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Targeted gene deletion in *Aspergillus fumigatus* using microbial machinery and a recyclable marker

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ABSTRACT

The emerging invasive fungal pathogen *Aspergillus fumigatus* causes very serious infections among immunocompromised patient populations. While the genome of this pathogen has been sequenced, a major barrier to better understanding the complex biology of this eukaryotic organism is a lack of tools for efficient genetic manipulation. To improve upon this, we have generated a new gene deletion system for *A. fumigatus* using yeast recombinational cloning and *Agrobacterium tumefaciens* mediated transformation (ATMT) employing a recyclable marker system. This system reduced the time for generating a gene deletion strain in our hands by two-thirds (12 weeks to 3 weeks) using minimal human labor, and we demonstrate that it can be used to efficiently generate multiple gene deletions within a single strain.

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1. Introduction

Aspergillus fumigatus is an opportunistic mould pathogen that is now the most common cause of invasive fungal infection among bone marrow/stem cell transplant patients (Kontoyiannis et al., 2010) and remains a significant pathogen in other immunocompromised patient populations (Pfaller and Diekema, 2010). Infections caused by this environmental mould are associated with mortality rates above 50% in some patient populations (Baddley et al., 2010). A large contributing factor to this high mortality rate is our inability to either diagnose these infections early or to treat advanced disease with currently available antifungals. Unfortunately, there are no new antifungal classes in the pipeline with activity against these moulds. This highlights the necessity for the development of new tools to help better understand the complex biology of these understudied emerging pathogens.

Genetic manipulation of members of the Aspergillus genus has been possible for some time (Tilburn et al., 1983), but the process of deleting genes in this haploid organism has classically been labor intensive, slow and wrought with complications. Recent advances have made the process easier and more amenable to single or multiple gene deletions. We have combined elements of these advances to produce a gene deletion system that is faster, more efficient and amenable for deletion of large groups of genes, either individually or in multiples.

Sugui et al. previously described the application of *Agrobacterium tumefaciens* Mediated Transformation (ATMT) of *A. fumigatus* (Sugui et al., 2005). *A. tumefaciens* is a gram negative bacillus that has been used to genetically manipulate plants for decades, but its ability to also transform fungi is now being realized (Michielse et al., 2005; Sugui et al., 2005). The process involves generating a strain of *A. tumefaciens* harboring a specialized ATMT plasmid containing a deletion construct for the specific *A. fumigatus* gene of interest, followed by co-cultivation of that strain with conidia derived from *A. fumigatus*. This process is more efficient and less laborious than classical gene deletion methods that require the formation of *A. fumigatus* protoplasts and electroporation of DNA into the protoplasts (Sugui et al., 2005). However, while ATMT is an upgrade, the bottle-neck in the ATMT process in our hands was the construction of the plasmid using traditional sub-cloning methods. To circumvent this bottle-neck, we turned to yeast recombinational cloning whereby *Saccharomyces cerevisiae* is chemically transformed with the DNA pieces of the construct that contain overlapping sequences. The yeast cell is able to recognize the specific overlaps and recombine the pieces to build the plasmid (Gietz and Woods, 2002) that can then subsequently be harvested and used for ATMT. Additionally, due to the paucity of good positive selection markers for *A. fumigatus*, the ability to recycle the selection marker after gene deletion is necessary to delete multiple genes. Hartmann et al. recently described the use of the β-rec/six system in *A. fumigatus* (Hartmann et al., 2010). This system first involves the addition of the recognition sites on both sides of the marker within the deletion construct, followed by simple passage of the *A. fumigatus* deletion strain on xylose containing media. The xylose stimulates the

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Table 1
Fungal strains used in this study.

Strain Name	Description
Af293	*Aspergillus fumigatus* reference strain 293
agtaΔ	*A. fumigatus* agtaΔ::hph3 in Af293
amy1Δ	*A. fumigatus* amy1Δ::hph3 in Af293
amy1Δ (hph3-)	*A. fumigatus* amy1Δ with hph3 recycled
agtaΔ amy1Δ	*A. fumigatus* agtaΔamy1Δ::hph3
S.c.6210	*Saccharomyces cerevisiae strain 6210*

Table 3
Primers used in this study.

