



# Heterologous Expression of Fungal Secondary Metabolite Pathways in the *Aspergillus nidulans* Host System

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## Contents

1. Introduction	128
2. Identification of Secondary Metabolite Genes in Fungal Genomes	130
3. Design Primers for Fusion PCR	130
4. Obtain Genomic DNA for PCR Template	131
4.1 Materials	132
4.2 DNA Extraction from Hyphae	132
4.3 Materials	133
4.4 DNA Extraction from Spores	133
5. Fusion PCR Construction	134
5.1 Materials	134
5.2 Genomic PCR	134
5.3 One Pot Fusion Reaction	135
5.4 Two Pot Fusion Reaction	135
6. Transformation	136
6.1 Materials	136
6.2 Protoplasting	136
7. Diagnostic PCR	138
8. Liquid Culturing of Mutant Strains	138
8.1 Materials	138
8.2 Culturing Strains in Glucose Minimal Media	139
9. Recipes	139
10. Conclusions	140
Acknowledgments	141
References	141

## Abstract

Heterologous expression of fungal secondary metabolite genes allows for the product formation of otherwise silent secondary metabolite biosynthesis pathways. It also allows facile expression of mutants or combinations of genes not found in nature. This capability makes model fungi an ideal platform for synthetic biology. In this chapter a detailed description is provided of how to heterologously express any fungal secondary metabolite gene(s) in a well-developed host strain of *Aspergillus nidulans*. It covers all the necessary steps from identifying a gene(s) of interest to culturing mutant strains to produce secondary metabolites.



## 1. INTRODUCTION

Fungal species have been widely used to study fundamental biology of eukaryotes since they grow faster and are easier to grow compared to higher order mammalian cells. Several species such as *Aspergillus fumigatus* and *Aspergillus flavus* are of particular interest because of their pathogenicity due to both invasiveness in immunocompromised patients and the toxic compounds they produce, for example, gliotoxin and aflatoxin. These compounds are often the product of the secondary metabolite (SM) pathways in these fungi. SM pathways offer a myriad of organic molecules and can also sometimes be medicinally useful such as most famously penicillin G, cyclosporine A, and lovastatin. Since many of these biosynthetic pathways present in the genomes of fungal species have not been linked to their product, this provides an opportunity for discovery as more (fungal) genomes are sequenced.

Fungal secondary metabolite biosynthesis pathways involve one core set of enzymes that is responsible for the backbone of the compound. These core enzymes could either be polyketide synthases (PKS), nonribosomal synthetases (NRPS), a hybrid PKS-NRPS, or a terpene cyclase (TC). Discovery of new secondary metabolite genes is often accomplished by homology searches using known genes of these core enzymes (Ingliš et al., 2013; Khaldi et al., 2010; Takeda, Umemura, Koike, Asai, & Machida, 2014). Conveniently, in many cases, the other genes (coding for tailoring enzymes, resistance proteins, transcription factors, or transporters) in the biosynthetic pathway are clustered in the genome so they can be identified easily once the core enzymes have been identified. There are several ways to link the identified secondary metabolite pathways with their products. One way is to

create knockouts in the producing organism (Chiang et al., 2008). In this approach the disappearance of one or more compounds in the secondary metabolite profile in the mutant strain compared to a control strain would indicate that the deleted gene in the mutant is necessary for product formation. This method is quite straightforward but would require that a culture condition can be identified under which the gene product is produced. In many cases secondary metabolite clusters identified from a sequenced genome are either silent or produce very small amounts, so that a condition to turn on the pathway cannot be identified easily. Alternatively, our lab and others have used a heterologous expression approach to link secondary metabolism genes with their respective compounds (Chiang et al., 2013, 2015). The heterologous expression approach is useful particularly when the secondary pathways are silent in the producing organism or when no genetic system is available.

