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Candida albicans: genetics, dimorphism and pathogenicity

Summary *Candida albicans* is a dimorphic fungus that causes severe opportunistic infections in humans. Recent advances in molecular biology techniques applied to this organism (transformation systems, gene disruption strategies, new reporter systems, regulatable promoters) allow a better knowledge of both the molecular basis of dimorphism and the role of specific genes in *Candida* morphogenesis. These same molecular approaches together with the development of appropriate experimental animal models to analyze the virulence of particular mutants, may help to understand the molecular basis of *Candida* virulence.

Key words *Candida albicans* · Dimorphism · Yeast genetics · Morphogenesis · Yeast pathogenicity

Introduction

Candida albicans is a dimorphic fungus that exists as a commensal of warm-blooded animals including humans. It colonizes mucosal surfaces of the oral and vaginal cavities and the digestive tract and is also able to cause a variety of infections, depending on the nature of the underlying host defect. Therefore, *C. albicans* infections (candidiasis) are very infrequent in healthy individuals. Candidiasis may be divided into superficial (such as oral and vaginal thrush and chronic mucocutaneous candidiasis) and deep-seated (such as *Candida*-due myocarditis and acute disseminated *Candida* septicaemia) and represent a major clinical problem. For several reasons (immunosuppressive treatments, long-term catheterization, use of broad-spectrum antibiotics and longer survival of immunologically compromised individuals), *Candida* infections have increased dramatically over the last two decades.

An interesting feature of *C. albicans* is its ability to grow in two different ways; reproduction by budding, forming an ellipsoid bud, and in hyphal form, which can periodically fragment and give rise to new mycelia, or yeast-like forms (Fig. 1). Transitions between the two phenotypes can be induced in vitro in response to several environmental cues such as pH or temperature, or different compounds such as N-acetylglucosamine or proline. However, perhaps the most critical criterion for pathogenicity is the induction of the mycelial form by serum or macrophages. In addition to the intrinsic biological interest of this dimorphism, its ability to switch between the yeast and the hyphal mode of growth has been implicated in its pathogenicity [22, 60, 63, 101].

In the light of the above, it is obvious that *C. albicans* is a major model of pathogenic yeast. Despite this, advances in the knowledge of the molecular genetics of *C. albicans* have proceeded very slowly if compared with those achieved in conventional yeasts. *C. albicans* lacks a sexual cycle and is a diploid organism, which has made it difficult to manipulate it genetically [78]. Fifteen years ago, it began to become apparent that *C. albicans* was a diploid organism and therefore it was very difficult to obtain mutant strains. However, in the following years many auxotrophic mutants were obtained and parasexual genetic methods to analyze them were developed. Also, this period saw the dawning of basic molecular techniques.

C. albicans molecular biology is mainly based on the molecular knowledge of *Saccharomyces cerevisiae* owing to their similarity and also because many *C. albicans* genes can be expressed in *Saccharomyces*, although not viceversa. Furthermore, *C. albicans* shows many peculiarities that do not have counterparts in *Saccharomyces*, such as its ability to grow in hyphal form [78] and its virulence [22], and this is another reason for the need to develop genetic tools for its study. It is also of great importance to define molecular targets for the development of new antifungal agents [28].

Review papers on the molecular biology of *C. albicans* are available [57, 64, 85, 111] which address the current advances made in the understanding of *C. albicans* molecular genetics. In addition, a very useful WWW Server on *C. albicans* research (http://alces.med.umn.edu/candida.html) has been created by Dr. Scherer (Univ. Minnesota, St. Paul, MN, USA) in which information on genetics, physical mapping, sequence data and other useful resources (methodology, metabolism, morphology, etc.) about this organism are included.

