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Gene essentiality in *Aspergillus fumigatus*

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Products of essential genes of *Aspergillus fumigatus* are seen as potential targets for antifungal drugs, and both functional screens and bioinformatics approaches have been used to help identify such genes. The random screening approach makes use of the deletion of one copy of a gene in a diploid, and failure to recover the deletant during haploidization. In order to investigate the function of putative essential genes identified by both screening and bioinformatics approaches, the conditional promoter of the *alcA* gene of *Aspergillus nidulans* has been used. In some cases, the genes identified are not absolutely essential, but their deletion leads to slow growth. Such deletants can be recovered and cultivated for phenotypic characterization following transformation of haploid strains.

Keywords *Aspergillus fumigatus*, essential genes, conditional promoter

Introduction

Completion of the genome sequence of *A. fumigatus* [1] offers new opportunities for understanding the biology of this fungus, particularly by means of functional genomics. The strongest argument for funding the sequencing project was to help provide the means of combating diseases caused by this opportunistic pathogen. In particular, there is a need for both improved diagnostic methods and for new antifungal drugs. For the latter, one common approach has been to identify processes which are essential to survival and/or growth of the organism, and this in turn led to the concept of essential genes. The genomes of filamentous fungi are 2–3 times larger than that of *Saccharomyces cerevisiae*, additional functions including asexual sporulation and secondary metabolism [2]. Filamentous fungi are also able to utilize a much wider range of nutrient sources than *S. cerevisiae* [3], and the greater metabolic capacity also includes a battery of secreted enzymes [4]. Nevertheless, many of these additional functions are conditional rather than essential, and such 'non-essential' functions including most of the secondary metabolism and excreted enzymes. For example, although *Aspergillus* species secrete proteases and can utilize proteins, amino acids and amides as nitrogen sources, these are not required if ammonium or nitrate is available. Essential processes therefore include much of the primary metabolism, and those associated with the cell cycle and mycelial maintenance and growth. Many of these latter functions are also common to *S. cerevisiae*.

Bioinformatics and essential genes

The recent publication of the genome sequence of *Aspergillus fumigatus* Af293 [1] included a table of putative essential genes which was based on deductions from bioinformatics, that is, genes with orthologues whose function is essential in *S. cerevisiae* and *C. elegans*. While this approach provides a useful starting point, genes which are essential in one species may not be essential in another species for a number of reasons, including functional redundancy. In addition, BLAST searching for putative orthologues has its limitations. Some essential genes present in filamentous fungi may not have obvious orthologues in yeast and mammals, either because the particular function and gene is absent in the latter, or because the primary sequence does not show a significant level of conservation between distantly-related species. There also may be an advantage in finding essential genes which are fungal-specific if the aim is to use the function as a drug target.

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Screening for essential genes

Because of the limitations of bioinformatics, methods have been devised to screen for essential genes in *A. fumigatus*. If a gene is absolutely essential for any growth, then its deletion will mean that viable deletants cannot be recovered. However recessive essential genes can be deleted from the diploid strains to yield viable heterozygous strains. Diploids can be obtained for many fungi, and the failure to recover haploid progeny carrying the deletion from the heterozygous diploid provides evidence for its essentiality. This approach to screening was developed independently for *A. fumigatus* by 2 groups [5,6]. Deletions in the diploid can be generated by transformation and random insertion, by transposition into a BAC vector, followed by transformation of *A. fumigatus* by the BAC insert to replace the modified region, or by transposition with a fungal transposon. However, random ectopic insertion by transformation of diploids can lead to large deletions and rearrangements, and is not an ideal approach. Benomyl is then used to haploidize the diploid transformants. The diploid recipient is homozygous for uridine requirement (*pyrG*/*pyrG*), and also carries complementary spore colour mutations present in the haploid parental strains. The resulting mixture of diploid and haploid derivatives is plated on media with and without uridine. Haploids are recognized by spore colour, and green-spored diploids discounted. Haploid colonies prototrophic for uridine result from insertion of the $pyrG^+$ selectable marker present in the transforming plasmid (or transposon) at a non-essential site in the genome, while failure to recover such haploids indicates insertion at an essential site in the genome. Since mitotic recombination can confound the result, comparison is made between media with and without uridine, and a drastic reduction in numbers of haploid colonies in the absence of uridine indicates insertion in an essential gene (Fig. 1).

