Keller, 2011). More recently, it has been shown that some SM cluster transcription factors can activate more than one SM cluster. In *A. nidulans*, induced expression of ScpR induced expression not only of *inp* cluster genes but also of the *afo* cluster genes (Bergmann et al., 2010), and *A. nidulans* *aflR* is involved in both ST and asperthecin biosynthesis (Yin et al., 2012).

2.3. Chromatin modifiers

LaeA impacts SM production through chromatin modification (Reyes-Dominguez et al., 2010). Complementary to this observation, several reports have indicated SM activation through modification of histone decorating enzymes, typically through deletion of heterochromatin enhancing enzymes usually involved in deacetylation and subsequent methylation of H3K9 residues. Histone modifiers shown to affect SM production include the histone deacetylase (HDAC), HdaA, heterochromatin protein 1 (HepA), and H3K9 methyltransferase (ClrD) (Lee et al., 2009; Palmer, Perrin, Dagenais, & Keller, 2008; Shwab et al., 2007). Epigenetic activating chemicals such as HDAC inhibitors also induce production of various new compounds. Trichostatin A, for example, induces production of penicillin and ST in *A. nidulans* (Shwab et al., 2007). Other HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) and 5-azacytidine have been used to induce production of cladochromes and oxylipins in *Cladosporium cladosporioides* (Wang et al., 2011; Williams, Henrikson, Hoover, Lee, & Cichewicz, 2008). Several recent reviews address this link between epigenetics and fungal secondary metabolism (Cichewicz, 2010; Palmer & Keller, 2010; Strauss & Reyes-Dominguez, 2011).

3. MINING FOR NOVEL COMPOUNDS AND THEIR BIOSYNTHETIC GENES

3.1. *In silico* genomic mining

Identifying SM biosynthetic genes is a crucial part of unlocking cryptic gene clusters. It also aids in the sustainable production of these compounds via heterologous expression in a compatible host and pathway engineering to improve yield, bioactivity, and bioavailability. Backbone genes such as PKSs, NRPSs, and DMATs are multimodular enzymes made out of conserved functional domains characteristic of each type of enzyme. These can be used to search the sequenced genome for putative open reading frames for these functional domains. Recently, a number of SM cluster prediction algorithms have been developed to achieve this (Khaldi et al., 2010; Medema et al., 2011). Here we discuss two pipelines for identifying putative SM clusters in fungi, namely, *Secondary Metabolite Unique Region Finder* (SMURF) and antibiotics and *Secondary Metabolite Analysis* *SHell* (antiSMASH).

SMURF is an algorithm that predicts PKS-, NRPS-, and DMAT-based SM gene clusters in fungi. SMURF cluster predictions are based on two key parameters: *d*, the maximum intergenic distance (in base pairs) permitted between two adjacent genes in the same cluster,

and y , the maximum number of SM domain-negative genes allowed within a cluster. SMURF can predict SM clusters from both nonannotated and annotated genomes. In addition, the SMURF database contains precomputed results of SM gene clusters from 27 sequenced fungal genomes that can be readily obtained and used for downstream validation and manipulation (Khaldi et al., 2010).

While SMURF is highly specific for predicting PKS-, NRPS-, and DMAT-based SM gene clusters in fungi, antiSMASH can be used to predict the whole repertoire of compound classes spanning the full tree of life. The antiSMASH pipeline is also designed to provide detailed analysis of PKS/NRPS functional domain architecture and predict core structures of their respective products as well as an analysis of orthologous gene clusters based on the identity of adjacent accessory genes (Medema et al., 2011). A comparison between antiSMASH and SMURF showed that both pipelines are of equal performance when tested against *A. fumigatus* AF293 and *Penicillium chrysogenum* Wisconsin 54-1255 (Medema et al., 2011).

