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Enhancing Nonribosomal Peptide Biosynthesis in Filamentous Fungi

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Abstract

Filamentous fungi are historically known as rich sources for production of biologically active natural products, so-called secondary metabolites. One particularly pharmaceutically relevant chemical group of secondary metabolites is the nonribosomal peptides synthesized by nonribosomal peptide synthetases (NRPSs). As most of the fungal NRPS gene clusters leading to production of the desired molecules are not expressed under laboratory conditions, efforts to overcome this impediment are crucial to unlock the full chemical potential of each fungal species. One way to activate these silent clusters is by overexpressing and deleting global regulators of secondary metabolism. The conserved fungal-specific regulator of secondary metabolism, LaeA, was shown to be a valuable target for sleuthing of novel gene clusters and metabolites. Additionally, modulation of chromatin structures by either chemical or genetic manipulation has been shown to activate cryptic metabolites. Furthermore, NRPS-derived molecules seem to be affected by cross talk between the specific gene clusters and some of these metabolites have a tissue- or developmental-specific regulation. This chapter summarizes how this knowledge of different tiers of regulation can be combined to increase production of NRPS-derived metabolites in fungal species.

Keywords

LaeA regulation; Cross talk; Chromatin regulation; Transcription factor

1 Introduction

In filamentous fungi, the genes required for biosynthesis of a given secondary metabolite are predominantly contiguously aligned in the genome [1]. These gene clusters typically contain one or more backbone gene(s), including nonribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), dimethylallyl tryptophan synthases (DMATs), and terpene cyclases (TCs). Adjacent to backbone genes are those genes encoding additional modifying enzymes (e.g., oxidoreductases, monooxygenases, etc.), as well as regulatory and resistance elements. The multiple levels of regulation involved in coexpression of these cluster genes can facilitate demarcation of cluster boundaries and can be used to facilitate activation of otherwise silent gene clusters, to be discussed in further detail below. The reader is encouraged to peruse recent reviews [2–6] for additional information.

NRPS-derived metabolites display a vast chemical diversity as they can incorporate proteinogenic and non-proteinogenic amino acids in their D- and L-configurations [7]. The incorporated amino acids can be connected in a linear or cyclic fashion that can undergo additional modifications by tailoring enzymes encoded in the respective gene cluster. This structural diversity of nonribosomal peptides is also reflected in their broad spectrum of biological activities utilized in medicinal and pharmaceutical research [8, 9], with penicillin, cephalosporin, and cyclosporin as the most prominent examples [10–13]. Despite the chemical variety produced by NRPSs, the standard NRPS enzyme itself is composed of three discrete canonical domains (adenylation (A), thiolation (T) or peptidyl carrier protein (PCP), and condensation (C) domains), each of which is referred to as one NRPS module. Each module is responsible for the recognition (via the A domain) and incorporation of a single amino acid into the growing peptide product. In general, an NRPS consists of one or more modules and can terminate in a condensation-like (C_T) domain that releases the peptide. Occasionally, epimerase (E) and *N*-methyltransferase (M) domains that convert L- to D-amino acids and *N*-methylate peptide bonds, respectively, are present within the NRPS [14, 15]. Deviations of the classical NRPS composition can be found in hybrid PKS/NRPS enzymes [16] (e.g., Fus1 responsible for fusarin C biosynthesis) and stand-alone monomodular NRPS-like enzymes [17] (e.g., TdiA responsible for terrequinone A biosynthesis) where not all canonical domains are present (Fig. 1).

The presence of at least one A domain in each NRPS enzyme facilitates identification in sequenced fungal genomes. However, in contrast to bacterial NRPS, knowledge about amino acid specificity of fungal A domains is limited up to date with only the anthranilate-activating A domain identified with some confidence [21]. Despite the variety of NRPS, NRPS-like, and PKS/NRPS hybrid enzymes predicted to be encoded by many fungal genomes (e.g., 20–30 predicted in *Aspergillus* and *Fusarium* spp. [22–26]), relatively little is known about the metabolites produced by these enzymes.