The site of insertion can be determined by rescue of the vector and flanking regions from the diploid parent in *Escherchia coli*, or by inverse PCR, and comparison of the recovered region with the genome sequence. While insertion within a coding region identifies the essential gene, in some cases the insertion can be in a non-coding region between genes, and gene identification is not so straightforward. Transformation of the diploid parent by the putative essential gene should restore function, detectable by subsequent haploidization.

Bioinformatics can then be used in many cases to deduce a predicted function for the essential gene. However, in some cases where the function of the essential gene is not obvious (e.g., where there are no characterized orthologues, and/or where the predicted gene protein product has no known domains or motifs) functional analysis of the essential gene is required.

Functional analysis of essential genes

Since the screen for essentiality is based on failure to recover haploid deletants, not only essential genes in the strict sense, but also those whose deletion leads to slow growth may also fail to be recovered after haploidization. Several approaches have been used to investigate further the function of the putative essential genes, including promoter exchange [7] and RNAi [8]. We have used a conditional promoter and direct deletion in haploids.

The *alcA* promoter of *Aspergillus nidulans* can be downregulated on high sugar, rich media, not only in *A. nidulans* [9] but also in *A. fumigatus* [7], in order to turn off gene expression. The initial validation of this

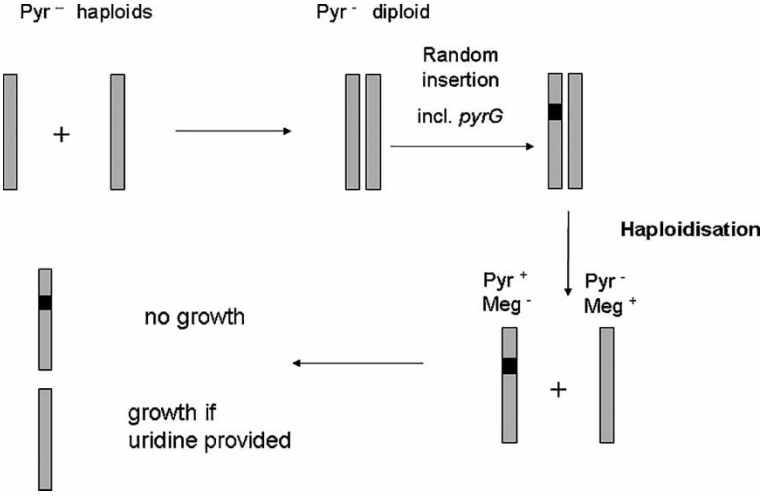

Fig. 1 Deletion of essential genes in diploids. A green spored, diploid strain homozygous for the $pyrG^-$ allele and heterozygous for brown and white spore colour mutations is transformed with a vector carrying the $pyrG^+$ selectable marker, and insertion can occur in an essential gene *meg* to give a heterozygous diploid with the genotype $pyrG^+/meg^-$. Haploidization of the diploid following treatment with benomyl gives rise to easily detectable brown and white spored, haploid colonies which are defective for the essential gene, and cannot grow ($pyrG^+$ meg^-), or require uridine for growth ($pyrG^-$ meg^+).

method made use of the *A. fumigatus* orthologue of a gene known to be essential in *A. nidulans*, *nudC*, which is required for nuclear migration. A further improvement is the use of a promoter exchange cassette, which can be used with fusion PCR in order to avoid time consuming vector construction. [10]. We have recently applied these methods to two genes. One of these, 3A4, was identified in a diploid screen at F2G as an ORF lying 160nt downstream of an insertion, and was therefore a candidate essential gene with a disrupted 5' region (Fig. 2). Inspection of the gene product revealed no Pfam domains or other informative motifs, and putative orthologues could be detected by BLAST searching only in other filamentous species.