3.2. Proteomic investigation of secondary metabolism: a proteomic approach

Although not designed for activating cryptic SM gene clusters, a recently developed technique that streamlined the workflow for new drug discovery is noteworthy. Proteomic Investigation of Secondary Metabolism (PriSM) allows investigators to identify both biosynthetic genes and their respective metabolites in tandem and bypassing the need for genome sequence information *a priori* (Bumpus, Evans, Thomas, Ntai, & Kelleher, 2009). Thus far, this alternative approach can only be applied to PKS- and NRPS-based SM clusters whose enzymes are both expressed and >100 kDa in size. PriSM operates on the premise that PKSs and NRPSs are large-molecular weight proteins (LMWPs; >200 kDa) that can be separated from the majority of cellular proteins by SDS-PAGE and subsequently selected for specific phosphopantetheinyl (Ppant)-containing peptides characteristic of such enzymes. The protocol is discussed in detail (Bumpus et al., 2009); briefly, LMWPs are isolated from total protein extracts and subjected to in-gel trypsin digestion and selective Ppant-containing peptide detection using a high-performance fourier transform mass spectrometer, followed by targeted PCR, cluster sequencing, and finally product discovery. Although in its infancy, PriSM sets forth an innovative and alternative approach of impressive potential, especially when used in combination with approaches that enable the expression of cryptic clusters.

4. GENETIC MANIPULATION OF BIOSYNTHETIC AND REGULATORY GENES

Many successful attempts to activate cryptic SM gene clusters involve altering the expression of genes encoding biosynthetic enzymes, developmental and pathway-specific transcription factors, and histone modifiers. Targeted gene deletions can be used to abolish expression and promoter replacements can be used to modify expression of a gene of interest (GOI). When an inducible promoter is chosen, the researcher can reversibly control the state of expression (on or off) and, in some cases, the level of expression itself. Here we discuss a rapid method for creating deletion/promoter replacement constructs, protoplast generation, and transformation in *Aspergillus* spp. This protocol is adapted from (Szewczyk et al., 2006; Yang et al., 2004; Yelton, Hamer, & Timberlake, 1984; Yu et al., 2004).

4.1. Design of transformation constructs

The 5' upstream and 3' downstream flanking regions serve as complementary sequences to allow homologous recombination at the locus of interest. These flanking regions range from 1.0 to 1.5 kb in size. For targeted gene disruption, you could replace the entire open reading

frame (ORF) of the GOI with a selectable marker of choice (Fig. 15.1A); replace a small piece of the ORF internal to the GOI with a selectable marker of choice (Fig. 15.1B); or insert a selectable marker of choice within the GOI and disrupt it without removing any ORF (Fig. 15.1C). For promoter replacement, the promoter of choice has to be inserted immediately before the transcriptional start site of the GOI. The selectable marker in this case is usually fused upstream of the promoter to be inserted (Fig. 15.1D). The double-joint PCR protocol below can also be used for other applications such as fluorescent tagging and epitope tagging of genes for microscopy and purification, respectively. When this is the case, the inserted/fused fragment must be translated in-frame to the GOI. Design of in-frame cassettes can be found in (Szewczyk et al., 2006).

4.2. Creation of linear transformation cassettes using double-joint PCR

1. Design eight primers (Fig. 15.2):

- 1.1 Four primers (P1–P4) for amplification of 5′ upstream and 3′ downstream flanking regions that are ~1.0–1.5 kb in size. These will be used in PCR Reaction I.
- 1.2 Two nested primers (P5 and P6) for selective amplification of full-length fusion product. These will be used in PCR Reaction III.
- 1.3 Two primers (PC1 and PC2) for amplification of the selection marker. These will be used in PCR Reaction I.

Note: The selection marker can either be a drug resistance gene or one that restores prototrophy to an auxotrophic mutant. In the latter case, it is important to not choose the wild type copy from the same species as it will reduce the frequency of homologous recombination at the locus of interest. For example, when transforming a *pyrG* mutant of *A. fumigatus*, it is advisable to use *Aspergillus parasiticus* (or another fungus) *pyrG*.

2. Isolate genomic DNA (gDNA) from the parental strain that will be used as template for amplifying flanking regions to the gene or locus of interest and plasmid DNA/ gDNA as template to amplify the selection cassette.
3. Set up PCR Reaction I. PCR amplify both 5′ upstream and 3′ down-stream flanking regions and the selectable marker using *PfuII* Ultra (Agilent Technologies). Refer to Fig. 15.2 for reaction setup.
Note: *PfuII* Ultra is a high fidelity polymerase that minimizes PCR errors during amplification. Other high fidelity polymerases can be used instead.
4. Clean up the PCR products from Reaction I of residual template, primers, nucleotides, and in some cases, nonspecific products via gel extraction using QIAquick gel extraction kit (Qiagen).
5. Quantitate the amount of DNA for PCR Reaction II using the Nanodrop (Life Technologies) or agarose gel electrophoresis.
6. Set up PCR Reaction II. Use a fragment copy number ratio of 1:2:1 (5′ flank:marker:3′ flank). Refer to Fig. 15.2 for reaction setup.
7. Run 5–8 μl of Reaction II product on a 0.8% agarose gel. You should be able to see three to four bands corresponding to both flanking regions, the selection marker, the single-joint product, and the double-joint product (the double-joint product may or may not be visible, Fig. 15.2).