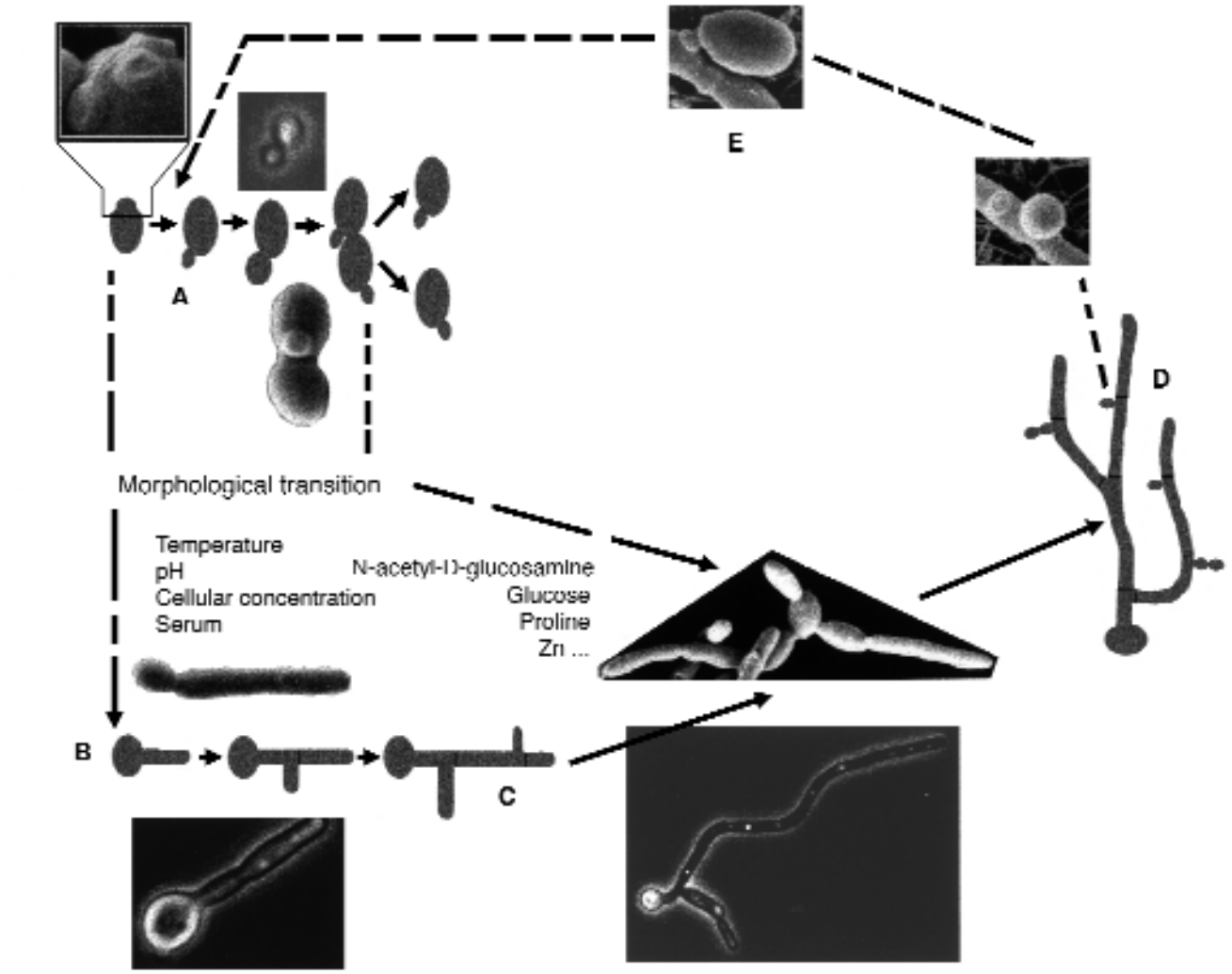

Fig. 1 *Candida albicans* dimorphism. (A) Blastospores are unicellular forms of the fungus that divide by budding. (B) In the presence of some environmental factors, cylindrical outgrowth is initiated on the surface of a blastospore forming a germ tube. (C) Germ tubes grow and septa are laid down behind the extending apical tip to form a hypha. (D) Hyphal branches and/or secondary branches are produced just behind newly laid-down septa, constituting a micelium. (E) Secondary blastospores become separated from the filament. The main factors that favour filamentation (yeast → hypha) are: temperature 35°C, pH 7.0, an inoculum of 1×10^6 blastospores/ml, and the presence of different compounds, such as N-acetylglucosamine, proline or serum