The three commonly used host system in the lab are *Saccharomyces cerevisiae* (yeast) (Tsunematsu, Ishiuchi, Hotta, & Watanabe, 2013), *Aspergillus oryzae* (Sakai, Kinoshita, & Nihira, 2012), and *Aspergillus nidulans*. Gene targeting is very efficient in yeast, but wild-type yeast is not rich in SMs and thus relevant building blocks may be absent. Introns also differ between yeast and filamentous fungi, so cDNA is necessary for heterologous expression. Both *A. nidulans* and *A. oryzae* are more suited in these respects since intron splicing is not an issue. In our lab we have focused on using *A. nidulans* as a host. *A. nidulans* has the advantage that its genetic system is well developed. Host strains with multiple nutritional markers are available. In collaboration with Berl Oakley's group at University of Kansas an *A. nidulans* strain, LO8030, with four mutations (*pyroA4*, *riboB2*, *pyrG89*, and *nkuA::argB*) and eight gene clusters knocked out (sterigmatocystin AN7804–7825, emericellamide AN2545–2549, asperfuranone AN1039–1029, monodictyphenone AN10023–10021, terrequinone AN8512–8520, austinol AN8379–8384/AN9246–9259, F9775 AN7906–7915, and asperthecin AN6000–6002) has been created (Chiang et al., 2015). The strain LO8030 wastes fewer resources on known secondary metabolites and has a clean background ideal for detecting new compounds as the result of heterologous expression. This chapter will explain step by step how to rapidly heterologously express SM genes in this highly optimized host without the need for cosmids, plasmids, or vectors. Parts of this protocol have also been described elsewhere in detail (Lim, Sanchez, Wang, & Keller, 2012), but will be included to enable readers to perform the experiments using this chapter alone.



## 2. IDENTIFICATION OF SECONDARY METABOLITE GENES IN FUNGAL GENOMES

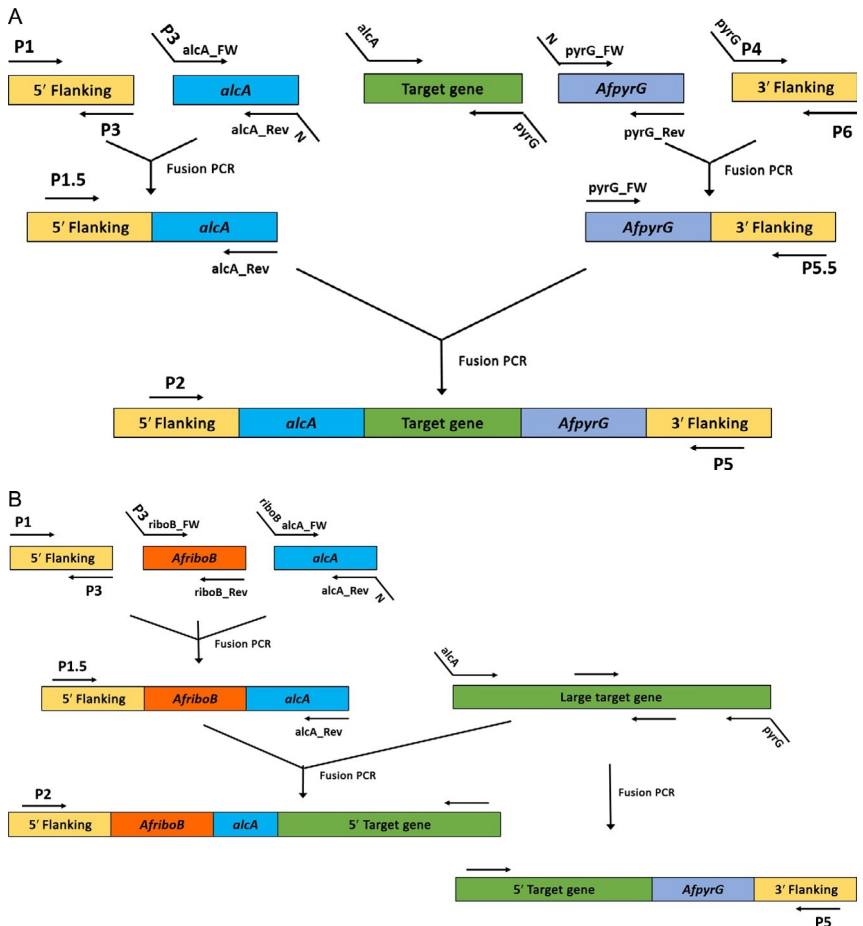
The first step is to identify the gene(s) you want to express. The Joint Genome Institute (JGI) is a great resource for (newly) sequenced genomes, but NCBI and AspGD also have genomes available. Genes of interest are usually found by blast homology analysis with known biosynthetic genes. The predicted function of hits and their surrounding genes can be used as an indication of validity. Once a gene is identified, the genomic sequence can be downloaded with introns included, since the *A. nidulans* system can recognize them.