The first characterized NRPS-derived compounds identified from fungal species are produced under standard laboratory conditions. Pioneering work using protein purification of NRPS enzymes demonstrated their involvement in cyclosporine, enniatin, and beauvericin biosynthesis [13, 27, 28]. With genetic manipulation of fungal genomes becoming available, genetic and chemical pathways leading to NRPS-derived metabolites could be identified through relatively straightforward gene deletions of NRPS-encoding genes coupled with analytical screenings for loss of compound production. Examples of compounds and pathways that have been identified in this traditional fashion include those of cyclosporine [27], HC-toxin [29], AM-toxin [30], peptaibols [31], ergotpeptine [32], fusarin C [33], equisetin [34], peramine [35], sirodesmin [36], gliotoxin [37], fumitremorgin [38], tenellin [39], pseurotin A [40], cytochalasin [41], cyclopiazonic acid [42], aureobasidin A [43], fumiquinazolines [44], apicidin [45], tryptopquialanine [46], ochratoxin A [47], fumigaclavines [48], ardeemin [49], nidulanin A [50], and pneumocandin [51]. Although many of the aforementioned NRPS-derived fungal secondary metabolites display biological activity, the need for discovery of new antibiotics is becoming more urgent [52] and calls for innovative avenues of uncovering cryptic metabolites.

In order to overcome the hurdle of activating silent gene clusters in fungal genomes, information about cluster boundaries and regulatory mechanisms is crucial. The discovery of a novel global regulator of secondary metabolism came in 2004 with the identification of the putative methyltransferase LaeA [53]. Since then, manipulation of LaeA has been used to identify and demarcate a number of NRPS-derived metabolites in a multitude of species [54]. Identified metabolites and corresponding gene clusters include penicillin [53, 55, 56], gliotoxin [53], terrequinone A [57, 58], NRPS9- and NRPS11-derived metabolites [59], fusarin C [60, 61], pseurotin [62], ochratoxin A [63], tyrosine-derived alkaloids [64], beauvericin [65], as well as lolitrem and ergot alkaloids [66].

Additionally, approaches of directly activating clusters through overexpressing the cluster backbone gene itself and/or any transcription factors associated within the cluster have been undertaken. Although not always successful, this approach has led to the identification of aspyridone A [67], apicidins [45, 68], microperfuranone [69], pyranonigrin E [70], and hexadehydroastechrome [71] and has confirmed the enzymes responsible for fumitremorgin [38], apicidin [45], fusarin C [19], and cytochalasin [72, 73] production.

In the protocol below, we will describe methods for identifying and characterizing fungal NRPS enzymes and methods for activating these NRPS and corresponding clusters through both cluster specific and global regulators.

2 Materials

1. YG medium: Prepare 20 g/L D-glucose, 5 g/L yeast extract, 1 mL/L trace elements, supplements as needed; other rich media may be substituted, sterilized by autoclaving.
2. KCl citric acid solution: Prepare 82 g/L KCl, 21 g/L citric acid monohydrate, pH to 5.8 with 1 M KOH.
3. Protoplasting solution: Prepare by mixing 8 mL KCl citric acid solution, 8 mL YG medium, and 1.3 g VintoTaste Pro.
4. KCl calcium chloride solution: 0.6 M KCl, 50 mM CaCl₂.
5. PEG solution: Prepare by making a solution of 44.7 g/L KCl, 5.5 g/L CaCl₂, 10 mM Tris pH 7.5, 250 g/L PEG (MW 3350) in water.
6. KCl Minimal Medium (KMM): Prepare 10 g/L D-glucose, 50 mL 20× nitrate salts, 1.0 mL/L trace elements, 44.7 g/L KCl, 15 g/L agar, supplements as needed, pH 6.5 sterilized by autoclaving.

3 Methods

3.1 Identification and Characterization of NRPS

1. Identification of NRPS-encoding genes in the sequenced fungal genome of choice can be achieved using BLAST-based algorithms [74]. Previously analyzed NRPS, PKS/NRPS, and NRPS-like sequences should function as archetypical input

sequences to ensure identification of all predicted gene calls in the sequenced genome.

