Chapter 10

Tools for Manipulation of Secondary Metabolism Pathways: Rapid Promoter Replacements and Gene Deletions in *Aspergillus nidulans*

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Abstract

Targeted gene deletions and promoter replacements are proving to be a valuable tool for awakening and analyzing silent secondary metabolism gene clusters in *Aspergillus nidulans* and, as molecular genetic methods for manipulating the genomes of other fungi are developed, they will likely be as valuable in those organisms. Here we describe procedures for constructing DNA fragments by PCR that can be used to replace genes or promoters quickly and on a large scale. We also describe transformation procedures for *A. nidulans* that allow these fragments to be introduced into target strains efficiently such that many genes or promoters can be replaced in a single experiment.

Key words: Secondary metabolism, Polymerase chain reaction, PCR, Gene deletion, Promoter replacement, Transformation, *Aspergillus*, Gene expression, Polyketide synthase, Non-ribosomal peptide synthetase

1. Introduction

The sequencing of fungal genomes has revealed that the genes involved in the production of a particular secondary metabolite are clustered in the genome and that, for each fungus, there are often many more gene clusters than known secondary metabolites (1–4). Many gene clusters are, thus, cryptic, not producing detectable products under normal lab growth conditions. Since secondary metabolites are a rich source of medically important compounds, it is important to obtain expression of these compounds. For compounds that are expressed normally, it is important to identify the genes involved and to decipher the biosynthetic pathways that produce them. Historically, these have been daunting tasks, but for

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Aspergillus nidulans they have been greatly facilitated by advances in gene targeting procedures (5, 6).

With respect to activating cryptic clusters, some are regulated at the chromatin level and they can be activated by deletion of certain genes involved in chromatin modification (7). Some gene clusters carry transcription factors within them that, when overexpressed, can activate expression of all the genes in the cluster resulting in the production of the product of the cluster (6, 8–10). Once expression of a pathway has been obtained, sets of targeted deletions can be used to determine which genes are part of the pathway (7, 10–15). Targeted deletions of polyketide synthase (PKS) genes or non-ribosomal peptide synthetase (NRPS) genes allow one to identify the gene cluster responsible for the synthesis of the compound. Targeted deletions of genes surrounding the responsible PKS or NRPS allow the genes required for the synthesis of the compound to be identified. In addition, many of the deletions will block the biosynthetic pathway and cause the accumulation of intermediate compounds in the pathway, and this allows the steps of the biosynthetic pathway to be determined. In addition, the intermediate compounds may be valuable themselves.

For these approaches to be maximally useful, it is important to be able to delete genes efficiently and to replace their promoters so that their levels of expression can be controlled. We have developed procedures that allow linear molecules to be created by fusion PCR (5, 16–18) that can be used to delete genes or replace their promoters (Fig. 1). This approach eliminates the need to clone genes in the conventional sense. Appropriate sequences can be amplified from genomic DNA and fused to genes or cassettes to construct transforming fragments. This approach is scalable in that transforming fragments to delete many genes (or replace the promoters of many genes) can be generated at the same time using a single PCR machine. It is also scalable in that one set of protoplasts can be generated for a strain and then aliquoted and transformed with the fusion PCR fragments. For example, if one has 10 candidate PKS genes that might be involved in the production of a particular secondary metabolite, one can create deletion constructs for all 10 of them by fusion PCR and, in a day, transform the constructs into an *A. nidulans* recipient strain, deleting each of the candidate PKS genes.

Our strategy for deleting genes in *A. nidulans* is to replace the target gene with a selectable marker as shown in Fig. 1. Sequences flanking the gene that one wishes to delete are amplified from genomic DNA. The primers are based on *A. nidulans* genomic sequences available at AspGD (http://www.aspergillusgenome.org/), CADRE (http://www.cadre-genomes.org.uk/index.html), or the Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). Primers P3 and P4 are designed with "tails" that are not part of the target

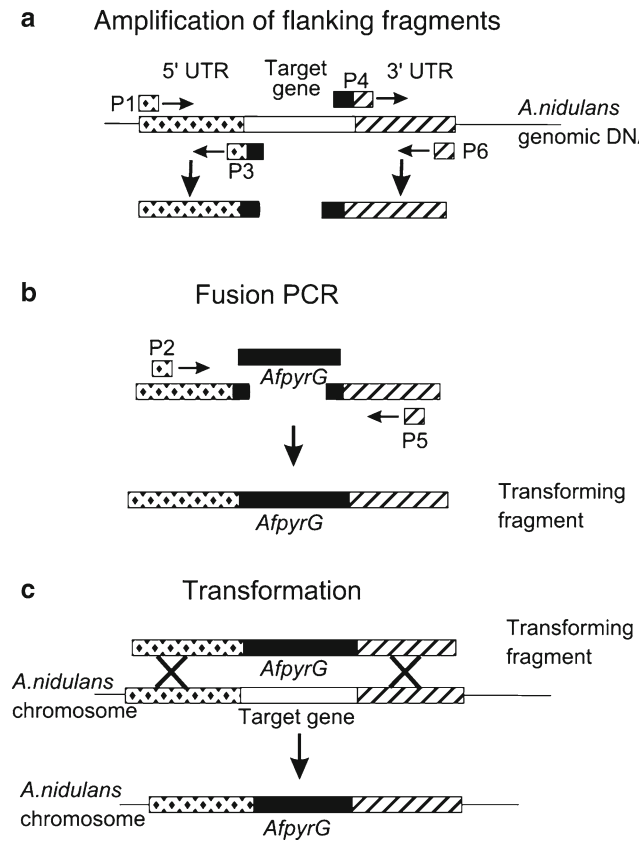

Fig. 1. Strategy for deleting genes. (**a**) Genomic sequences flanking the target gene are amplified from genomic DNA. Primers are designed based on the genomic sequences in the databases. Primers P3 and P4 are designed to have "tails" (*black region*) that are identical to sequences at the end of the fragment to which they will be fused, in this case the *A. fumigatus pyrG* gene (*AfpyrG*) which is used as a selectable marker. (**b**) Fusion PCR is used to amplify a transforming fragment. (**c**) Homologous recombination during transformation results in replacement of the target gene by the selectable marker.

