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Review

# *Candida albicans* mutant construction and characterization of selected virulence determinants

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ABSTRACT

*Candida albicans* is a diploid, polymorphic yeast, associated with humans, where it mostly causes no harm. However, under certain conditions it can cause infections ranging from superficial to life threatening. This ability to become pathogenic is often linked to the immune status of the host as well as the expression of certain virulence factors by the yeast. Due to the importance of *C. albicans* as a pathogen, determination of the molecular mechanisms that allow this yeast to cause disease is important. These studies rely on the ability of researchers to create deletion mutants of specific genes in order to study their function. This article provides a critical review of the important techniques used to create deletion mutants in *C. albicans* and highlights how these deletion mutants can be used to determine the role of genes in the expression of virulence factors in vitro.



**Contents**



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## 1. Introduction

*Candida albicans* is a diploid, polymorphic yeast that grows as budding cells which can extend to the mycelial state (i.e. hyphae and/or pseudohyphae) under favorable conditions (Kurtzman, 2011). *C. albicans* is readily isolated from human clinical samples due to its commensal lifestyle (Cooper, 2011), and, unlike non-pathogenic yeasts, is less frequently isolated from the environment outside its host. As a pathogen, it accounts for a large number of fungal infections occurring in the digestive tract, mucocuteneous tissues and skin as well as in the bloodstream. Furthermore, *C. albicans* is responsible for 40% of device-associated infections in the United States of America due to biofilm development on medical devices and their inherent resistance to antimicrobial therapy (Wenzel, 1995; Rueping et al., 2009). Infected individuals are, in most cases, immune deficient and/or immune suppressed due to conditions such as AIDS or critical illness. The ability of *C. albicans* to cause infections is largely attributed to a number of virulence factors including acquired resistance towards antimicrobial drugs such as the azoles and polyenes, and to a broad extent, its polyphenic nature which allows it to navigate the dynamic host environmental conditions such as varying temperatures, pH, stress (e.g. oxidative, nitrosative, osmotic, heavy metal and cell wall stress), carbon dioxide levels and available nutrient sources (e.g. carbon, nitrogen, phosphorus or sulfur sources). In addition, other virulence factors such as attachment to the human mucosa and secretion of lipolytic or proteolytic enzymes further contribute to the ability of *C. albicans* to invade host tissues and cause infections (Calderone and Fonzi, 2001; Nobile et al., 2012).

Gene deletion studies contribute largely to constructed mutant libraries utilized by researchers to study the genetic components underlying virulence of *C. albicans* (Homann et al., 2009; Pérez et al., 2013). Since construction of these mutants, it is now possible to conduct an assessment of specific genetic determinants and their effect on cellular activities employed by *C. albicans* to thrive in the mammalian host. In this review the emphasis will be placed on the common gene deletion methods routinely used to create *C. albicans* mutant strains. Furthermore, in vitro characterization of some of the important genetic determinants in *Candida* biology will also be highlighted.

## 2. Difficulty in genetic analyses of *C. albicans*

Although different phenotypes of *C. albicans* are visible and can be assessed by routine assays, the underlying genetic mechanisms which coordinate varying pathogenic traits of clinical isolates, are difficult to assess. One of the barriers hampering *C. albicans* genetic analysis is the diploid nature of this yeast. To genetically manipulate these strains, two rounds of allelic alterations are required (Jones et al., 2004; Noble et al., 2010). *C. albicans* also lacks natural plasmids required for transformation and its genome does not display the traditional codon usage in which the CUG codon encodes a serine residue. This results in the need for codon optimization for heterologous markers to be functional in transformation experiments (Santos and Tuite, 1995). Furthermore, expression and mutagenic studies have demonstrated that the use of selectable, especially auxotrophic, markers negatively impacts virulence in *C. albicans* (Lay et al., 1998; Brand et al., 2004). In addition, wild type strains are potentially resistant to dominant markers such as Geneticin (G418) and Hygromycin, generally used for selection in other yeasts (Griffiths, 1995; Santos and Tuite, 1995). These difficulties have led to the development of techniques suited to the genetic manipulation of this important opportunistic pathogen.