*Note:* Since JGI provides genome sequences prior to publication, consult the JGI website for their most recent policy regarding data usage and acknowledgments.



## 3. DESIGN PRIMERS FOR FUSION PCR

For genes smaller than 4000 base pairs, one forward and one reverse primer can be designed. The forward primer typically starts at the start codon and has a 5' sticky end of ~20 base pairs for fusion PCR to an upstream promoter. The powerful alcohol inducible promoter *alcA* (Waring, May, & Morris, 1989) is most often used in our lab, but other (noninducible) promoters like Tet-on system (Meyer et al., 2011) or *gpdA* (Punt, Kramer, Kuyvenhoven, Pouwels, & Vandenbondel, 1992) are also possibilities. The reverse primer is designed at roughly 300 base pairs after the stop codon to allow proper transcription termination. It has a 3' sticky end of ~20 base pairs for a selection marker. The gene *pyrG* from *A. fumigatus* is most often used to complement the *A. nidulans pyrG89* background strain. *AfriboB* and *AfpyrA* markers can be used for subsequent transformations. More genes can be inserted by marker recycling where a marker of the first construct is replaced by a second construct with a different marker. *AfpyrG* deletion can also be selected for with 5-fluoroorotic acid. Genes larger than 4000 base pairs require two or more DNA fragments for transformation due to PCR limits for large (8 + kb) constructs. The 5' and the 3' primers are designed in the same way and additional primers are designed to generate fragments with a 1000 base pair overlap. Additionally a second marker is fused to the 5' fragment before the promoter (see Fig. 1B).



**Fig. 1** Fusion PCR overview for genes smaller (A) and larger than 4000 base pairs (B). (A) Individual fragments are amplified from genomic DNA. Flanking fragments and the *alcA* promoter from the *A. nidulans* host, *AfpYrG* from *A. fumigatus* genomic DNA, and the target gene from its respective source. The "N" sticky ends of the *alcA*\_Rev and *pyrG*\_FW primers on the first line are an arbitrary number of nucleotides added to make the fusion PCRs on the middle line have nested primers on both sides. (B) The target gene is amplified in two separate pieces with 1000 base pairs overlap. In addition to the promoter, an additional marker is added to the 5' fragment. The two fragments are mixed and added to the protoplasts for transformation.

## 4. OBTAIN GENOMIC DNA FOR PCR TEMPLATE

The ability of *A. nidulans* to recognize introns, allows the use of unmodified genomic DNA for gene amplification. Genomic DNA is

preferably extracted from hyphae, since they presumably contain less metabolites that may inhibit polymerase. Usually genomic DNA from spores also works though. For hazardous species, it is possible to purchase only the genomic DNA from commercial sources such as ATCC. Since many fungal species are either plant or human pathogens sometimes obtaining the necessary permits will take several months and purchasing only genomic DNA can then greatly speed up the project. Lastly, with the significant improvements in commercial gene synthesis, it is also possible to obtain secondary metabolite genes via commercial DNA synthesis.

## 4.1 Materials

*Note:* In this section we provide the supplier and the item number we use in the Wang lab. Equivalent items can be used instead unless specifically noted below.

1. Miracloth (EMD Millipore, #475855)
2. Mortar and pestle
3. Sterile toothpick (Forster)
4. Lysis buffer hyphae (see [Section 9](#))
5. Phenol/chloroform/isoamyl alcohol (25:24:1) adjusted to pH 8 (EMD Millipore, #516726)
6. 3 M NaOAc, pH 5 (AMRESCO, #0602)
7. Isopropanol (J.T. Baker, #9084-01)
8. Ethanol, biotechnology grade (IBI scientific, # IB15720)
9. TE buffer (see [Section 9](#))