2. In order to parse and assign specific domains in the predicted protein sequences, a variety of Web-based tools are available and listed in Table 1. These tools use more precise and refined statistical models for functional assignment of domains and substrate predictions (*see* Note 1).

3.2 Creation of Transformation Cassettes

1. Amplify flanking regions, marker gene, and promoter region (if used) using expand long template polymerase. Refer to Fig. 2 for reaction setup (*see* Note 2).
2. Use agarose gel electrophoresis to confirm PCR products and purify using gel extraction (such as Qiagen QIAquick Gel Extraction Kit).
3. Quantify the concentration of each PCR fragment. This may be done either by spectrophotometry or by visual comparison to a known quantity of DNA during electrophoresis.
4. Set up second PCR reaction using a fragment copy number ratio of 1:2:1 (for deletion cassettes, 5' flank/marker gene/3' flank) or 1:2:2:1 (for overexpression cassettes, 5' flank/marker/promoter/3' flank).
5. Amplify the final product using nested primers (*see* Note 3).
6. Confirm desired PCR product through agarose gel electrophoresis of a small amount (~2 µL) of the reaction (*see* Note 4).
7. Remove buffer components and salts from the final PCR product by using a gel filtration column such as Sephadex G-50 superfine resin (GE Healthcare) (*see* Note 5).
8. Purified product should be kept on ice and may be stored at –20 °C for several months.

3.3 Transformation Procedure (See Note 6)

1. In a sterile 125 mL Erlenmeyer flask, inoculate 1×10^8 spores into 10 mL rich medium plus appropriate supplements (e.g., YG medium, supplement if needed).

¹None of the bacterial substrate codes for A domains matches the fungal A domains. (An exception is the penicillin NRPS, which is likely derived by horizontal gene transfer from bacteria.) Fungal species most likely use a degenerated specificity code since different A domain codes have been reported for binding the same amino acid.

²Alternative high-fidelity polymerases may be used for all reactions, but note that SDS in the PCR buffer is not easily removed by gel filtration and may affect transformation efficiency.

³Using nested primers decreases amplification of nonspecific products, although in most cases the outermost primers may also be used successfully for amplification.

⁴The final product should be easily visualized. Additional bands may be present, but if over 50 % of final concentration, the product may be purified using gel extraction. Restriction digests may be used to further confirm the correct assembly of the final PCR product.

⁵Although gel extraction may be used to purify products, in our hands efficiency is decreased relative to gel filtration purification.

⁶This procedure has been optimized for *Aspergillus nidulans* adapted from [75]; adjust as appropriate for your fungus.

2. Incubate overnight at room temperature shaking at 150 rpm depending on strain background and type of auxotrophy (if present). Extended, actively growing hyphae should be present in the sample.
3. Collect mycelia by filtration through sterile miracloth (Calbiochem).
4. Rinse with additional medium.
5. Transfer mycelia into sterile 125 mL flask.
6. Add 16 mL fresh protoplasting solution.
7. Incubate 2–4 h at 30 °C at 100 rpm (*see* Note 7).
8. Add 10 mL 1.2 M sucrose solution to a sterile 50 mL centrifuge tube.
9. Gently overlay protoplast solution, taking care not to disrupt layer formation.
10. Centrifuge at $1800 \times g$ for 10 min. Protoplasts will appear as a cloudy layer at the interphase.
11. Gently collect the protoplasts with a pipette and transfer to a fresh sterile tube.
12. Add at least 1 volume of 0.6 M KCl. Mix gently.
13. Centrifuge at $1800 \times g$ for 10 min to pellet the protoplasts.
14. Resuspend protoplasts in 1 mL of 0.6 M KCl.
15. Centrifuge at $2300 \times g$ for 3 min to pellet the protoplasts. Carefully remove supernatant.
16. Resuspend protoplasts in an appropriate volume of 0.6 M KCl, 50 mM citric acid solution. 100 μ L per transformation (including negative control) should be used. Typically up to 10 transformations may be performed per batch of protoplasts.
17. Add DNA (10 μ L of PCR product; 1–2 μ g) to 100 μ L protoplasts.
18. Vortex four to five times (1 s each time) at maximum speed.
19. Add 50 μ L of PEG solution (*see* Note 8).
20. Vortex four to five times (1 s each time) at maximum speed.
21. Incubate on ice for 25 min.
22. Add 1 mL PEG solution. Mix by gently pipetting up and down.
23. Incubate at room temperature for 25 min.
24. Plate transformation mixture on selection KCl Minimal Medium (KMM) with appropriate selection supplements by spreading transformation sample over selection plates with a glass spreader (*see* Note 9).