8. Set up PCR Reaction III. Selectively amplify the full-length double-joint product using nested primers P1 and P6 using Expand Long Template Polymerase (Roche). Refer to Fig. 15.2 for reaction setup.

Note: This is a high fidelity polymerase suitable for amplifying large double-joint cassettes with high product yield. It has worked well in our hands, but other polymerases may be used instead. Using nested primers also decreases amplification of nonspecific products.

9. Check the quality of PCR product from Reaction III using agarose gel electrophoresis. If multiple bands are observed, the band of interest can be purified from nonspecific bands via gel extraction.

Note: You can also confirm that the generated cassette is specific to the GOI by performing 1–2 restriction digests.

10. Clean up the PCR product from Reaction III by subjecting it through Sephadex™ G-50 DNA-grade resin (GE Healthcare).
11. Double-joint product can be stored at –20 °C for a few months and –80°C indefinitely.

4.3. Protoplast generation

In this method, protoplasts are generated from hyphae (Szewczyk et al., 2006) rather than germlings (Yelton et al., 1984). Thus, it is crucial to allow sufficient growth of the strain. The length of incubation varies with the type of auxotrophy and strain background. For instance, tryptophan auxotrophs never recover full growth even with supplemented tryptophan, so such mutants may have to be grown longer than a pyrimidine auxotroph. Generated protoplasts are extremely sensitive to detergent and much care has to be taken to ensure that glassware, filters, and tubes are free of residual detergent.

1. Obtain spore suspension of parental strain in distilled water +0.1% Tween-80.

Note: Using parental strains deficient for NkuA or NkuB increases the frequency of transformants carrying the correct integration of the transformation cassette (da Silva Ferreira et al., 2006). In this case, you may screen a mere 10+ transformants for gene deletion or 20+ transformants for promoter replacement, as opposed to 60+ transformants when using wild-type strains for NkuA and NkuB.

2. In a sterile 50-ml Erlenmeyer flask, inoculate 1×10^8 spores into 20 ml YG medium (5 g yeast extract, 20 g D-glucose, 400 µl trace element solution (Szewczyk et al., 2006) in 1 l distilled water).
3. Incubate for 12–14 h depending on strain background and type of auxotrophy at 30°C and shaking at 120–150 rpm.
4. Prepare fresh 2× protoplasting solution by adding 1.3 g Vinotaste Pro [Novozymes] into 10 ml for KCl, citric acid solution (8.2 g KCl, 2.1 g citric acid monohydrate, pH, to 5.8 with 1.1 M KOH, in 100 ml distilled water). Filter-sterilize using 0.22-µm Millex GV filter (make sure to discard the first 0.5 ml of pass-through to remove residual detergent).

Note: For *A. nidulans*, 1.3 g Vinotaste Pro is sufficient. For *A. fumigatus* and *A. flavus*, the protoplasting solution should also be supplemented with 0.1 and 0.2 g of lysing enzymes from *Trichoderma harzianum*, respectively (Sigma–Aldrich, Catalog number: L1412).