Primer Name	Primer Sequence
AGTA OL1	CCCGGGAAAGAGTCAAGCCAATGCTG
AGTA OL2	GGGCCCTCAGAAACGAATGATCCGGTT
AGTA OL3	TCTAGAAGTCCTCGTGTACTGTTCGGAACATTGATCTAAA
AGTA OL4	GTCACTGTACAGAGCTATCACTGTGCATACCCTATCTATT
AGTA OL5	TTTAGATCAATGTTCCGAACAGTACACGAGGACTTCTAGA
AGTA OL6	AATAGATAGGGTATGCACAGTGATAGCTCTGTACAGTGAC
AGTA 7	AATCAGCAAGAATCATCAGG
AGTA 8	GAGTCGTGTGCTAATATTCT
AGTA 9	CGTCCAAAGCATTCTAGAGA
AGTA 10	CGAAGAGGATGTTGAACGTA
AGTA 11	AAACACGTCAACACGGGCTT
AGTA 12	AAGTACATTCTTTGCGAGCT
Hyg 5′	GCTCCTCTTCTTTACTCTGA
Hyg 5′-2	AACCATGCATGGTTGCCTAG
Hyg 3′	CACATTCTCCTTCGCTTACT
Hyg 3′-2	TTACTCAGCCCTTCTCTCTG
AMY1-OL1	CCACCGCGGTGGCGGCCGCTCTAGAACTAGTGG ATCCCCCATAATGCAGGTTGCTGCTCA
AMY1-OL3	GTATACTCTATTGACCTATATGGGAGACCTAAAGCAATTG
AMY1-OL2	CAATTGCTTTAGGTCTCCCATATAGGTCAATAGAGTATAC
Brec-OL5	GACAAATGGTGTTCAGGATCAAGAAGGATTACCTCTAAAC
Brec-OL4	GTTTAGAGGTAATCCTTCTTGATCCTGAACACCATTTGTC
AMY1-OL5	ACCCTCTTCTGTTCTCGCATTATTATGCTCAACTTAAATG
AMY1-OL4	CATTTAAGTTGAGCATAATAATGCGAGAACAGAAGAGGGT
AMY1-OL6	CGACGGTATCGATAAGCTTGATATCGAATTCCTGCAG CCCGATATCATGTTGACTATATC
AMY1-7	TGGCTTCACATTCTCCTTCG
AMY1-8	CAAGAGGCAAGATCGACTAC
AMY1-9	GGCAGAAGTGGAGAATCTAG
AMY1-10	ATCTGGCATACTGTTGCTCG
AMY1-11	GGAGATAATGGAACACCGAG
AMY1-12	CGTCGAAATAATCCCGCTGC

2. Materials and methods

2.1. Strains and growth conditions

The ATCC *Aspergillus fumigatus Strain 293* (*Af293*) was used as the wild-type strain for all experiments. *Af293* was maintained on *Aspergillus* Minimal Media (AMM) (http://www.aspergillus.org.uk/indexhome.htm?secure/laboratory_protocols/index.php~main) or Sabourand Dextrose (SabDex) media. All fungal strains used or generated in this study are listed in Table 1. All plasmids are listed in Table 2.

2.2. A. fumigatus genomic DNA isolation

Conidia were inoculated into SabDex broth and grown overnight at 37 °C, 200 RPM. A portion of the resulting hyphae were removed to a microcentrifuge tube. Approximately 50 mg of 0.2 mm silica beads (BioSpec) were added to the tube. The mixture was then ground briefly with a motorized pestle (Fisher). 500 μL of extraction buffer (100 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% β-mercaptoethanol, 1% cetyltrimethylammonium bromide (Sigma), 0.7 M sodium chloride) and 500 μL 25:24:1 phenol:chloroform:isoamyl-alcohol were added to the microcentrifuge tube. After inverting the tube until the mixture became milky, the sample was centrifuged at top speed in a table top microcentrifuge. After removing the aqueous phase to a clean microcentrifuge tube, 500 μL 24:1 chloroform:isoamylalcohol was added. The sample was centrifuged as above. The aqueous phase was moved to a clean microcentrifuge tube and 1.5 volumes of isopropanol were added. The tubes were inverted gently to spool out the DNA; then centrifuged for 10 min in a microfuge at top speed. The supernatant was discarded and the pellet was resuspended in 50 μL sterile ddH_2O. 1 μL of RNAaseA (20 mg/ml) was added and the mixture incubated for 30 min at 37 °C. 150 mL of cold ethanol was added and then the mixture was placed at −20 °C for 30 min. The sample was centrifuged as above, the supernatant removed, DNA dried and resuspended in 50 μL of ddH_2O.

2.3. Molecular cloning for generation of Aspergillus fumigatus agtaΔ strain

For Af*AgtA*, two ~1 kb sequences directly adjacent to both 5′ and 3′ ends of the *AfAgta* locus were amplified from *Af293* genomic DNA using overlapping primer pairs AGTA OL7/AGTA OL3 and AGTA OL4/AGTA OL8 in separate PCR reactions with Phusion ultra high fidelity polymerase (see Table 3 for all primer sequences). The HPH3 hygromycin resistance

Table 2
Plasmids used in this study.

Plasmid Name	Description	Reference
PSK485	β-Rec Cassette containing the upstream β-rec six recognition sequence	Hartmann et al
Topo-ßrec/HygR	Hygromycin cassette with the downstream β-rec six recognition sequence flanking 3′ end of cassette in pCR2.1	This Study
pDHT-SK	*Ann tumifaciens* mediated transformation vector containg VIR ORF's for inserting tDNA	Sugui et al
pDHT-Y	Modified pDHT-SK to include the Cen/Ars yeast origin of replication and URA3 inserted at the SfoI restriction site	This Study
pDHT-Y-agtaΔ	pDHT-Y with the agtaΔ deletion insert cloned in at the SmaI site	This Study
pDHT-Y-amy1Δ	pDHT-Y with the amy1Δ deletion insert cloned in at the SmaI site	This Study