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Tools and techniques

Rapid mapping of insertional mutations to probe cell wall regulation in *Cryptococcus neoformans*

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ABSTRACT

Random insertional mutagenesis screens are important tools in microbial genetics studies. Investigators in fungal systems have used the plant pathogen *Agrobacterium tumefaciens* to create tagged, random mutations for genetic screens in their fungal species of interest through a unique process of trans-kingdom cellular transconjugation. However, identifying the locations of insertion has traditionally required tedious PCR-based methods, limiting the effective throughput of this system. We have developed an efficient genomic sequencing and analysis method (AIM-Seq) to facilitate identification of randomly generated genomic insertions in microorganisms. AIM-Seq combines batch sampling, whole genome sequencing, and a novel bioinformatics pipeline, AIM-HII, to rapidly identify sites of genomic insertion. We have specifically applied this technique to *Agrobacterium*-mediated transconjugation in the human fungal pathogen *Cryptococcus neoformans*. With this approach, we have screened a library of *C. neoformans* cell wall mutants, selecting twenty-seven mutants of interest for analysis by AIM-Seq. We identified thirty-five putative genomic insertions in known and previously unknown regulators of cell wall processes in this pathogenic fungus. We confirmed the relevance of a subset of these by creating independent mutant strains and analyzing resulting cell wall phenotypes. Through our sequence-based analysis of these mutations, we observed "typical" insertions of the *Agrobacterium* transfer DNA as well as atypical insertion events, including large deletions and chromosomal rearrangements. Initially applied to *C. neoformans*, this mutant analysis tool can be applied to a wide range of experimental systems and methods of mutagenesis, facilitating future microbial genetic screens.

1. Introduction

Genetic screens, including those performed by random insertional mutagenesis, have elucidated the functions of many genes in varied species. *Agrobacterium tumefaciens*-mediated transconjugation (AMT) is one method of trans-kingdom DNA delivery that has been increasingly used to generate insertional mutants in a variety of organisms. While *A. tumefaciens* is a plant pathogen in nature, it has been adapted for DNA transformation into a number of species, including several fungi. Fungi that have been studied using AMT include the yeast *Saccharomyces cerevisiae;* the plant pathogens *Fusarium oxysporum, Leptosphaeria maculans, and Magnaporthe oryzae*; and the human pathogenic fungi *Aspergillus fumigatus, Blastomyces dermatitidis, Histoplasma capsulatum,* and *Cryptococcus neoformans* (Betts et al., 2007; Blaise et al., 2007; Bundock et al., 1995; Idnurm et al., 2004; Meng et al., 2007; Mullins et al., 2001; Sugui et al., 2005; Sullivan et al., 2002; Youseff et al., 2009).

The DNA transferred during transconjugation, or T-DNA, is flanked by short border repeats that serve as recognition sequences for processing and transfer by *A. tumefaciens*. When used for AMT,

Abbreviations: AMT, *Agrobacterium*-mediated transconjugation; AIM-HII, *Agrobacterium* Insertional Mutagenesis High-throughput Insert Identification; AIM-Seq, *Agrobacterium* Insertional Mutagenesis Sequencing; AS, acetosyringone; CDS, coding DNA sequence; CFW, calcofluor white; DIC, differential interference contrast; EY, eosin Y; IGV, Integrative Genomics Viewer; IM, induction medium; NAT, nourseothricin; SDS, sodium dodecyl sulfate; T-DNA, transfer DNA; WGA, wheat germ agglutinin.

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the portion of the T-DNA between the border repeats is replaced with species-specific selectable markers to allow selection of transformed isolates (Michielse et al., 2005). In contrast to other common methods such as transposon mutagenesis, AMT insertions have been reported to occur frequently as single genomic integration events with less bias towards integration at particular loci (de Groot et al., 1998; Michielse et al., 2005).

C. neoformans is an opportunistic fungal pathogen that causes over 500,000 deaths per year worldwide primarily in immunocompromised populations (Park et al., 2009). An environmental fungus, *C. neoformans* is inhaled into the lungs, where it establishes a primary infection. In immunocompromised individuals, it can disseminate from this primary site of infection to the central nervous system to cause life-threatening meningitis. *C. neoformans* has emerged as a model organism of fungal pathogenesis, and molecular genetic techniques are well established in this species. Importantly, the genome sequence has recently been published for a clinically derived isolate used widely in many research laboratories (Janbon et al., 2014). AMT has been an important tool for determining the function of a number of genes in *C. neoformans*, including those required for melanin production, sexual development, metal homeostasis, and capsule production (Chun and Madhani, 2010; Feretzaki and Heitman, 2013; Fu et al., 2011; Hu et al., 2013; Idnurm et al., 2004; Lin et al., 2010; Walton et al., 2005).