## 4.2 DNA Extraction from Hyphae

1. Harvest the hyphae by filtration through Miracloth.
2. Transfer to Eppendorf tube, lyophilize, and grind dry mycelia with a sterile toothpick or transfer to mortar and freeze with liquid nitrogen and grind to powder.
3. Add 700  $\mu\text{L}$  lysis buffer and vortex for 30 s.
4. Incubate at 65°C for 1 h (tube with cap-lock).
5. Add 700  $\mu\text{L}$  (an equal amount of lysis buffer in step 3) phenol/chloroform/isoamyl alcohol (25:24:1) adjusted to pH 8 and vortex for 1 min.
6. Microcentrifuge at max speed (or at least  $10,000 \times g$ ) for 15 min at R.T.
7. Transfer aqueous phase ( $\sim 400 \mu\text{L}$  in 100  $\mu\text{L}$  steps to avoid disturbing separation) to a new Eppendorf tube.

8. Add 13.3  $\mu\text{L}$  of 3 M NaOAc, pH 5, followed by 223  $\mu\text{L}$  ( $0.54 \times V$ ) of isopropanol and invert gently. DNA clots may be visible.
9. Microcentrifuge at max speed (or at least  $10,000 \times g$ ) for 3 min at R.T.
10. Remove supernatant with a pipette and rinse the pellet twice with 70% ethanol.
11. Remove supernatant with a pipette and place the tube in a dry bath at  $50^\circ\text{C}$  for  $\sim 10$  min.
12. Resuspend in 100  $\mu\text{L}$  TE buffer. If the DNA does not dissolve, place in dry bath at  $65^\circ\text{C}$  for 15 min.
13. Take 0.5  $\mu\text{L}$  for a 50  $\mu\text{L}$  PCR reaction.
14. Genomic DNA can be stored at  $-20^\circ\text{C}$  for months or at  $-80^\circ\text{C}$  for longer.

### 4.3 Materials

1. Miracloth (EMD Millipore, #475855)
2. Lysis buffer spores (see [Section 9](#))
3. Glass beads, acid washed (Sigma, #G8772)
4. Phenol/chloroform/isoamyl alcohol (25:24:1) adjusted to pH 8 (EMD Millipore, #516726)
5. 3 M NaOAc, pH 5 (AMRESCO, #0602)
6. Isopropanol (J.T. Baker, #9084-01)
7. Ethanol, biotechnology grade (IBI scientific, # IB15720)
8. TE buffer (see [Section 9](#))

### 4.4 DNA Extraction from Spores

1. Take 500  $\mu\text{L}$  of spore suspension in an Eppendorf tube and centrifuge at  $8000 \times g$  for 5 min.
2. Remove supernatant and resuspend in 100  $\mu\text{L}$  lysis buffer, then add 150 mg/100  $\mu\text{L}$  of 0.45–0.5-mm glass beads.
3. Vortex for 30 s.
4. Incubate for 30 min at  $65^\circ\text{C}$  vortexing 30 s every 10 min (tube with cap-lock).
5. Add 100  $\mu\text{L}$  of phenol/chloroform/isoamyl alcohol (25:24:1).
6. Vortex for 5 min.
7. Microcentrifuge at max speed (or at least  $10,000 \times g$ ) for 5 min at R.T.
8. Collect 50–60  $\mu\text{L}$  from the upper phase and transfer to a new tube.
9. Add 5  $\mu\text{L}$  of 3 M NaOAc, pH 5, mix, add 100  $\mu\text{L}$  ethanol, mix. Put in  $-20^\circ\text{C}$  for 20 min.

10. Microcentrifuge at max speed (or at least  $10,000 \times g$ ) for 30 min at RT.
11. Discard supernatant and wash with 100  $\mu\text{L}$  of 70% ethanol.
12. Microcentrifuge at max speed (or at least  $10,000 \times g$ ) for 15 min at RT.
13. Remove supernatant and place the tube in a dry bath at  $50^\circ\text{C}$  for  $\sim 5$  min.
14. Dissolve in 30  $\mu\text{L}$  TE buffer.
15. Take 0.5  $\mu\text{L}$  for a 50  $\mu\text{L}$  PCR reaction.



## 5. FUSION PCR CONSTRUCTION

Individual fragments are amplified using the designed primers in 50  $\mu\text{L}$  PCR reactions (Fig. 2). Note: Accuprime™ high-fidelity *Taq* is the commercial polymerase we use due to its accuracy, high yield, and ability to generate large DNA fragments. Even though it is more expensive than other polymerases commercially available, in our experience those have performed significantly worse in terms of both success and yield. Therefore, we highly recommend using the same polymerase we use in the Wang lab for the fusion PCR construction.