genomic sequence, but, rather, are identical to the ends of the sequence that one wishes to fuse with the flanking sequences. In the case of gene replacements, the flanks are fused to a selectable marker. Selectable markers can be genes that complement *A. nidulans* mutations that inhibit growth or they can confer resistance to compounds toxic to *A. nidulans*. It is best to use a marker that is not an *A. nidulans* gene because *A. nidulans* genes can recombine by homologous recombination with the mutant allele in the genome and complement the mutation without integrating at the target gene. We routinely use the *Aspergillus fumigatus pyrG* gene (*AfpyrG*) (19), *A. fumigatus riboB* (*AfriboB*), and *A. fumigatus pyroA* (*AfpyroA*) genes as selectable markers (5) complementing *A. nidulans pyrG*89, *riboB*2, and *pyroA*4 mutations.

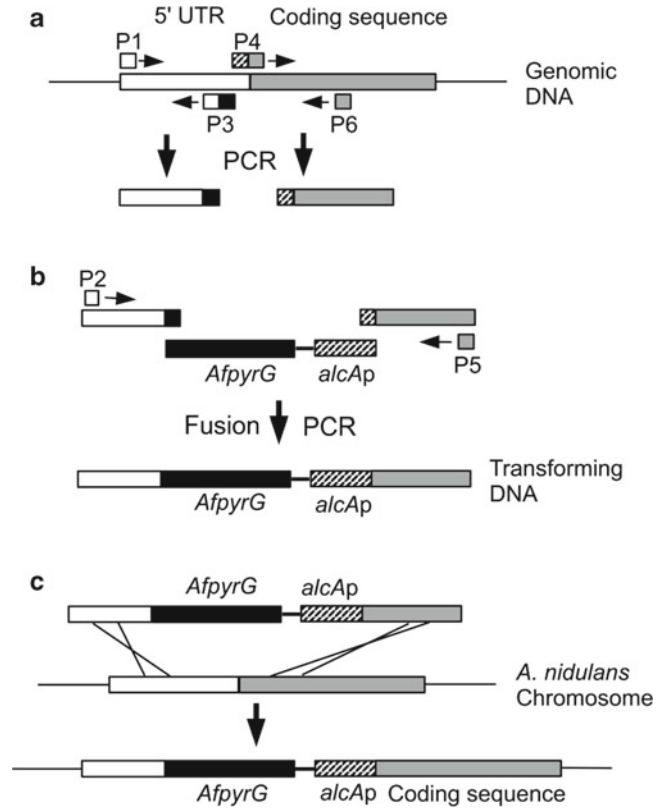

Fig. 2. Strategy for replacing promoters. (**a**) Flanking sequences for the target gene are amplified from genomic DNA. Primers are designed based on genomic sequences in the databases. Primers P1 and P3 amplify a region of the 5′ untranslated region (5′ UTR). The full extent of the endogenous promoter is usually unknown. Any portion of the promoter that is not deleted will be moved far away from the start codon in any case. P3 does not have to be immediately upstream of the start codon because the region between P3 and the start codon will be deleted upon integration (**c**). P4 amplifies beginning with the start codon of the coding sequence. P3 and P4 are designed with tails to allow them to be fused to a cassette containing a selectable marker and promoter as shown in **b**. In this case the selectable marker is *AfpyrG* and the promoter is from the *A. nidulans alcA* gene. (**c**) Transformation and homologous integration of the fusion PCR product result in the coding sequence of the gene being put under control of the *alcA* promoter.

Promoters can be replaced using a similar strategy (Fig. 2). A flanking fragment is amplified from the 5′ untranslated region. As drawn in Fig. 2, the flanking fragment does not include promoter sequences, but there is no harm in including promoter sequences. In the final construct, they will be separated from the coding sequence and should not affect transcription. A second fragment is amplified that begins with the start codon and extends approximately 1,000 bp. The fragment can end within the coding sequence or downstream if the coding sequence is less than 1,000 bp. The two amplified fragments are fused by PCR to a cassette containing

a selectable marker and the promoter of choice. In Fig. 2, *AfpyrG* (19) is the selectable marker and the promoter is from the *A. nidulans alcA* gene (20, 21), but the procedure is flexible with respect to choice of promoter and selectable marker. The cassette can, itself, be constructed by fusion PCR and the construct can be subcloned into a plasmid for long-term maintenance if desired.