5. Harvest mycelium by gravity filtration over sterile Miracloth (Calbiochem).

6. Wash away residual detergent from mycelium with fresh YG medium.
7. With a sterile spatula, transfer mycelium from Miracloth into a fresh 125-ml Erlenmeyer flask with 16 ml protoplasting solution (1:1, v/v, YG medium: 2× protoplasting solution).
8. Incubate for 2–4 h at 30°C and shaking at 80–100 rpm.
Note: Hyphae tend to clump together in the first hour of protoplasting. Break the clump by gently pipetting up and down every now and then with a sterile serological pipette. Do not pipet after the first hour of protoplasting as now the cell walls are almost fully digested and protoplasts are sensitive to physical shearing. Monitor protoplast formation microscopically. Small or few protoplasts beyond the first hour to two of protoplasting indicate that most protoplasts are lysing.
9. In a sterile 50-ml centrifuge tube (or glass round-bottom centrifuge tube), gently overlay protoplast solution over chilled 10 ml 1.2 M sucrose solution.
10. Centrifuge at $1800 \times g$ for 10 min at 4°C in a swinging-bucket rotor. Make sure to set brakes to low or turn the brake off entirely. Hyphae will pellet and protoplasts will collect at the interphase (top of sucrose layer).
11. Collect the protoplasts with a pipette, making sure not to disturb the interphase and disperse the protoplasts. A small amount of protoplasts may still be dispersed throughout the protoplasting solution. You can collect the entire protoplasting solution if this occurs.
12. Transfer the collected protoplasts to a new sterile centrifuge tube and add at least an equal volume of 0.6 M KCl. Mix thoroughly by gently inverting the centrifuge tube several times.
13. Centrifuge at $1800 \times g$ for 10 min at 4 °C to pellet the protoplasts. They will form a loose pellet, and care must be taken while decanting the supernatant post-centrifugation.
14. Resuspend protoplasts in 1 ml of 0.6 M KCl and transfer to a sterile microcentrifuge tube.
15. Centrifuge at $2400 \times g$ for 2 min to pellet the protoplasts. Pipette away supernatant being careful not to dislodge the protoplast pellet.
16. Wash protoplasts twice by repeating steps 14 and 15.
17. Resuspend protoplasts in an appropriate volume of 0.6 M KCl, 50 mM citric acid solution (for KCl-based transformation), or STC buffer (for sorbitol-based transformation; see transformation protocol for recipe).

Note: This protocol, when performed optimally, can yield up to 2×10^7 protoplasts and is sufficient for 10 transformations (Szewczyk et al., 2006). Thus, the pellet can be resuspended in up to 1 ml of the above solutions using ~100 µl/transformation. Do not forget to also account for an additional 100 µl for the negative control.

4.4. Transformation

Both sorbitol- and KCl-based transformations have been used to generate transformants from protoplasts obtained by the above method. In our hands, the efficiency of sorbitol-based transformation is much higher than KCl-based transformation for *A. fumigatus*. Though a comparison has not been made between these two methods for *A. nidulans* and *A. flavus*, both have produced transformants from these two species. These protocols were adapted from Szewczyk et al. (2006) and Yelton et al. (1984).

4.4.1 Sorbitol-based transformation

1. Dilute DNA (3–5 µg) in <100 µl total volume of STC (1.2 M sorbitol, 10 mM CaCl₂, 10 mM Tris–HCl, pH 7.5) in a 15-ml round bottom Falcon tube.
2. Add 100 µl of protoplasts, vortex four to five times (1 s each time) at maximum speed to thoroughly mix protoplasts and DNA and incubate for 50 min on ice.
3. Add 1.25 ml of room temperature, freshly filtered 60% PEG solution (60%, w/v, PEG 3350, 50 mM CaCl₂, 50 mM Tris–HCl, pH 7.5). Mix gently by placing the tube on its side and rotating it multiple times. Incubate at room temperature for 20 min.

Note: PEG must be stored at room temperature, as it will precipitate at 4°C. PEG precipitate will lyse protoplasts and reduce transformation efficiency. Hence, you could filter PEG solution right before using with a 0.22-µm Millex GV filter to remove precipitate.

4. Add 5 ml STC buffer and mix gently as before.
5. Plate transformation mixture on selection Sorbitol Minimal Medium (SMM) medium (10 g D-glucose, 50 ml 20× sodium nitrate salts (120 g NaNO₃, 10.4 g KCl, 10.4 g MgSO₄·7H₂O, 30.4 g KH₂PO₄ in 1 l distilled water), 1 ml trace element solution (Szewczyk et al., 2006), 218.6 g sorbitol, 1.5% agar, and selection supplements) by pipetting ~1 ml transformation mixture onto each plate and overlay with 5 ml of selection SMM top agar (SMM with 0.75% agar). Alternatively, you may spread the transformation mixture with a glass spreader.

Note: During overlay with top agar, make sure that it is cooled to 50°C.

6. Incubate right side up at 30°C overnight to let dry and then transfer the plates to 37°C to expedite growth of transformants (*A. nidulans* and *A. fumigatus*).