### 5.1 Materials

1. Techne Prime thermocycler (Techne, #5PRIMEG/02)
2. Accuprime™ *Taq* high fidelity with buffer I and II (Thermo Fisher Scientific, #12346094)
3. Agarose (IBI scientific, #IB70071)
4. Ethidium bromide (IBI scientific, #40075)
5. QIAquick gel extraction kit (Qiagen, #28704)
6. Zymo-Spin I columns (Zymo Research, #C1003)

### 5.2 Genomic PCR

PCR reactions are analyzed on a 1% agarose gel with 0.01% ethidium bromide and correct bands are cut out and purified using a gel extraction kit (Qiagen) and eluted in 30  $\mu\text{L}$  elution buffer. The concentration of eluted DNA is determined by nanodrop. For fusion PCR, each fragment is mixed at a fixed molar ratio (1:1, 1:2:1, or 1:2:2:1). Fusion PCR with more than four fragments should be split up into two separate fusions of which the products can be fused again. The amount of each fragment needed depends on each size, but a minimum of 30 ng of each fragment is recommended. Fusion PCR can be done in one or two pots. In one pot the primers are



Reaction components		Thermocycler program		
Genomic DNA	0.5 µL	<i>T</i>	Time	Ramp rate
Forward primer (10 µM)	2 µL	94°C	5 min	3.4 °C/s
Reverse primer (10 µM)	2 µL	94°C	20 s	3.4 °C/s
10× buffer II (accuprime™)	5 µL	70°C	1 s	3.4 °C/s
Accuprime™ Taq high fidelity (5u/µL)	0.2 µL	55°C	30 s	0.1 °C/s
ddH <sub>2</sub> O	40.3 µL	68°C	1 min/kb + 1 min	0.2 °C/s
		68°C	20 min	–
		4°C	∞	1 °C/s

Fig. 2 Genomic PCR conditions.

added with the separate fragments, which is the quickest way of obtaining the fusion construct (Fig. 3). In two pots the fragments are first fused without primers and then an aliquot is amplified in a second round with primers, which takes more time than the one pot reaction, but it is easier to generate more fusion construct, since the amplification step can be scaled up easily (Fig. 4).

5.3 One Pot Fusion Reaction

Reaction components		Thermocycler program		
5' Flanking fragment	30+ ng	<i>T</i>	Time	Ramp rate
DNA fragment (gene)	30+ ng	94°C	5 min	3.4 °C/s
3' Flanking fragment	30+ ng	94°C	20 s	3.4 °C/s
Forward primer (10 µM)	2 µL	70°C	1 s	3.4 °C/s
Reverse primer (10 µM)	2 µL	55°C	30 s	0.1 °C/s
10× buffer I (accuprime™)	5 µL	68°C	1 min/kb + 1 min	0.2 °C/s
Accuprime™ Taq high fidelity (5u/µL)	0.2 µL	94°C	20 s	3.4 °C/s
ddH <sub>2</sub> O	up to 50 µL	70°C	1 s	3.4 °C/s
		55°C	30 s	0.1 °C/s
		68°C	1 min/kb + 1 min + 5 s/cycle	0.2 °C/s
		94°C	20 s	3.4 °C/s
		70°C	1 s	3.4 °C/s
		55°C	30 s	0.1 °C/s
		68°C	1 min/kb + 1 min + 20 s + 15 s/cycle	0.2 °C/s
		68°C	20 min	–
		4°C	∞	1 °C/s

Fig. 3 One pot fusion PCR conditions.