2. Materials

2.1. Polymerase Chain Reaction

1. PCR enzymes: AccuPrime Pfx DNA polymerase and AccuPrime *Taq* High Fidelity (Invitrogen), KOD Hot Start DNA Polymerase (EMD Chemicals, Inc.), Phusion Hot Start II High Fidelity DNA polymerase (Thermo Scientific). Some enzyme kits include nucleotides; in other cases they must be purchased separately.
2. PCR cleanup kit: QIAquick PCR purification kit (Qiagen).
3. Thermocycler with programmable ramp rates (see Note 1).
4. PCR tubes: 200 μl thin-wall PCR tubes appropriate for thermocycler.
5. PCR primers (design of primers discussed under Subheading 3).
6. *A. nidulans* genomic DNA (can be prepared by a number of procedures but must be clean enough to allow long-term storage and reliable PCR). We generally prepare *A. nidulans* DNA in large amounts, purifying it over two CsCl density gradients (22). It can be stored for years at 4°C in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA).
7. Microcentrifuge for PCR cleanup.

2.2. Growth and Transformation (See Note 2)

1. 1.2 M KOH solution: Boil 200 ml of deionized distilled water. Allow it to cool to room temperature. Add 50 ml to a clean beaker. Add 6.73 g of KOH and allow it to dissolve. Transfer it to a clean graduated cylinder and add boiled deionized distilled water to a final volume of 100 ml. This solution will absorb CO_2 and should, consequently, be made fresh before use.
2. KCl/citrate solution (~1.2 M to be diluted to make protoplasting solution): Dissolve 8.2 g of KCl and 2.1 g of citric acid monohydrate in approximately 50 ml of deionized distilled water. Adjust to pH 5.8 with 1.2 M KOH solution. Transfer to a clean graduated cylinder and add deionized distilled water to a final volume of 100 ml. Mix thoroughly. This solution can be autoclaved, making sure that there is no volume change which will alter the molarity of the solution, or it can be stored at 4°C for up to 2 weeks in a tightly closed container.

3. 0.6 M KCl solution: Dissolve 4.47 g of KCl in 100 ml of deionized distilled water. This solution can be autoclaved, making sure that there is no volume change, and can be stored at room temperature in a tightly closed container (see Note 3).
4. 0.6 M KCl, 50 mM $CaCl_2$ solution: Dissolve 4.47 g of KCl and 0.74 g of $CaCl_2$ dihydrate in 100 ml of deionized distilled water. Sterilization and storage are the same as for 0.6 M KCl.
5. Polyethylene glycol (PEG) solution: Dissolve 4.47 g of KCl and 0.74 g of $CaCl_2$ dihydrate in 20 ml of deionized distilled water in a clean, rinsed 100 ml graduated cylinder. Add 0.804 ml of 1.0 M Tris–HCl solution and 0.196 ml of 1.0 M Tris base solution. Mix thoroughly. Add 25 g of PEG approximate molecular weight 3,350 (Sigma). Add deionized distilled water to approximately 90 ml. Seal the top with parafilm and mix by inversion. The PEG will dissolve slowly and the suspension can be mixed by inversion at 10-min intervals until the PEG dissolves. Gentle warming speeds the dissolving of the PEG. After the PEG goes into solution, add deionized distilled water to 100 ml. Seal the top with parafilm and mix thoroughly by inversion. Aliquot the solution into clean, rinsed bottles or vials (~20 ml with large tops to allow easy access to the solution) and autoclave, making sure that the volume does not change. After the vials have cooled, seal their tops with Parafilm M and store at room temperature. Do not store at 4°C or below as the PEG will precipitate.
6. 1.2 M Sucrose solution: Dissolve 41.1 g of sucrose in 50 ml of deionized distilled water. When dissolved make up to a final volume of 100 ml. Aliquot into conveniently sized bottles. Sterilize by autoclaving making sure that there is no volume change. Store in containers tightly sealed with Parafilm M. Storage can be at room temperature or 4°C, but it will be used at 4°C.
7. Protoplasting enzyme: VinoTaste Pro (Novo Nordisk). In the United States VinoTaste Pro is available from Gusmer Enterprises (www.gusmerenterprises.com) (see Note 4).
8. Miracloth (Calbiochem): We normally cut miracloth into 4 in. squares, place them into a glass Petri dish, and autoclave.
9. Strains: Correct gene targeting is greatly facilitated by deletion of the *nkuA* gene (5). *nkuA* deletion strains are available from the Fungal Genetics Stock Center (http://www.fgsc.net/).
10. Millex-GV 0.22 μm pore size PVDF (Durapore) filter units (Millipore) (33 mm syringe-driven disposable) or Stericup-GV or Stericup-GP 0.22 μm pore size vacuum-driven disposable filter units (150 ml) (Millipore). All these units have low protein binding filter membranes (see Note 5).