4.4.2 KCl-based transformation

1. Add DNA (10 µl of PCR Reaction III product) in <15 µl TE buffer to 100 µl protoplasts.
2. Vortex four to five times (1 s each time) at maximum speed to thoroughly mix protoplasts and DNA.
3. Add 50 µl of room temperature, freshly filtered 25% PEG solution (25%, w/v, PEG 3350, 0.6 M KCl, 50 mM CaCl₂, 50 mM Tris–HCl, pH 7.5).
4. Vortex four to five times (1 s each time) at maximum speed.
5. Incubate on ice for 25 min.
6. Add 1 ml room temperature, freshly filtered 25% PEG solution, and gently mix by turning the tube on its side and rotating it multiple times.
7. Incubate at room temperature for 25 min.
8. Plate transformation mixture on selection KCl Minimal Medium (KMM) medium (10 g D-glucose, 50 ml 20× sodium nitrate salts, 1 ml trace element solution (Szewczyk et al., 2006), 0.6 M KCl, 1.5% agar, and selection supplements) by spreading transformation sample over selection plates with a glass spreader.
9. Transformants will be visible from 2 to 7 days post-transformation depending on the species and types of selection marker. Typically, transformants bearing a

nutritional marker will be visible 2–3 days post-transformation while those bearing a drug resistant marker will be visible 6–7 days post-transformation.

10. The transformants can then be confirmed. Mutant screening and confirmation procedures are beyond the scope of this chapter. Briefly, we typically perform an initial PCR screen for transformants bearing the insertion at the correct locus. We then follow up with Southern analysis using two different restriction digest patterns to choose mutants with a single integration event (at the desired locus) and not ectopic integrations elsewhere in the genome.

5. SECONDARY METABOLITE ANALYSIS

With the ability to rapidly generate fungal mutants, a reliable protocol is needed for the purification and identification of their SMs. Here we describe methods to isolate and characterize fungal SMs using liquid chromatography/mass spectrometry (LC/MS) and NMR.

5.1. Small-scale cultivation for initial analysis

1. Under sterile conditions, transfer 50 ml of the liquid medium of choice into a sterile 125-ml flask. Inoculate culture to a final concentration of 5×10^6 spores/ml (or 2.5×10^8 spores in total).
2. Place the flask in a shaker at ~100 rpm. for 4 days at 37°C (duration and temperature can vary with strain). By this time, the mycelium should have grown and appear as light-colored spheres.

Note: If an inducible promoter is used, it should be added after 18 h cultivation. For *alcA* promoters, 133 μ l of cyclopentanone is added to 50 ml cultivation. The volume can be scaled accordingly.

3. After cultivation, 25 ml of filtered medium is typically collected in a 50-ml Falcon tube via filtration through Miracloth (Calbiochem).
4. In the same Falcon tube, extract the medium twice with an equal amount of ethyl acetate and transfer the ethyl acetate organic layer by a glass pipette into a tared vessel.

Note: When necessary, add concentrated HCl to the filtered medium until a pH of 2 is reached, typically when analyzing compounds with a carboxylic acid group. ApHpaper can be used. Extract and evaporate as usual.

5. Evaporate the organic layer. We use a Turbovac LV (Caliper Lifesciences) to evaporate several vessels in parallel; alternatively, one can use a rotoevaporator to concentrate the organic layer.
6. After evaporation, the mass of the residue is determined and the residue is then dissolved in methanol at a concentration of 1 mg/ml for LC/MS analysis.
7. The solution should always be syringe-filtered to remove particulates that can clog an LC/MS column. Place ~100 μ l of solution into a disposable 1-ml syringe fitted with a 13-mm filter with a PTFE membrane. With the syringe plunger, push the solvent through the filter and into an LC/MS vial. The sample is then ready to be submitted to the LC/MS. For LC/MS, we typically use two buffers, a polar “A” buffer, and a nonpolar “B” buffer. For “A” buffer, 950 ml H₂O, 50 ml acetonitrile, 0.5 ml formic acid, and for “B” buffer, 1 l acetonitrile, 0.5 ml formic acid.
8. A gradient with an increasing amount of the “B” buffer over time is used to separate and elute the molecules. A typical gradient is 0% B from 0 to 5 min, 0% to

100% B from 5 to 35 min, 100% B from 35 to 40 min, 100% B to 0% B from 40 to 45 min, and reequilibration with 0% B from 45 to 50 min. Set the flow rate to 125 $\mu\text{l}/\text{min}$.