5.4 Two Pot Fusion Reaction

The fusion PCR is again analyzed on 1% agarose gel and correct product is cut out and purified using a gel extraction kit. Fusion PCR construct is eluted in 10–15 µL elution buffer or ddH<sub>2</sub>O from Zymo-Spin I columns that allow elution with volumes as low as 6 µL and can be directly used for transformation or stored at –20°C. The fusion PCR product is not quantified, but the amount of DNA is correlated with transformation success: more DNA gives more transformed colonies. Also, for larger constructs

Reaction components		Thermocycler program		
DNA fragment 1	30+ ng	T	Time	Ramp rate
DNA fragment 2	30+ ng	94°C	5 min	3.4 °C/s
10× buffer I (accuprime™)	2.5 µL	94°C	30 s	3.4 °C/s
Accuprime™ Taq high fidelity (5 u/µL)	0.1 µL	55°C	10 min	0.1 °C/s
ddH <sub>2</sub> O	up to 25 µL	68°C	1 min/kb + 1 min	0.2 °C/s
		68°C	20 min	–
		4°C	∞	1 °C/s
Fusion template	2 µL	94°C	5 min	3.4 °C/s
Forward primer (10 µM)	2 µL	94°C	20 s	3.4 °C/s
Reverse primer (10 µM)	2 µL	70°C	1 s	3.4 °C/s
10× buffer II (accuprime™)	5 µL	55°C	30 s	0.1 °C/s
Accuprime™ Taq high fidelity (5 u/µL)	0.2 µL	68°C	1 min/kb + 1 min	0.2 °C/s
ddH <sub>2</sub> O	38.8 µL	68°C	20 min	–
		4°C		1 °C/s

Fig. 4 Two pot fusion PCR conditions.

and transformation with more than one fragment, DNA amounts need to be higher to be successful.

6. TRANSFORMATION

Protoplasts of the *A. nidulans* background strain are always freshly prepared for transformation (Szewczyk et al., 2006).

6.1 Materials

1. Supplemented yeast glucose (YG) media (see Section 9)
2. Gyrotory shaker (Barnstead 4000 MaxQ)
3. 2× Protoplasting solution (see Section 9)
4. Miracloth (EMD Millipore, #475855)
5. 1.2 M sucrose (sterile) (Sucrose, Omnipur, VWR, #EM-8550)
6. 0.6 M KCl (sterile) (VWR, #EM-PX1404-5)
7. 0.6 M KCl, 50 mM CaCl<sub>2</sub> (sterile) (CaCl<sub>2</sub>·H<sub>2</sub>O, VWR, #CX0130-2)
8. PEG solution (see Section 9)
9. Glucose minimal media agar plates (see Section 9)
10. Sterile cotton swab (Puritan, #25-806 1WC)
11. S/T buffer (see Section 9)

6.2 Protoplasting

1. Fresh spores (1e8, less than 1 week old) are inoculated in 20 mL YG media with supplements pyridoxine, riboflavin, uridine, and uracil.
2. Spores are shaken at 30°C, 135 rpm for 14 h.
3. 2× Protoplasting solution made 20 min before.

4. The hyphae are filtered with sterile Miracloth.
5. Hyphae are washed with 2 mL of YG media and transferred with a sterile spatula to a sterile 50-mL conical flask with 8 mL YG media.
6. 8 mL of  $2\times$  protoplasting solution is filtered into the flask.
7. Protoplasting at  $30^{\circ}\text{C}$ , 135 rpm for 2–2.5 h, beware of overdigestion.
8. 4 mL of protoplasting solution is carefully layered on 8 mL of 1.2 M sucrose in a 15-mL tube and centrifuged with the brake off at  $1800\times g$  for 10 min.
9. Protoplasts are collected from slightly above the resulting interface with a sterile dropper. Avoid disturbing the interface or collecting from below the interface. Collect 1–1.5 mL from each tube in a new 15-mL tube.
10. Add  $2\times$  volume of 0.6 M KCl and centrifuge with brake at  $1800\times g$  for 10 min.
11. Remove supernatant with a serological pipette and resuspend in  $2\times 1$  mL of 0.6 M KCl. Resuspend by pipetting the first 300  $\mu\text{L}$  slowly up and down a few times, then add the rest and transfer to a sterile Eppendorf tube (1 mL in each tube).
12. Centrifuge at  $2400\times g$  for 3 min. Then wash each tube with 1 mL of 0.6 M KCl two more times. Material that does not resuspend after a few times of pipetting up and down should be removed by keeping the suspension sideways in the tip for 20 s. Unsuspended clogs will stick to the tip wall.
13. All protoplasts are resuspended in 1 mL of 0.6 M KCl, 50 mM  $\text{CaCl}_2$  and centrifuged at  $2400\times g$  for 3 min.
14. Supernatant is discarded and protoplasts are resuspended in 100  $\mu\text{L}$  of 0.6 M KCl, 50 mM  $\text{CaCl}_2$  per transformation. Up to 10 transformations can be done with one batch.
15. Protoplast solution is added to the DNA solution (10–15  $\mu\text{L}$ ) and vortexed once for 1 s.
16. 50  $\mu\text{L}$  of freshly filtered PEG solution is added and vortexed once for 1 s.
17. Put on ice for 25 min.
18. Add 100  $\mu\text{L}$  PEG solution and mix by pipetting up and down a minimum of 10 times.
19. Keep at RT for 25 min.
20. Plate each transformation mixture gently with a sterile spreader on two 10-cm glucose minimal media (GMM) plates with 0.6 M KCl, riboflavin, and pyridoxin (roughly 130  $\mu\text{L}$  per plate).