11. YG medium (made with distilled H_2O): 5 g/l yeast extract, 20 g/l D-glucose, 400 μl/l of trace element solution (23).
12. Uridine stock solution (100×): 24.42 g uridine dissolved in 100 ml final volume of deionized distilled water. Filter sterilize with a 0.22 μm pore size filter unit. Can be stored at room temperature.
13. Riboflavin stock solution (100×): 25 mg/100 ml of deionized distilled water. Filter sterilize with a 0.22 μm pore size Stericup-GV or a Stericup-GP filter unit. Can be stored at room temperature, but riboflavin is light sensitive and must be stored in the dark.
14. Pyridoxine stock solution (1,000×): Dissolve 50 mg of pyridoxine in 100 ml of deionized distilled water. This solution may be autoclaved and stored at room temperature.
15. Gyratory water bath shaker (e.g., New Brunswick Innova 3100 or C76).
16. Sterile transfer pipets (1 ml volume).
17. Variable speed refrigerated centrifuge with a swinging bucket rotor capable of centrifuging 15 and 50 ml disposable centrifuge tubes.
18. Variable speed microcentrifuge (preferably with swing-out head).
19. 50 ml sterile disposable centrifuge tubes or equivalent sterilized, reusable, capped centrifuge tubes.
20. 15 ml sterile disposable centrifuge tubes.
21. Phase contrast microscope.
22. Vortex Genie vortex mixer.
23. Selection plates for transformation: Selection plates allow for the selection of transformants. To prevent protoplast lysis, they contain 0.6 M KCl. To select for complementation of *pyrG*89, we generally use YAG (5 g/l yeast extract, 20 g/l D-glucose, 400 μl/l of trace element solution (23), 15 g/l agar) plus 0.6 M KCl although minimal medium with appropriate supplements can also be used. For other selections we use minimal medium (below) with appropriate supplements plus 0.6 M KCl (see Note 6).
24. Minimal medium (24).

Add 10 g D-glucose, 2 ml of a 26% w/v solution of $MgSO_4$ $7H_2O$, 15 g Agar, and 400 μl of trace metal solution (23) to 900 ml distilled water. Autoclave. Cool to 60°C. Add 100 ml of separately autoclaved 10× stock salts. Mix thoroughly before pouring plates. 10× stock salts: 60 g/l $NaNO_3$, 5.2 g/l KCl, 15.2 g/l KH_2PO_4, adjust pH to 6.0–6.5 with saturated KOH solution.

3. Methods

3.1. Design PCR Primers (See Note 7)

Primers P1, P2, P5, and P6 (Figs. 1 and 2) are designed based on the genomic sequences available in the databases. We have used three different databases, AspGD (http://www.aspgd.org/), CADRE (http://www.cadre-genomes.org.uk/index.html), and the Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). The genome sequences are available on each database, but there are differences in the way they are presented and retrieved that individual researchers may prefer (see Note 8).

1. To design primers to replace a gene (Fig. 1) first download, from a database, a sequence extending from 1,100 bp 5′ to (upstream of) the start codon of the target gene to 1,100 bp 3′ to (downstream of) the stop codon of the gene.
2. Choose approximate positions of primers in the downloaded sequence. Primer P1 is from a region between 1,000 and 1,100 bp upstream of the start codon. P2 is a sequence approximately 1,000 bp upstream of the start codon. A portion of P3 corresponds to a sequence near to, and slightly upstream of, the start codon and the remainder corresponds to the cassette that will be fused to the flanking fragment. A portion of P4 corresponds to a sequence near to but slightly downstream of the stop codon and another portion corresponds to the cassette. P5 corresponds to a sequence approximately 1,000 bp downstream of the stop codon and P6 corresponds to a sequence between 1,000 and 1,100 bp downstream of the stop codon.
3. Design primers with appropriate melting temperatures. Use the following formula to calculate melting temperature (T_m): $T_m = 4 \times$ (total number of C's and G's) $+ 2 \times$ (total number of A's + T's) (see Note 9). For primers P1, P2, P5, and P6 use a melting temperature of approximately 60°C. If the G + C content is 50% the primer would be a 20mer. Primers 3 and 4 each have two regions. One region anneals to genomic DNA and this portion of the primer should be designed to have a melting temperature of approximately 60°C. The other region of primers 3 and 4 corresponds to a cassette (either a selectable marker or a selectable marker plus the promoter that will be introduced). For the gene replacement shown in Fig. 1, the black region of P3 corresponds to one end of the cassette containing *AfpyrG* and the black region of P4 corresponds to the other end of *AfpyrG*. These primer regions should also have a melting temperature of approximately 60°C. Each region of P3 and P4 should, thus, have a melting temperature of 60°C,

but the melting temperatures of the entire primers will, of course, be much higher (see Note 10).

4. For a promoter replacement (Fig. 2), download a sequence extending from 1,200 bp upstream of the start codon of the target gene to 1,100 bp downstream of the start codon. Precise choice of primer position depends on how much of the native promoter one wishes to remove. Since the procedure moves any remnant of the native promoter far away from the coding sequence of the target gene, the amount of the promoter that will be removed is generally not important. Be careful, however, not to remove control sequences for adjacent genes. In our example, we will assume that approximately 50 bp of the native promoter will be removed. A portion of P3 will thus correspond to a sequence approximately 50 bp upstream of the start codon and the remainder will correspond to the end of the selectable marker in the cassette. P1 will be from a region between 1,050 and 1,150 bp upstream of the start codon and P2 will correspond to a sequence approximately 1,050 bp upstream of the start codon. A portion of P4 will correspond to the end of the promoter in the cassette and the other portion will correspond to a portion of the coding sequence of the target gene beginning with the start codon. P5 will be approximately 1,000 bp downstream of the start codon and P6 will be 1,000–1,100 downstream of the start codon.
5. Design primers to have the same T_m as for gene replacements. For promoter replacements the sequence of primer 4 is constrained by the fact that the start codon has to be included. This means that in some cases T_m has to be 58°C or 62°C.

3.2. Amplify Flanking Sequences

This amplification uses *A. nidulans* genomic DNA. We use AccuPrime *Pfx* DNA polymerase (Invitrogen) (see Note 11). For a given target gene, primers P1 and P3 are used to amplify one flanking fragment and P4 and P6 are used to amplify the other fragment. Flanking fragments for several genes can be amplified at the same time in the same thermocycler.