In this review we shall briefly summarise the work on *Candida* classical genetics (mutant isolation, parasexual genetics and chromosomal reorganisations), the general strategies used for gene isolation and the molecular tools currently being developed in *C. albicans* (transformation systems, gene disruption strategies and gene reporter systems), and the use of animal models for studies on its pathogenesis and the immune response against this yeast. We aim at offering a concise, basic review of the tools available for studying the biology and pathogenicity of this yeast. We have purposefully left out of this review a major aspect for the pharmaceutical industry: the need for the development of really effective, non toxic, fungicidal therapeutical agents, since those currently available in clinical practice are confined to relatively few chemical classes [28] and resistances are being developed. It is therefore necessary to find new, selective antifungal targets, and significant efforts are currently being focused in this direction.

Genetics

Classical genetics *C. albicans* classical genetics had a great impact in the 1980s. Now, in spite of its being out of fashion, it may still offer clues to the understanding of this yeast. The diploid nature of *C. albicans* was deduced from the determination of its DNA content and the kinetics of reassociation of denatured total DNA. In addition, it was found

that many clinical isolates of *C. albicans* displayed a strongly biased auxotroph spectrum after ultraviolet (UV) irradiation [123]. This was interpreted as the consequence of the natural heterozygosity of many *C. albicans* strains for some loci. This hypothesis was later confirmed from the analysis of sectored colonies obtained after UV treatment of putative natural heterozygotes or revertants from mutants isolated by chemical mutagenesis [92, 94, 124].

Mutant isolation *C. albicans* diploidy accounts for the difficulties in obtaining mutant strains. In fact, most mutant alleles are recessive and therefore should be present in homozygosity for the mutant phenotype to be shown. Accordingly, after mutagenesis of a *C. albicans* wild type strain (+/+), a mutant heterozygous strain (+/–) for several loci is generated which must be rendered homozygous (–/–) by either spontaneous or induced (mainly with the use of UV irradiation) mitotic recombination. A second round of mutagenesis could lead also to the homozygous state.

Different mutagenic agents have been used to obtain *C. albicans* mutants. The most common are UV irradiation, methyl-nitro-nitrosoguanidine, ethylmethane-sulfonate and nitrous acid [45, 93, 106]. Several mutant enrichment procedures for auxotrophs have been developed, such as protocols based on amphotericin B treatment [92], inositol starvation [21] and folate pathway inhibitors [38]. In addition, morphological mutants have been isolated by using differential filtration enrichment techniques [10, 31]. With these procedures, a wide range of mutants has been isolated [91]. Although molecular protocols have now replaced classical mutant isolation in *C. albicans*, the former approach may still provide a source of host strains for direct cloning in *C. albicans* [34, 75], especially as regards the above-reported processes where *S. cerevisiae* and *C. albicans* are clearly different.

Parasexual genetics There are many examples of the use of protoplast fusion of *C. albicans* [30, 68], and all published protocols are very similar with regard to the use of calcium salts and polyethylene glycol. Protoplasts from strains to be analyzed that present appropriate markers (normally auxotroph markers) can be fused to give fusion products which are selected in a medium that does not allow the growth of any of the parental strains. Thus, two kinds of colonies appear on the regeneration plates: fast-growing stable colonies, corresponding to uninucleate tetraploid hybrids (probably resulting from the fusion of two nuclei from both parental strains), and slow-growing multinucleate colonies or heterokaryons, which are unstable on complete medium [108]. On minimal medium, these slow growing colonies develop faster growing sectors, which could be tetraploid hybrids or aneuploids [107]. These hybrids are stable and the loss of chromosomes necessary to come back to the diploid state can be induced by heat shock treatment [42] or by using the antimicrotubule agent methyl benzimidazole carbamate [3]. In this way, the full parasexual cycle of *C. albicans* can be induced ($2n \times 2n \rightarrow 4n \rightarrow 2n$).