21. Incubate face up at 30°C overnight.
22. Flip plates and transfer to 37°C.
23. Colonies should be visible 2 days after transfer.

By choosing the flanking DNA of the fusion construct (~1000 base pairs each side) to target the *γA* or the *wA* locus, the visible colonies after transformation have changed color from green to either yellow or white. When picking colonies for diagnostic PCR, the green ones, if any, can be disregarded. Three colonies per transformation are picked and scratched on selective plates. After 2 days a single colony is picked with a sterile cotton swab and replated on selective plates to ensure genetic homogeneity. Spores are harvested by covering the plate in 7 mL S/T buffer by rubbing a sterile cotton swab over the surface and collecting the suspension.



## 7. DIAGNOSTIC PCR

Genomic DNA of the transformants is extracted as described above. Diagnostic PCR (same as genomic PCR except 10 µL reactions) is performed by using primers outside of the insert on both background and new strains. An increase or decrease in size according to the fragment inserted shows correct transformation. For large, multiple fragment insertions the whole insertion is too large for a single PCR. Therefore, shorter fragments both within and on the border are chosen and compared with absence in the background. Diagnostic PCR usually shows success rates near 90%. Southern blots can be performed if necessary to double confirm. In addition (part of) the genes can be sequenced to confirm insertion and identify potential mutations not detected by PCR.



## 8. LIQUID CULTURING OF MUTANT STRAINS

### 8.1 Materials

1. Glucose minimal media (see [Section 9](#))
2. Methyl ethyl ketone (or 2-butanone) (Sigma-Aldrich, #34861)
3. Ethyl acetate (EMD Millipore, #EX0240-5)
4. 6 M HCl (J.T. Baker, #5619-03)
5. Dimethyl sulfoxide (DMSO) (J.T. Baker, #9224-06)
6. Methanol (EMD Millipore, #MX0488-1)
7. 0.2-µm PTFE filter (VWR, #28145-491)

## 8.2 Culturing Strains in Glucose Minimal Media

Genetically correct strains are cultured alongside the background strain in 30 or 50 mL of liquid GMM in 125-mL conical flasks at 37°C, 180 rpm for 42 h. This ensures most if not all glucose is used up and will not inhibit subsequent *alcA* induction. The temperature is lowered to 30°C and the *alcA* promoter is induced by adding 134.4 or 224  $\mu$ L methylethylketone (MEK). Cyclopentanone can also be used, but is more toxic to the fungus. After 3 days of induction, the cultures are filtered with filter paper and extracted with an equal volume of ethyl acetate. Aqueous phase is acidified to pH 2 with 6 M HCl and again extracted with an equal volume of ethyl acetate. Ethyl acetate extractions are dried, weighed, and redissolved in 20% DMSO in methanol at a concentration of roughly 1 mg/mL or as desired. After filtration with a 0.2- $\mu$ m PTFE filter, 10  $\mu$ L is injected for LC-MS analysis. New peaks compared to the background are identified. Hit strains will be scaled up to liter scale. Extracts are first purified by flash chromatography followed by semipreparative HPLC. Purified compounds are characterized by HighRes MS and NMR. Detailed protocols for secondary metabolite analysis can be found in our earlier *Methods in Enzymology* review ([Lim et al., 2012](#)).