1. Set up a 50 μl reaction in a thin-walled 200 μl PCR tube according to the enzyme manufacturer's instructions with 100 ng *A. nidulans* genomic DNA, 300 nM final concentration of each primer, and 1.25 Units of AccuPrime *Pfx*.
2. Use the following PCR amplification profile (thermocycler lid heated to 105°C).
   (a) 94°C for 2 min to activate the enzyme.
   (b) 30 cycles with the following repeating steps:
   - 94°C for 20 s.
   - Cool to 70°C at the maximum ramp rate allowed by the thermocycler. Remain at 70°C for 1 s.

- Ramp down from 70°C to the annealing temperature at a rate of 0.1°C/s (see Note 12). The annealing temperature is 5°C below the T_m for the primers. With respect to primers P3 and P4 use the T_m that corresponds to the region of annealing of the primer to the genomic DNA. If the T_m for the two primers is different, use an annealing temperature that is 5°C below the lower T_m.
- Anneal for 30 s.
- Ramp from the annealing temperature to an extension temperature of 68°C at a rate of 0.2°C/s.
- Extend at 68°C for 1 min/kb of anticipated product.
- Ramp from 68 to 94°C at maximum rate.

(c) Final extension at 68°C for 4 min.

3. Purify PCR products. It is necessary to remove excess primers, etc. before using the amplified molecules for fusion PCR. Residual primers can cause extra bands during the fusion PCR amplification. We use a QIAquick PCR purification kit (Qiagen) and follow the manufacturer's instructions except that we elute with TE buffer instead of the buffer EB supplied in the kit. Examine fragments by agarose gel electrophoresis to evaluate purity and concentrations of fragments. Note, however, that successful fusion PCR can be carried out with DNA preparations that have some spurious amplified bands.

3.3. Create Transforming Fragments by Fusion PCR

1. Choose an appropriate thermostable DNA polymerase. We have used three DNA polymerases successfully for fusion PCR: AccuPrime *Taq* High Fidelity, KOD Hot Start DNA Polymerase, and Phusion Hot Start II High Fidelity DNA polymerase (see Note 13). We have published a detailed protocol for fusion PCR with AccuPrime *Taq* High Fidelity DNA polymerase (18) and will not repeat it here. Phusion and KOD are unusually heat-resistant, rapid, and processive enzymes. This allows relatively short extension times even when one is amplifying long fragments. In our hands, KOD and Phusion give essentially identical results, and we use similar PCR profiles for them.
2. Set up 50 µl reactions in 200 µl thin-walled PCR tubes. The reactions are set up according to the manufacturers' instructions. We use 0.5 µl of each DNA fragment (both flanks and the cassette). Use nested primers (P2 and P5 in Figs. 1 and 2). The use of nested primers greatly reduces the amplification of extraneous bands.
3. Use the following fusion PCR amplification profile (thermocycler lid heated to 105°C). The profile is for KOD. Values in parentheses are for Phusion.

(a) 94°C for 2 min to activate the enzyme (98°C for 30 s).

(b) 25 cycles repeating the following steps:

- 94°C for 10 s (98°C for 10 s).
- Ramp down to 75°C at the maximum ramp rate allowed by the thermocycler. Remain at 75°C for 1 s.
- Ramp down from 75°C to the annealing temperature at 0.1°C/s. The annealing temperature is the lowest primer $T_m + 2$°C ($T_m + 3$°C).
- Anneal for 10 s.
- Ramp up to an extension temperature of 68°C (72°C) at 0.2°C/s.
- Extend for 30 s/kb of anticipated final product.
- Ramp up at maximum ramp rate to 94°C (98°C).

(c) Final extension at 68°C for 4 min (72°C for 5 min).

4. Purify PCR products as discussed in Subheading 3.2, step 3. Evaluate fusion PCR products by agarose gel electrophoresis. Note: Accurate gene targeting can be achieved even if the fusion PCR product has multiple bands (5). Products can be stored frozen.

3.4. Prepare Protoplasts for Transformation

One of the advantages of using fusion PCR to generate transforming fragments is that many can be generated in parallel using a single thermocycler. If several fragments are to be transformed into the same strain, it is convenient to make enough protoplasts to allow parallel transformations with each fragment. The procedure below easily allows ten parallel transformations.

1. Inoculate 20 ml of nonselective media in a 50 ml Erlenmeyer flask with 1×10^8 conidia. We normally use YG medium. For strains carrying the *pyrG*89 allele, we supplement YG with 10 mM uridine and 9 mM uracil (final concentrations) (see Note 14).
2. Incubate for 16 h at 30°C on a gyratory shaker shaking at 120 rpm. This can be an air shaker or a water bath shaker (e.g., New Brunswick Innova 3100 or C76).
3. Cool variable speed centrifuge to 4°C. Make sure that 1.2 M sucrose is at 0–4°C.
4. Prepare 2× protoplasting solution. Approximately 60 min before hyphae are to be harvested, prepare protoplasting solution. Dissolve 2 g of VinoTaste Pro in 10 ml of 1.2 M KCl/citrate solution in a clean centrifuge tube that holds at least 15 ml. VinoTaste Pro often contains lipid. To remove it centrifuge the solution at 5,000 × *g* for 15 min at room temperature and remove the lipid layer from the top of the solution. The solid enzyme can be stored for long periods at 4°C prior to

preparation of the protoplasting solution, but after long storage a higher concentration of enzyme may be required.