Molecular biology

Transformation systems The introduction and stable maintenance of DNA in *C. albicans* cells has been one of the major problems to solve in the genetic manipulation of this yeast, and only recently have strains and methods been developed.

C. albicans seemed to be refractory to any transformation protocol other than protoplast formation, which has been the preferred (and for some time the only) method to transform it. The protocol is similar to that used with *S. cerevisiae* but with several modifications [11, 41, 55], due to the stronger nature of *C. albicans* cell wall. In recent years, other *S. cerevisiae* protocols have been successfully adapted to *C. albicans*, such as the lithium acetate [32, 62] or electroporation [51, 79] techniques. However, they are considerably less efficient than the protoplast transformation method.

The ideal for *C. albicans* researchers, at least for those in clinical practice, would be the ability to manipulate and study clinical isolates in order to determine their characteristics. To date, only two dominant selectable markers have been described: one of them is based on the use of the *TCM1* gene, encoding a *S. cerevisiae*-modified ribosomal protein, which confers resistance to the protein synthesis inhibitor trichodermin [41]; the other one is the mycophenolic acid resistance method, more recently described, which uses the putative *C. albicans* IMP dehydrogenase gene (*IMH3*) in plasmids [51]. Nevertheless, the classical approach involving the use of auxotrophic mutants has also been used for *C. albicans*. These mutants were initially obtained by multiple rounds of either chemical or physical mutagenesis. Therefore, they probably bore other mutations due to the impossibility of crossing strains to isolate the desired mutation in a clean genetic background. The most widely used auxotrophs are *ura3* strains such as 1006 [35], SGY243 [47] or cMD716 [32] and *ade2* strains such as SGY129 [47] or hOG300 [94]. Strains bearing multiple markers are SGY484 (*ura3 leu2*) [46], 792-WC3 (*ura2 ade2*) [32], 1006 (*ura3 lys1 arg4 ser57 MPA1*) [35] or 1161 (*ura3 lys1 arg4 ser57 gal1 MPA1*) [34]. Only when a genetic method was used to obtain *ura3* strains it was possible to compare the phenotypes of strains bearing different mutations. The generation of *C. albicans* CAI4 strain as well as an appropriate method for systematic deletion of the two alleles of each gene present in the genome of *C. albicans* (see later) by Fonzi and Irwin has favoured the work of many other researchers [26]. This strain was constructed by deleting the *URA3* loci by genetic means in a clinically isolated strain, SC5314, and is currently being used as the standard strain to generate deletions. More recently, other strains bearing multiple markers using CAI4 as a basis have been obtained: RM1000 (*ura3 his1*), CNC44 (*ura3 arg5,6*) or CNC43 (*ura3 his1 arg5,6*) [75].

The first experiments allowing the introduction of DNA into *C. albicans* were achieved by homologous integration at

Table 1 *Candida albicans* autoreplicative transformation vectors

Name	ARS[a]	Gene markers	Reference
pMK22	ARS1	*URA3*	56
pCARS1	ARS1	*URA3*	46
pRC3925	ARS2	*URA3,LEU2*	11
pCR2312	ARS2	*URA3, LEU2*	12
p1041	ARS1	*URA3*	34
p1110	ARS1	*URA3, ARG4*	34
p1109	ARS1	*URA3, ARG4*	34
p1113	ARS1	*URA3, LYS1*	34
p1334	ARS1	*URA3, LYS1*	34
pEH7	ARS3	*TCM1*	41
pCB4	ARS2	*ADE1*	79
pRM1	ARS2, ARS3	*URA3, LEU2*	86
pRM10	ARS2, ARS3	*URA3*	86
pAN8	ARS2, ARS3	*ARG5,6*	75