## 3. Genetic analyses of *C. albicans*: from traditional to current methods

Traditionally, genetic manipulation was achieved randomly via the use of chemical mutagens, transposable elements and UV-enhanced mitotic recombination techniques (Fonzi and Irwin, 1993). Such mutagenic methods often resulted in additional genetic alterations that are difficult to reverse when identified. Therefore, mutants would usually display vague phenotypic outputs which ultimately complicated interpretation of results. Due to this, genetic manipulation efforts, many of them based on principles used in the study of *Saccharomyces cerevisiae*, have been improved over the years.

In this review, deletion techniques have been divided into spontaneous and induced recombination systems as well as PCR techniques, which in turn can comprise spontaneous or induced systems applied in the presence of auxotrophic and/or dominant markers, as summarized in Table 1. Induced and/or spontaneous systems imply that a particular system does/does not incorporate any additional recombinase (or recombinase-like) protein to recycle the deletion cassette for a second round of genetic deletion.

### 3.1. Spontaneous recombination systems

#### 3.1.1. URA blaster

The development of the URA blaster technique was a step forward in *C. albicans* genetic research. This technique was developed from studies involved in the genetic manipulation of *C. albicans* clinical isolates in efforts to make a strain nutritionally competent for growth on medium lacking uracil (Fonzi and Irwin, 1993). URA blaster entails the deletion of the target gene, using a construct that carries a *URA3* gene containing two *hisG* sequences from *Salmonella typhimurium*. After an allele of the target locus is deleted, *C. albicans* isolates, including those carrying the disruption fragment, are plated on uracil-deficient medium for selection. Isolates which lack the deletion fragment cannot synthesize uracil since they also lack *URA3* gene essential for the production of orotidine-5′-monophosphate (OMP) decarboxylase (ODCase). The short distance between the *hisG* sequences allow for spontaneous homologous recombination to occur, leading to subsequent removal of the *URA3* gene. As a result, a copy of the *hisG* sequence is placed at the target locus. Transformants are then passaged onto medium containing 5-fluoroorotic acid (5-FOA) to screen for those with an excised *URA3* gene ($URA^-$). These isolates are resistant to the toxicity of the metabolic product of 5-FOA, 5-fluorouracil. As a result, the URA3 marker is spontaneously recycled for a second round of transformation. After the final transformation step, the *C. albicans* strain will be homozygous for the specific deletion (Fonzi and Irwin, 1993; Berman and Sudbery, 2002).

The URA blaster method has been employed and documented in a number of published papers aimed at determining the underlying genetic aspects of virulence of *C. albicans* (Smith et al., 1992; Huh et al., 2001; Inglis and Johnson, 2002). However, other studies have indicated that the auxotrophic marker, *URA3*, has a negative impact on virulence of auxotrophic strains (Kirsch and Whitney, 1991; Lay et al., 1998). This gene is required for hyphal growth and virulence in *C. albicans* and it was also discovered that *ura3*/*ura3* mutants do not form biofilms on Spider medium (Lay et al., 1998). In addition, the final transformation step in the URA blaster method does not involve selection using medium containing 5-FOA, increasing the possibility of using $URA^+$ strains that have not undergone recombination. This also suggests that a copy of the *URA3* gene is left and could be expressed at the locus of the target gene at insufficient levels. This non-native or ectopic *URA3* expression has been reported to greatly decrease *C. albicans* virulence as it represses biofilm formation, complicating interpretation of phenotypes (Fig. 1) (Lay et al., 1998).

In a study involving the re-evaluation of *C. albicans* mutant strains constructed using the URA blaster method, in comparison with wild type strains, ODcase activities were determined (Lay et al., 1998). This study revealed a double to 18-fold decline in ODcase activity and that this significant decline results in reduced germ tube formation by constructed mutant strains. This is a clear indication that sufficient *URA3* mRNA levels are required, ultimately suggesting that virulence of *C. albicans* strains partly depends on ODcase expression levels. Therefore, previously detected decreased virulence activities measured by



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