5. Sterilize the solution by filtering it through a sterile, disposable, syringe-driven 0.22 μm low protein binding filter unit (Millex GV PVDF filter material). It is useful to run a volume of 1.2 M KCl/citrate solution through the filter to wash away any contaminants that might be associated with the filter before filtering the protoplasting solution. To filter 10 ml of protoplasting solution, it may be necessary to use more than one filter unit. An alternative (particularly for larger volumes of protoplasting solution) is to use a 150 ml sterile, vacuum-driven unit (Stericup-GV or Stericup-GP).
6. Harvest the hyphae by filtering the culture through sterilized Miracloth. Wash with at least 3× the culture volume of sterile deionized distilled water or growth medium. This helps to remove any residual detergent. Use a flamed spatula to press residual liquid from the hyphal mass.
7. Resuspend the hyphal mass in 8 ml of nonselective medium (YG with any necessary supplements). Add 8 ml of sterile 2× protoplasting solution. Mix thoroughly.
8. Incubate at 30°C shaking at 100 rpm on a gyratory water bath shaker.
9. Mix thoroughly at 15-min intervals to break up clumps. It is important to break up hyphal clumps to maximize protoplast production. We do this by squirting the protoplasting solution and hyphae in and out of a 1 ml transfer pipet at 15-min intervals. We squirt once for each ml of protoplasting solution. Vigorous squirting is unlikely to damage protoplasts, but do not squirt so vigorously as to create a foam because this will result in denaturing the protoplasting enzyme.
10. Monitor protoplast formation by phase contrast microscopy (Fig. 3). Monitoring protoplast formation is important in that it enables one to determine when sufficient protoplasting has occurred and in rare instances in which there are problems with protoplasting, one can determine the nature of the problem (e.g., hyphae are not digested or protoplasts lyse immediately when they form). Protoplasting should be optimal after 1–1.5 h. Longer protoplasting will usually not result in more protoplasts and regrowth of cell wall may occur.
11. Place flask with protoplasts on ice for 5 min with gentle agitation to cool the suspension.
12. Pipet 15 ml of 1.2 M sucrose into a sterile 50 ml centrifuge tube.
13. Place a centrifuge tube containing 1.2 M sucrose in an ice bucket and use a sterile 10 ml pipet to layer the protoplast suspension onto the sucrose cushion (Fig. 4).

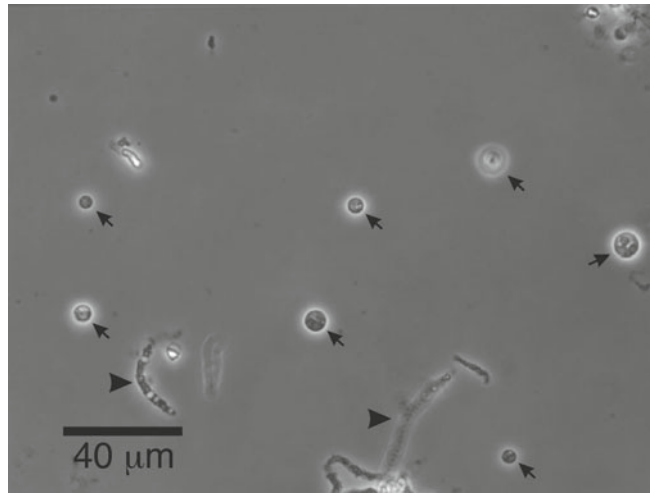

Fig. 3. Phase contrast image of protoplasts. The image was taken slightly more than 1 h after protoplasting was begun. Protoplasts are indicated by *arrows* and are of various sizes as is typical. Remnants of digested hyphae are present, some of which are indicated with *arrowheads*.

Fig. 4. Layering of a protoplast suspension onto a 1.2 M sucrose cushion. *Left*: The protoplast suspension is allowed to flow slowly from the pipet onto the sucrose cushion in a centrifuge tube in an ice bucket. The suspension, cushion, and interface (*arrow*) are visible at the *right*.

14. Centrifuge at 2,800 × *g* (RMax) at 4°C for 10 min. Accelerate and decelerate gently to prevent disturbing the interface between the sucrose and the protoplast suspension. After centrifugation set the temperature of the centrifuge to 25°C.
15. Harvest the protoplasts. During centrifugation most of the hyphal debris will sediment to the bottom of the sucrose cushion. The protoplasts will form a layer at the top of the sucrose

cushion. The thickness of the layer will depend on how much the interface is disturbed during handling. The protoplasts can be harvested by gently removing the protoplast solution down to the protoplast layer and then removing the protoplast layer. Alternatively, a pipet can be extended through the protoplasting solution down to the bottom of the protoplast layer and the layer gently pulled into the pipet by suction with a pipet bulb.