## 9. RECIPES

### *Lysis buffer hyphae (200 mL)*

Add 1.211 g Tris, 2.922 g EDTA, and 6 g SDS to a final volume of 200 mL ddH<sub>2</sub>O. Adjust pH to 7.2 with NaOH. Add 1% 2-mercaptoethanol right before use.

### *Lysis buffer spores (200 mL)*

Add 4 mL Triton X-100, 2 g SDS, 1.1688 g NaCl, 0.0584 g EDTA, and 0.3152 g Tris-HCl to a final volume of 200 mL ddH<sub>2</sub>O. Adjust to pH 8.0 with NaOH.

### *TE buffer (200 mL)*

Add 0.3152 g Tris-HCl and 5.84 mg EDTA to a final volume of 200 mL ddH<sub>2</sub>O. Adjust to pH 8.0 with NaOH. Autoclave.

### *Yeast glucose (YG) rich media (300 mL)*

Add 1.5 g yeast extract, 6 g dextrose, 300  $\mu$ L of Hutner's trace element solution to a final volume of 300 mL. Pyridoxine-dependent strains can grow on this medium. Riboflavin-dependent strain requires the addition

of 750  $\mu\text{g}$  riboflavin (from stock solution). Uridine<sup>a</sup> and uracil-dependent strains require 0.7326 and 0.3 g, respectively. Autoclave.

*Glucose minimal media (500 mL)*

Add 5 g dextrose, 500  $\mu\text{L}$  KOH (5.5 M), 500  $\mu\text{L}$  Hutner's trace element solution and 25 mL of 20 $\times$  salt solution (see below) to a final volume of 500 mL. Optional supplements: 0.25 mg pyridoxine, 1.25 mg riboflavin, 500 mg uracil, and 1.221 g uridine.<sup>a</sup> For agar plates, add 7.5 g agar. For 0.6 M KCl plates, add 22.36 g KCl. Autoclave. Agar media is kept at 55°C in a water bath before pouring 25 mL per 10-cm plate.

*20 $\times$  salt solution (2000 mL)*

Add 240 g  $\text{NaNO}_3$ , 20.8 g KCl, 20.8 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 60.8 g  $\text{KH}_2\text{PO}_4$  to a final volume of 2000 mL.

*2 $\times$  Protoplasting solution (10 mL)*

Dissolve 8.2 g KCl and 2.1 g citric acid monohydrate in 50 mL ddH<sub>2</sub>O. Adjust pH to 5.8 with 1.1 M KOH solution. Make the volume up to 100 mL. Autoclave. Take 10 mL of KCl/citric acid and add 2 g VinoTaste<sup>®</sup> Pro. Vortex and let it dissolve for 20 min. Filter sterilize into flask for immediate use (see step 6 of protoplasting).

*PEG solution (100 mL)*

Add 4.47 g KCl and 0.74 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  to about 20 mL ddH<sub>2</sub>O and 0.802 mL 1 M Tris-HCl and 0.196 mL 1 M Tris. Add 25 g PEG (average molecular weight 3350). Add ddH<sub>2</sub>O approximately up to 90 mL. Use a magnetic stirrer to dissolve PEG. Remove stirrer and make up to 100 mL. Autoclave. Before use, filter necessary amount with 0.2- $\mu\text{m}$  filter to remove detrimental PEG precipitates.

*S/T buffer (1000 mL)*

Add 8.5 g NaCl and 1 mL Tween 80 to 1000 mL ddH<sub>2</sub>O. Autoclave.



## 10. CONCLUSIONS

Gene transfer using this method is very facile and mutant strains with correct gene inserts are easily obtained. The functionality of the heterologously expressed genes is hard to predict and often requires additional experiments, like the addition of genes that only work in tandem. However, the ability to generate 10 or more different mutants per transformation and the use of several orthogonal markers, make it easy to screen many gene mutants and combinations. The use of the powerful *alcA* promoter in this system

<sup>a</sup> Add filter-sterilized uridine stock solution (1 M) after autoclaving.

allows for the detection of secondary metabolites from less than optimal enzymes and even protein purification for in vitro studies. The artificial recreation and subsequent manipulation of biosynthetic pathways that this system enables, will greatly contribute to synthetic biology and drug discovery in the next decade.

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