[a]ARS sequences are numbered chronologically; thus, ARS1 refers to the CARS sequence isolated by Dr. M. B. Kurtz [56], ARS2 to ARS obtained by Dr. R. D. Cannon [12] and ARS3 to Dr. E. Herreros [41].

the *ADE2* locus in the genome of the hOG300 strain [55]. The efficiency per microgram of DNA is normally low (around 0.5–10 transformants/μg DNA) and may, to a certain extent, be affected by the transcriptional state of the gene of interest [118]. Although integrative transformation is very useful for introducing stable traits or for disrupting genes in *C. albicans*, an autoreplicative system is necessary for library screening and DNA recovery. Due to the lack of natural plasmids and to the inability of 2-micron sequences to replicate autonomously in *C. albicans*, the key to the construction of replicative plasmids has been the isolation of sequences able to promote autonomous replication in *C. albicans* (called ARS, for Autonomously Replicating Sequences). This task was accomplished by following two opposite directions. Some researchers isolated ARS by direct screening in *C. albicans* by using a genomic library cloned in an integrative vector. Plasmids able to replicate independently and to produce higher transformation frequencies were isolated and their DNA fragments analyzed. This led to the isolation of CARS (*C. albicans* ARS) or ARS1 [56]. Plasmids based on this ARS increase the transformation efficiency up to 10^3 transformants/μg DNA. Nevertheless, these plasmids seem to replicate in multimers and need to be resolved in *S. cerevisiae* as an intermediate host [34]. Other authors isolated *C. albicans* ARS by using genomic *C. albicans* DNA libraries constructed in integrative *S. cerevisiae* plasmids and searching for stable plasmids in *S. cerevisiae*. This strategy allowed the isolation of two ARS: ARS2 [11] and ARS3 [41]. Both of them stimulate autonomous replication and increase the transformation efficiency of plasmids in *C. albicans*, obtaining frequencies similar to those obtained with CARS-derived plasmids. Typically, two types of transformants arise with these plasmids: cells with faster growth rates, which contain seven to eight copies of integrated plasmids, and slowly-growing cells with two or three copies of replicative plasmids [12, 41]. Recently, a new set of plasmids has been developed by joining both the ARS2 and ARS3 sequences in a single vector. These plasmids do not increase the transformation efficiency to a large extent, but do increase their copy numbers and their mitotic stability, thus facilitating the use of this yeast in direct gene cloning (see later). In addition, they do not form multimers as frequently as others do, and can be isolated directly in *Escherichia coli* from *C. albicans* [86]. To date no *C. albicans* centromeric regions have been reported. Table 1 shows several *C. albicans* replicating plasmids described until now.

Gene disruption strategies Gene disruption is an essential tool in host strain construction, gene mapping and functional analysis. For many years, some biological characteristics of *C. albicans* (diploidy and lack of sporulation) hindered the development of disruption systems. Initially, some strategies were described that involved the use of UV-enhanced mitotic recombination on a heterozygous strain (obtained through homologous recombination with an appropriate gene marker) to generate homozygosity at the desired *locus* [47]. In this way, a *leu2 ura3* strain was constructed and its availability allowed the design of disruption strategies based on both markers that avoid the random mutagenesis inherent to UV irradiation [58].

A major advance in *C. albicans* genetic manipulation was achieved when Fonzi and Irwin [26] adapted a disruption strategy used in *S. cerevisiae*. The method involves the use of *C. albicans URA3* flanked by *Salmonella typhimurium hisG* genes to provide flanking recombination regions. Following homologous recombination in the first chromosomal allele, intrachromosomal excision of the URA3 marker is selected with the antimetabolite 5-fluoroorotic acid (5-FOA), thus allowing the recovery of *ura3* auxotrophy and enabling a second round of homologous recombination to disrupt the second chromosomal allele.