16. Transfer protoplasts to a 15 ml or a 50 ml sterile centrifuge tube. Add a volume of 0.6 M room-temperature KCl that is equal to or greater than the volume of the protoplasts.
17. Centrifuge for 10 min at 2,300 × *g* (RMax). The centrifuge was set to 25°C (step 14 above) and gradually warms during the centrifugation. During this centrifugation, shake the PEG solution (the PEG stratifies during storage and some may come out of solution) and filter it using a 33 mm Millex-GV disposable filter (syringe mounted, 0.22 μm pore size). Filtered PEG solution should not be used for subsequent transformations without re-filtering.
18. Decant supernatant. Either remove the supernatant gently by pipetting taking care not to disturb the pellet or pour off gently making sure not to dislodge the pellet.
19. Resuspend the pellet in 2 ml of 0.6 M KCl. Transfer to 2 × 1.5 ml sterile microcentrifuge tubes (1 ml in each tube).
20. Wash three times at room temperature with 0.6 M KCl. Using a variable speed microcentrifuge, preferably with a swing-out head, centrifuge at 2,400 × *g* for 3 min. After centrifugation remove the supernatant carefully with a 1,000 μl micropipettor. Add 1.0 ml of fresh, sterile 0.6 M KCl with a micropipettor. Resuspend each pellet by squirting it in and out of the 1,000 μl pipet tip six to ten times (see Note 15).
21. After the third centrifugation remove the supernatant and resuspend each pellet in 500 μl of 0.6 M KCl, 50 mM $CaCl_2$ solution. Combine the two suspensions into one tube. Centrifuge at 2,400 × *g* for 3 min. Remove the supernatant and resuspend the pellet in a volume appropriate for the number of transformations to be carried out. We normally use 40 μl of protoplasts per transformation. Thus for ten transformations resuspension of the pellet in 450 μl would be appropriate. This gives enough extra volume to compensate for losses during pipetting into individual tubes.
22. Transfer 40 μl of protoplast solution into individual 1.5 ml sterile microcentrifuge tubes (one tube per transformation).

3.5. Transform

1. To each tube add DNA in no more than 6 μl of TE buffer. Mix thoroughly by vortexing (6–7 × 1-s bursts with a Vortex Genie). The protoplasts are relatively resistant to physical shearing and

it is important to mix the transforming DNA thoroughly with the protoplasts.

2. Add 20 μl of PEG solution (at room temperature). Mix by vortexing as in step 1.
3. Place tubes in an ice-water bath for 25 min.
4. Add 400 μl of room-temperature PEG solution. Mix by squirting in and out of a micropipet tip approximately ten times. It is important to mix thoroughly.
5. Incubate at room temperature for 30 min.
6. Spread transformation mixture onto selection plates (100 μl/plate).
7. Incubate. Transformation rates are higher if one incubates at 30°C overnight before shifting plates to 37°C. However, if plates are initially incubated at 37°C, more than adequate number of transformants are usually obtained and the colonies grow more quickly.

3.6. Determine if Transformants Have Correct Gene Replacements

This can be done with Southern hybridizations using standard methods or by diagnostic PCR. A diagnostic PCR procedure is as follows.

1. After streaking transformants into single colonies, prepare DNA by the method of Lee and Taylor (25). This procedure is somewhat lengthy because it requires lyophilization of hyphae, but it is reliable.
2. Choose appropriate primers to verify correct gene targeting (Fig. 5). In many cases, primers P1 and P6 can be used. Because nested primers (P2 and P5) are used for fusion PCR amplification, P1 and P6 are not in the transforming fragment but they are in the genome just outside of the targeted region. The size of the band amplified by P1 and P6 can be easily predicted for the untransformed gene and for the correctly targeted gene. Correct targeting can be further verified by designing a primer in the DNA flanking the target gene, but outside of the transforming sequence, and a second primer within the selectable marker of the transforming sequence (PA and PB in Fig. 5c). This primer pair will amplify a band of a predictable size in correct transformants but will not give specific amplification in wild-type controls. A similar amplification can be carried out with another primer in the selectable marker and a primer outside of the transforming sequence in the coding sequence or 3′UTR (Fig. 5d). Use the primer design principles stated in Subheading 3.1.
3. Diagnostic PCR: The fidelity of the thermostable polymerase is not important for diagnostic PCR. This allows a variety of enzymes to be used. In general, the manufacturer's instructions should be followed. We normally use the PCR profile in Subheading 3.2, step 2 above.