5.2. Large-scale cultivation for isolation of metabolites

1. Once a new unknown peak of interest is identified by LC/MS, it will be necessary to grow the strain again in a larger scale to acquire enough material for NMR analysis. The amount of medium needed will depend on the titer of the metabolite. A good starting point is to use about 2 l of medium.
2. The volumes of ethyl acetate involved will mean that separatory funnels must be used in the place of Falcon tubes, and the organic material should be collected in a large round-bottom flask. Extract at least twice; four times may be optimal. Use a rotoevaporator to evaporate the organic solvent.
3. A small amount (~0.5 mg) of dried material can be dissolved in methanol and submitted to the LC/MS to determine if the scaled-up cultivation was successful. Remember to syringe filter the solution.

5.3. Silica gel column chromatography for semipurification

The next step is to isolate the metabolite in question. Because a crude mixture is likely to contain a number of different products, multiple purification steps may be necessary, and careful planning is needed. Typically, we use standard organic chemistry silica gel chromatography as the first round of purification in order to obtain a semipure mixture that can be further purified using other methods and to get rid of salts and lipids that could potentially complicate further purification steps.

1. Weigh out two portions of silica (particle size 0.063–0.200 mm). One portion will be two to three times the mass of the crude material. The other portion will be 30 times the crude mass.
2. Dissolve the crude material. If common organic solvents such as dichloromethane and methanol fail to dissolve the material, try adding water and then sonicate.
3. Add the smaller portion of silica into the dissolved material and evaporate the solvents completely. A substance resembling sand should be achieved. Remove any clumps with a spatula.
4. Add the larger portion of silica to a large Erlenmeyer flask. Add enough hexanes to form a slurry.
5. Pour the slurry into a glass chromatography column. Make sure to add a small amount of cotton taken from a cotton ball to the bottom of the column to prevent the silica from leaking out.

Note: Do not use too much cotton, as it will significantly slow the flow of the solvent. The Erlenmeyer flask must be rinsed several times with hexanes to transfer all of the silica.

6. Allow solvent to drain out of the column. Using air pressure via an adapter with a regulator and release valve will greatly facilitate the running of the column, but always be careful that the valve at the bottom of the column is open before applying pressure. Remove pressure when there is no longer solvent above the silica to prevent the formation of air bubbles in the column that can jeopardize the purification process.

7. Lightly tap the edge of the column with a blunt object such as the back of a pair of scissors. This will cause the silica to settle and release air bubbles trapped in the column. Some solvent will emerge above the level of the silica. Drain until there is no solvent above the silica. Tap the edge of the column again to further pack the column. Drain.
8. Repeat tapping and draining until the silica settles no more (indicated by the lack of solvent emerging above the silica layer). The column is now packed.
9. Add more hexanes to the top of the silica so that it is ~1 cm in height. Be careful not to disrupt the silica. This can be done by slowly pipetting the solvent onto the inner side of the column in order to preserve the integrity of the packed silica.
10. Pour in the small amount of silica that was mixed with the crude fungal extract. Make sure that all of this material is off the sides of the column. If necessary, extra hexanes can be used to completely transfer the material. If so, drain the solvent until it reaches the top of the material.
11. Add standard, commercially available sand so that it is ~1 cm in height. The sand serves as a physical barrier and will help prevent disruption of the packed silica when solvent is added.
12. Elute with the solvent systems necessary to drain the metabolite(s) of interest. For every 1.5 g of crude material, use 1 l of solvent mixture. A suitable solvent system is a mixture of ethyl acetate and hexanes.

Note: The most nonpolar metabolites will be eluted from a mixture of 10% ethyl acetate in hexanes. The more polar the metabolite, the more ethyl acetate will be needed, all the way up to 100% ethyl acetate. For especially polar metabolites, it may be necessary to use methanol in ethyl acetate. Do not exceed 10% methanol, and start gradually since each percentage of methanol added will significantly increase the polarity of the mixture. If you are unsure which solvent composition is appropriate, start with 10% ethyl acetate/hexanes, follow with a 10% increment in ethyl acetate (e.g., 20% ethyl acetate/hexanes), and so forth. Collect each fraction separately and evaporate.