With this system, many *C. albicans* genes have been disrupted in their two alleles [2, 32, 33, 36, 62, 69, 74, 81, 82, 102]. Phenotypic studies of the mutants constructed have been carried out to gain insight into their relationship with

certain processes of great interest such as dimorphism or virulence [4, 23, 36, 109].

Some modifications of this disruption strategy have been reported [105]. This system has been improved [75] with the utilization of a Ura- His- *C. albicans* strain (RM1000) as host strain for the disruption host of the *ARG5,6* gene. This allowed the cotransformation with two different constructions for simultaneous disruption of both alleles, each with a different selection marker (*URA3* or *HIS1*). This enabled the construction of a *arg5,6* null mutant in a single step.

Gene reporter systems A suitable gene reporter system is essential for the study of the function and regulation of *C. albicans*-cloned genes. *C. albicans* decodes the CUG codon into serine during mRNA translation while in most species it is decoded into leucine [104]. The use of the non-universal genetic code by *C. albicans* represents an important handicap for the use of heterologous gene reporter systems in this yeast (*E. coli* β-galactosidase or *Photinus pyralis* luciferase do not work in *C. albicans*). However, some heterologous gene reporter systems have been developed. Thus, β-galactosidase activity encoded by *Kluyveromyces lactis LAC4* gene was the first gene reporter system described in *C. albicans* [61]. *LAC4* mRNA contains two CUG codons [87], but this does not affect the enzymatic activity of this reporter. More recently, *Renilla reniformis* luciferase, whose gene lacks CUG codons, has been described as a bioluminescent gene expression reporter in *C. albicans* [117]. Furthermore, although the native green fluorescent protein (GFP) from *Aequorea victoria* is not expressed in *C. albicans*, it has been genetically optimised with regard to its codon usage, and the GFP-derivative obtained is efficiently expressed in *C. albicans* [19]. Another possible strategy to avoid the genetic code problem is the use of homologous reporter genes: the *URA3* [72] gene as well as the major exoglucanase gene from *C. albicans*, *XOG1* [33], have been described.

These gene reporter systems have different sensitivities and employ different host strains and detection methods [19, 85]. This makes them more or less useful in each specific application. In addition, some of them have been adapted to the construction of in-frame proteins (e.g. *LAC4* [61] and *URA3* [72]) and in some cases they may be useful in protein localization studies (e.g. GFP [39]).

Isolation of *C. albicans* genes Many strategies have been used for the isolation of *C. albicans* genes: screening with heterologous probes, immunological screenings, reverse genetics, cDNA differential gene expression, and complementation of *S. cerevisiae* or *C. albicans* mutants [85]. Below we shall focus a little more attention on the latter two methods. Complementation in *S. cerevisiae* was chosen among the early strategies used to isolate *C. albicans* genes because it is a straightforward system that usually ensures the functionality of cloned genes [71, 74]. Nevertheless, this strategy may be unsuccessful due to differences in the transcription and translation machinery. Complementation of phenotypes of *C. albicans* mutants is an arduous task due to the lack of libraries in efficient vectors. This problem has been overcome although to date, only nutritional genes have been isolated [34, 75, 86]. In view of the virulence and hyphal formation capacities of *C. albicans*, it is clear that *S. cerevisiae* is probably not the best model to study these processes.