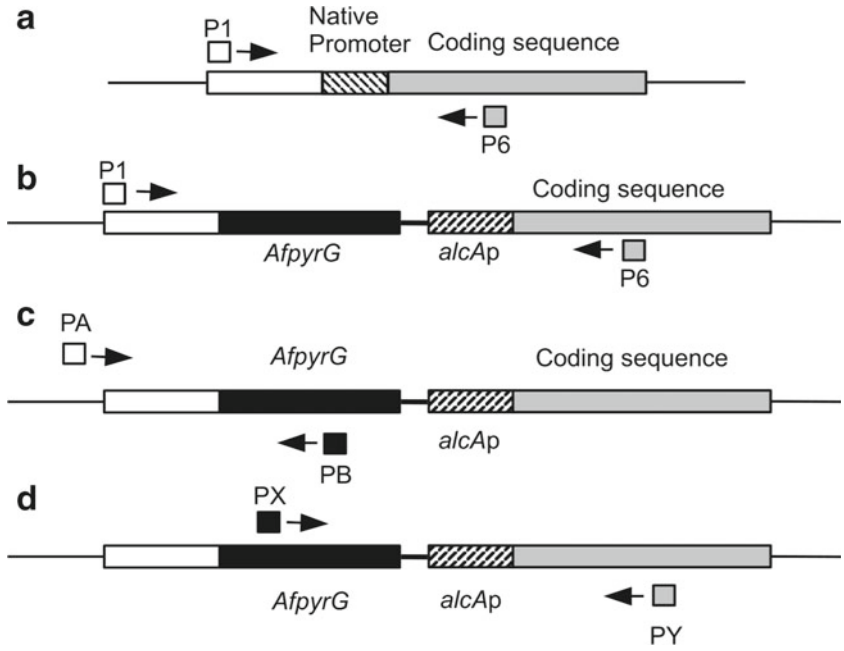

Fig. 5. Diagnostic PCR for a promoter replacement. Similar strategies (not shown) can be used for gene replacements. In **a** and **b**, P1 and P6 are the same as the P1 and P6 primers in Figs. 1 and 2. (**a**) shows the wild-type locus and (**b**) shows a correct promoter replacement. If a correct promoter replacement has occurred the band amplified by P1 and P6 will be longer than in the wild type and of a size that can be calculated precisely from sequence information of the locus and inserted sequences (*AfpyrG* and *alcA*p). (**c**) For correct promoter replacements, a primer that binds within the *AfpyrG* gene (PB) and a primer outside of the locus (PA) will amplify a band, the size of which can be calculated precisely. If the locus is untransformed, no amplification will be obtained from these primers. The same logic applies for (**d**), but in the opposite direction.

4. Notes

1. Thermocycler: In our experience the brand of the thermocycler is not important. It is, however, important that ramp rates be programmable. This allows the same cycling conditions to be used on different machines and this allows consistent results to be obtained.
2. *A. nidulans* protoplasts are *extremely* sensitive to detergents. All glassware must be clean and thoroughly rinsed to remove all traces of detergent.
3. We generally seal the containers with Parafilm M to eliminate the possibility of evaporation which would change the molarity of the solution. It is often convenient to aliquot the solution into clean, rinsed small bottles or vials before autoclaving. One or two vials can, thus, be used for each experiment. If larger containers are used for multiple experiments, the chances of contamination increase.

4. In the past we have used Vinoflow FCE (Novo Nordisk), an enzyme used in the wine industry (5, 18). This enzyme is no longer commercially available, but we have found that VinoTaste Pro (Novo Nordisk) works as well (although we generally need to use a higher concentration of VinoTaste Pro than Vinoflow FCE).
5. Low protein binding filters are important for filtering the protoplast solution to prevent removal of the protoplasting enzyme.
6. Other osmotic balancers may be used instead (e.g., 1.0 M sucrose).
7. We use primers of standard purity. We do not find it necessary to use HPLC or gel-purified primers. A 10 nM scale synthesis provides a more than adequate amount of primers.
8. We generally design our flanking DNA fragments to be approximately 1,000 bp in length. These are the fragments that flank the cassettes and are essentially amplified regions of genomic DNA except for short tails that allow them to be fused to cassettes. A length of 1,000 bp allows efficient transformation and accurate gene targeting (5), but it is short enough to allow linear transforming DNA molecules to be constructed easily by fusion PCR (i.e., the final product will not be too long to amplify efficiently).
9. A number of formulas are used to calculate T_m. In the absence of information about ionic conditions, calculation of precise T_m is impossible, but the formula given works reliably for our purposes.
10. With primers of the T_m we specify, one does not need to worry about secondary structure at the annealing temperature and concerns about repeats are minimal (i.e., they will be short enough to minimize spurious annealing at the temperatures used for PCR). It is important not to design primers that are too long. Their melting temperatures may be high enough to allow incorrect annealing and this may result in extra amplified bands.
11. The AccuPrime buffer system greatly reduces nonspecific priming and *Pfx* is a high-fidelity polymerase which minimizes the possibility of the introduction of mutations during PCR amplification.
12. Ramp rates are important. The specified ramp rates allow correct primers to anneal and extend before incorrect primers. The use of maximum ramp rates for all steps can result in amplification of spurious bands.
13. AccuPrime *Taq* High Fidelity is actually a mixture of two thermostable DNA polymerases, *Taq* DNA polymerase as well as

an enzyme from *Pyrococcus* sp. that has 3′ to 5′ exonuclease activity that allows proofreading. The advantage of AccuPrime *Taq* High Fidelity DNA polymerase over the other two enzymes is that the AccuPrime buffer system minimizes amplification of bands in addition to the desired fusion PCR product. AccuPrime *Taq* High Fidelity DNA polymerase has two disadvantages relative to the other polymerases. First, its fidelity is considerably lower than the other two enzymes. This gives an increased chance for PCR-induced mutations. Second, the AccuPrime *Taq* High Fidelity DNA polymerases are less thermostable and have lower rates of polymerization than KOD or Phusion. Extension times have to be longer with AccuPrime *Taq* High Fidelity DNA polymerases than the other two enzymes, and they have to be extended even more in later rounds of polymerization because of the denaturing of the enzymes.

14. Uridine is maintained as a stock solution and is added after autoclaving. Uracil is added directly to the medium before autoclaving. For strains carrying *riboB*2 we supplement with riboflavin (added after autoclaving from a sterile stock solution) to a final concentration of 7 μM. Pyridoxine is added to a final concentration of 2.43 μM and can be added before or after autoclaving. The conidia should be free of detergent if possible. If they are harvested in a tween + saline solution, as is often the case, the conidia should be washed in sterile deionized distilled water by centrifugation and resuspension three times before inoculation.
15. During protoplast washing steps centrifugation of protoplasts at higher g forces or longer times than specified can lead to protoplast lysis.

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