The process of AMT has been streamlined so that generating and screening large mutant libraries is quite straightforward. However, identifying the sites of AMT insertion continues to be a time- and resource-consuming task. Classical molecular methods, such as inverse PCR, splinkerette PCR, and vectorette PCR often require many rounds of optimization to identify a limited number of AMT insertion sites (Arnold and Hodgson, 1991; Devon et al., 1995; Leoni et al., 2011; Triglia et al., 1988). The effort required to identify AMT-generated mutations creates a significant bottleneck in an otherwise efficient system. Furthermore, current PCR-based methods regularly fail to identify many induced mutations.

Here we report a new method to rapidly identify sites of insertion generated by techniques such as AMT. AIM-Seq (*Agrobacterium*-mediated Insertional Mutagenesis Sequencing) is a sequencing and analysis method that combines batch sampling, whole genome sequencing, and a new bioinformatics pipeline, AIM-HII (*Agrobacterium*-mediated Insertional Mutagenesis High-throughput Insert Identification), to identify the sites of insertion in mutants generated by AMT. This method has several key advantages over established PCR-based identification methods, as well as other insertion sequencing methods such as Tn-Seq or INSeq (Barquist et al., 2013; Goodman et al., 2009; van Opijnen et al., 2009). Previously generated AMT libraries can be used with no additional steps or optimization required. This is in contrast to transposon-insertion sequencing methods that require additional steps to enrich for insert-flanking fragments prior to sequencing (Barquist et al., 2013). Although higher throughput than traditional PCR-based insertion identification methods, these previously described deep sequencing methods still pose technical challenges especially biases introduced by initial PCR-enrichment steps. While applicable in theory to eukaryotes, many of these established techniques were designed with smaller, prokaryotic genomes in mind. Additionally, AIM-Seq is capable of identifying non-canonical insertion events that cannot be assessed by standard PCR-based and insertion sequencing methods. Furthermore, when considering the overall cost, including labor, of performing PCR-based identification methods for each mutant selected, the cost of the DNA extraction and whole genome sequencing for AIM-Seq is less expensive on a per mutant basis.

The *C. neoformans* cell wall plays a key role in immune recognition and avoidance. Given this important cellular function, we sought to characterize mutants with changes in the cell wall that may affect its interaction with the host. We screened a library of random AMT mutants for altered cell wall phenotypes, applying the AIM-Seq method to map and confirm the mutations. In this screen we identified "typical" T-DNA insertions in genes known to be involved in cell wall homeostasis, as well as in genes not previously associated with the fungal cell wall. We also identified atypical AMT-induced events that would be missed by traditional mutation identification methods. We believe that these types of events are responsible for previously reported mutations of interest that could not easily be identified by standard means (Feretzaki and Heitman, 2013; Fu et al., 2011; Hu et al., 2013).

While the proof of concept experiments discussed here were conducted in *C. neoformans* using AMT generated mutants, this method is applicable more generally: it can be used with any insertional mutagenesis method (e.g. transposon-mediated mutagenesis) and in any mutable target organism. The only requirements are that the insert sequence and target genome sequences are known (or can be sequenced). Once mutants are generated and screened, organism-specific adjustments to this analysis tool can be easily made by choosing a sequencing depth appropriate to the genome size and the number of mutations expected.

2. Materials and methods

2.1. Strains, media, and growth conditions

C. neoformans strains used in this study are listed in Table 1. *A. tumefaciens* strain EHA105/NAT was used for AMT (Walton et al., 2005). Unless otherwise noted, all strains were created in the *C. neoformans* H99 *MAT* background (Janbon et al., 2014). Strains were cultured on YPD (yeast extract 1%, peptone 2%, dextrose 2%) agar plates or in YPD liquid media with shaking at 150 rpm at 30 °C, unless otherwise stated (Sherman, 1991). To induce capsule for India ink visualization, cells were incubated in CO_2-independent tissue culture media (Gibco) for 3 days with shaking at 150 rpm at 37 °C. Melanin was assessed at 30 °C and 37 °C on Niger seed (*Guizotia abyssinica*) medium prepared as described previously (Kwon-Chung and Bennett, 1992). Congo red (0.5%) was added to YPD medium prior to autoclaving. Caffeine (1 mg/mL) and calcofluor white (1 mg/mL) were filter sterilized and added to YPD after autoclaving. Quinolinic acid (Sigma Aldrich) was filter sterilized and spread onto yeast nitrogen base (YNB) plates at the indicated concentrations.

For cell staining and imaging, seed cultures were grown overnight in YPD medium with shaking at 150 rpm at 30 °C. Cells were then diluted into YPD or CO_2-independent tissue culture medium and incubated with shaking at 150 rpm at 30 °C or 37 °C for 18 h.

2.2. Molecular biology

All primers used in this study are listed in Table 2. Independent confirmatory mutants were generated by amplifying the insert T-DNA cassette from the original AMT mutant, followed by genomic integration by biolistic transformation into the H99 *MAT* background (Toffaletti et al., 1993). All new deletion strains were confirmed by Southern blot analysis (data not shown), using the nourseothricin (NAT) resistance gene as a probe (McDade and Cox, 2001).