⁷Protoplasts are normally visible after 1 h of incubation. Protoplast clumping can be inhibited by gentle pipetting several times during incubation. Do not overdigest.

⁸PEG solution should be freshly filtered prior to transformation to remove precipitates which may rupture protoplasts.

⁹We typically plate ~100 μ L per medium size (15 cm) plate. This allows for sufficient separation of transformants.

25. Incubate at 37 °C. Transformants will be visible after 2–3 days. Proceed with your desired method of transformant confirmation and growth for metabolite production.

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Fig. 1.

Domain architecture of select fungal NRPS and their chemical products. **(a)** Domain organization of the SimA from *Tolypocadium inflatum* responsible for cyclosporine A production modified from Hoffmann et al. [18]. C* represents a truncated and presumably inactive C domain. **(b)** Domain architecture of the hybrid PKS/NRPS Fus1 from *Fusarium fujikuroi* responsible for fusarin C production modified from Niehaus et al. [19]. KS β -ketoacyl synthase domain; AT malonyl coenzyme A-acyl carrier protein transacylase domain; DH hydratase domain; ER enoyl reductase domain, most likely nonfunctional in Fus1; KR keto reductase domain; R reductase domain; Fus2-9, tailoring enzymes encoded in the fusarin C gene cluster of *F. fujikuroi*. **(c)** Domain organization of the NRPS-like TdiA involved in terrequinone A production in *Aspergillus nidulans* modified from Balibar et al. [17]. TE thioesterase domain, TdiB-D, tailoring enzymes encoded in the terrequinone A gene cluster of *A. nidulans*. **(d)** Domain organization of the NRPS GliP responsible for the first committed step in gliotoxin production in *Aspergillus fumigatus* modified from Scharf et al. [20]

Fig. 2. Schematic overview for gene manipulation strategies. **(a)** Schematic depiction of construction of an overexpression construct for targeted promoter replacement at the gene locus. **(b)** Schematic depiction of construction of a knockout construct for targeted gene replacement

Table 1

Web-based tools for NRPS domain analysis

Tool	Website	Reference
Conserved Domain Database (CDD)	www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi	[76]
Universal Protein Resource (UniProt)	http://www.uniprot.org	[77]
Protein sequence analysis and classification (InterPro)	http://www.ebi.ac.uk/interpro/	[78]
PKS/NRPS analysis website	http://nrps.igs.umaryland.edu/nrps/	[79]
NRPSpredictor	http://ab.inf.uni-tuebingen.de/software/NRPSpredictor/	[80]
NRPSpredictor2	http://nrps.informatik.uni-tuebingen.de/Controller?cmd=SubmitJob	[81]
Norine	http://bioinfo.lifl.fr/norine/index.jsp	[82]
Nonribosomal peptide synthetase substrate predictor (NRPSsp)	http://www.nrpssp.com/	[83]
Antibiotics and secondary metabolite analysis shell (antiSMASH)	http://antismash.secondarymetabolites.org/	[84]
Secondary metabolite unique regions finder (SMURF)	www.jcvi.org/smurf	[85]
Natural product domain seeker (NaPDos)	http://napdos.ucsd.edu/napdos_home.html	[86]