Promoter replacement and downregulation resulted in slow growth, suggesting that the gene was not absolutely essential, or that repression of the conditional *alcA* promoter is incomplete. Complete deletion in a haploid recipient also resulted in very slow growing colonies, confirming that the gene was not absolutely essential. A second gene, *pptB*, was identified by bioinformatics as a putative mitochondrial phosphopantotheinyltransferase, required for addition of a cofactor to the mitochondrial acyl carrier protein Acp1 [11]. Acp1 is associated with mitochondrial complex I (NADH dehydrogenase) in some species, and is required for normal respiration and for lipoic acid biosynthesis. Deletion of the gene in yeast leads to a petite mutant phenotype, and its function might be essential in an obligate aerobe such as *A. fumigatus*. Both downregulation and deletion resulted in slow growing colonies.

In both examples, further investigation of the defect in these deletants is in progress. Localization of a gene may also provide further information about its function, and a series of GFP/RFP cassettes was recently developed for tagging [12]. For example, we have recently been investigating the function of a gene *hbrB* in *A. nidulans*. This gene is required for polar growth, and downregulation leads to a severe growth defect [13]. However, as with gene 3A4 in *A. fumigatus*, bioinformatics gives no clues as to its function. Tagging *hbrB* with GFP has revealed its presence in unidentified subcellular compartments within the hyphae [A. Leeder, G. Turner, unpublished results]. Tagging of appropriate known genes can provide markers for subcellular compartments, and use of GFP and RFP tags could then facilitate identification of the compartments.

Further improvements in functional analysis

In addition to the use of diploids for deletion of recessive essential genes, use has previously been made of the heterokaryons which arise from transformation. Protoplasts are usually multinucleate, such that deletion of an essential gene by transformation of a *pyrG* mutant can lead to a balanced heterokaryon which can grow on minimal medium lacking uridine. Nuclei deleted for an essential gene but carrying a functional *pyrG* gene are maintained in a heterokaryon alongside untransformed nuclei which retain the essential gene but are Pyr^{-}. The resulting heterokaryotic colonies arising from lethal deletions can be then purified by streaking the uninucleate asexual conidia to single colonies, revealing the phenotype of the deletant [7,14]. This approach was made difficult by the relatively high frequency of ectopic integration events found in the aspergilli, complicating interpretation of the Southern blot data carried out on heterokaryons to confirm deletion. However, the recent use of ku70/ku80 deletion strains of *Aspergillus* to eliminate ectopic integration and complex rearrangements during integration [12], which result from non-homologous end joining, has made this approach much more attractive.

A combination of approaches to gene function on genes identified by primary screening in diploids including conditional promoters, RNAi, deletion in haploid ku strains, combined with phenotype analysis, localization of gene product, should aid characterization of potential drug targets and facilitate screens for new drugs.

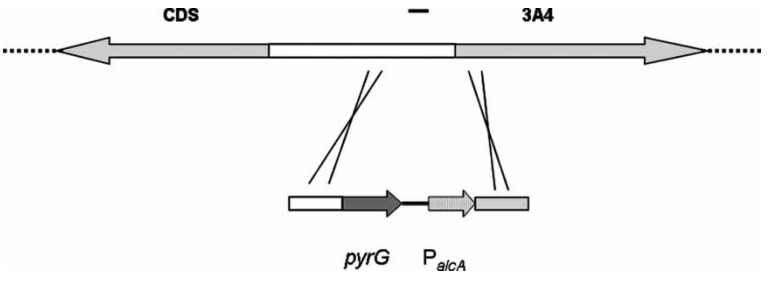

Fig. 2 Promoter exchange using a conditional promoter. Plasmid pGA1a carries the *pyrG* gene and the *alcA* promoter of *Aspergillus nidulans*. Using fusion PCR [10], the *pyrG*-P_{alcA} cassette is joined to approximately 1kb flanking regions, consisting of the 5′ upstream region of the essential gene (left), and the 5′ end of the coding region of the gene (right). *A. fumigatus* is transformed with this linear fragment, and homologous integrants detected by PCR and confirmed by Southern blot. Repression of transcription of the essential gene is achieved on YEPD medium [7].

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