13. A small amount of material can be prepared for LC/MS analysis to keep track of the status of the purification.

5.4. Final HPLC purification of the metabolite

Provided that the metabolite was eluted, you can proceed to HPLC purification. Dissolve the evaporated fraction(s) from silica gel chromatography completely in methanol. Try to keep solvent volumes to a minimum because it will affect the loading of the sample onto the HPLC.

1. Make sure to filter the sample prior to HPLC purification.
2. We typically use 950 ml H₂O, 50 ml acetonitrile, and 0.5 ml trifluoroacetic acid for buffer "A" and 1 l acetonitrile, 0.5 ml trifluoroacetic acid for buffer "B." Use a flow rate of 5.0 ml, with a UV detector at 250 nm.
3. The gradient depends on the polarity of the compound to be purified and its proximity to other metabolites. Typically, the closer the metabolites are to each other, the slower the gradient.
4. Fractions containing the product should be combined and evaporated, but first a sample should be submitted to the LC/MS to verify that it is indeed the product in question.

5. Frequently, the metabolite is now pure and, if a sufficient quantity was obtained, it is ready to be analyzed by NMR. However, sometimes the LC/MS and/or NMR reveal impurities, and further purification is necessary. Two strategies can be employed: (1) repeat the HPLC but employ a slower gradient or abolish the gradient entirely (isocratic); (2) perform a preparative TLC (See Section 5.5.).

5.5. Preparative TLC

Preparative TLC is appropriate for compounds that can be visualized under UV. It is also cost-effective and particularly useful as the final step of a purification scheme. However, it is not useful for purifying large amounts of compounds.

1. Dissolve the sample in a small amount of methanol. Pack a little cotton down partly through the end of a glass pipette. This “paint brush” is used to apply the material as a line about 2 cm above the bottom of a precoated 20×20 cm TLC plate. No more than 10 mg of material should be applied to a single plate. Try to keep the line as thin as possible as it will affect the separation.
2. The appropriate solvent system can be determined by performing an initial analytical TLC. The right solvent composition will be similar, but not necessarily identical, to the composition used to elute the product in column chromatography.
3. Place the plate in the developing chamber. Remove the plate when the solvent is about 2 cm from the top of the plate. This will take about an hour. Let dry for another hour.
4. If the product is colorless, you will need to place the plate under a UV lamp (~250 nm) to visualize the product. With a pencil, trace the outside of the product and then remove the plate from the UV lamp.
5. Using a razor blade, scrape off the silica containing the product and collect in a flask.
6. Add 10 ml of 95:5 dichloromethane:methanol and stir for an hour. Make sure not to inhale the silica that is being scraped off.
7. Collect the product by pouring the solvent over a filter atop a funnel and into a tared flask. Evaporate the solvent.

5.6. NMR characterization of the metabolite

Once pure product is obtained in sufficient quantity (as much as 5 mg may be needed), you can now subject it to NMR analysis for structure elucidation. The types of NMR experiments needed depend on the nature of the product.

1. A proton NMR should be run first and preferably also a carbon NMR. Be sure that the NMR tubes are clean and dry and that deuterated solvent is used.
2. If you have an idea of the metabolite's identity you can compare the acquired spectra with published data.
3. If the metabolite's identity is still unknown, additional experiments, such as DEPT, HSQC, HMBC, COSY, and ROESY, may be needed to deduce the structure. The analysis of NMR data is beyond the scope of this chapter.
4. Often, it is not necessary to fully solve the structure of the compound of interest. Rather, you may use programs such as SciFinder Scholar to determine if there are any published compounds with similarities to the partially solved structure.

Note: Other software programs such as ACD/Labs can be used to either compare the acquired NMR data with those in its databases or attempt to logically solve the structure. Some computer programs also will predict the NMR spectra for a given chemical, so if you have an idea of the structure, you can see if the data match the computer prediction. A close match does not guarantee that the structure is correct, but it may help to develop an understanding of the identity of the molecule. Solving a structure through NMR can be a “back and forth” process. That is, the NMR data may provide some clues about the structure. You can then consult journal articles, use software programs, or consult with peers, which may provide inspiration for the choice of further NMR experiments to advance in solving the structure.

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Figure 15.1.

Design of transformation cassette: (A) whole deletion of ORF from GOI, (B) internal deletion of ORF from GOI, (C) insertion of selection marker disrupting the ORF of GOI, and (D) native promoter replacement with either a constitutive or inducible promoter.

Figure 15.2.
Double-joint PCR reaction setup and cycling conditions.