Expression controlled systems One of the most powerful tools in the study of gene functions are regulatable promoters. These allow the construction of mutant strains in which the protein of interest is overexpressed or, alternatively, underexpressed. Four *C. albicans* promoters have been studied using luciferase as the reported gene. Two of them are phase-specific (*WH11* and *OP4*); one is constitutive (*EF1*-2p), and the other one is regulated by galactose (*GAL1*) [117]. Other *C. albicans* carbon source-regulatable promoters have been also used in the study of new *C. albicans* cloned genes: *MAL*2p (maltose-inducible/glucose-repressible promoter [7,19]), *HEX1*p (glucose-repressible/N-acetylglucosamine-inducible promoter [70]) or *PCK1*p (glucose-repressible [120]). The principal goal in this study would be the description of promoters able to repress totally the gene under their control. Promoters able to switch off completely the expression of a given gene would enable the study of *C. albicans* essential genes, which are potential new antifungal targets. Recently, the construction of a conditional *TOP1 C. albicans* null mutant strain has been reported [44]. In this strain, an internal fragment of the first copy of the *TOP1* gene was replaced by the *hisG-CaURA3-hisG* cassette, while the second copy was placed under a maltose-inducible/glucose-repressible promoter (*MRP1*p). Under *MRP1*p repression conditions (glucose), DNA topoisomerase I activity was undetectable, which suggested total repression of the promoter. This system could be useful in the construction of different conditional null mutants of putative *C. albicans* essential genes, although more studies about *MRP1* regulation (using reporter genes or mRNA level studies) are necessary.

A look into the future of *C. albicans* manipulation The characteristics of *C. albicans* make it an interesting model to understand the hyphal development, pathogenicity and virulence of fungi. In fact, more and more laboratories are committed to its study. Nevertheless, it is still necessary to use *S. cerevisiae* as a model for the understanding of *C. albicans*. Unquestionably, we need more genetic tools to use *C. albicans* with the same degree of versatility as *S. cerevisiae* has been used over the last twenty years. The completed *S. cerevisiae* sequencing and the EUROFAN projects will help to achieve this aim by providing new tools developed to understand its biological functions. A physical map of the chromosomes of *C. albicans* [16, 73] has been obtained, and some projects involving the sequencing of the whole genome of this yeast are currently under way throughout the world, although not in a joint effort, as it was the case of *S. cerevisiae*.

Another approach very promising is two-dimensional analysis of *C. albicans* proteins. Since the whole of the

S. cerevisiae sequence is now known, the sequence of the different *Candida* gel spots in many cases should allow us to identify *C. albicans* genes which have homologues in *S. cerevisiae*. We are presently carrying out this type of analysis using the proteins secreted by *C. albicans* regenerating protoplasts.

Dimorphism

Like other pathogenic fungi, *C. albicans* is dimorphic. This yeast exhibits a number of different morphological forms under different environmental conditions; such forms include budding yeast cells (blastospores, blastoconidia), pseudohyphae (elongated cells which appear as filamentous cell chains), true hyphae, and clamydospores [78] (Fig. 1). A blastospore is a unicellular form of the fungus that divides by budding. This process of budding involves the growth of new cellular material from a small, selected site on the blastospore surface. The new bud usually develops at the pole distal to the birth scar and initiates a growth phase. Then, nuclear division takes place and a septum is laid down between the parent and daughter cell units. The two cell units separate to form blastospores. A hypha is a long microscopic tube comprising multiple fungal cell units divided by septa. Germ tube formation is the initial stage in the yeast-hyphal transition. A blastospore gives rise to new cellular material in the shape of a cylinder, called a germ tube, which grows continuously by extension of the distal pole. Mitotic cell division occurs within the extending hypha and septa are formed at intervals along the hyphae without interrupting the rate of extension. Hyphae may arise as branches of already existing hyphae or by germination of spores. A mycelium is an entire fungal cellular aggregate that includes hyphae with all their branches.

Experimental conditions for germ-tube formation There are several features essential for in vitro morphological transition of *C. albicans* [78]. The earliest workers with *C. albicans* found that changes in the growth environment of *C. albicans* led to changes in cell shape. However, no one has ever been able to discover any environmental "morphogen" that acts alone and under all conditions to provoke the growth of cells from any *C. albicans* isolate in only one morphological form. Some environmental factors that favor the filamentation in *C. albicans* are: temperature of 37–40°C, pH around 7.0 and an initial blastospore concentration not exceeding 10^6/ml. Also, certain chemicals, such as N-acetyl-D-glucosamine, amino acids, biotin, sulfhydryl compounds, heme group, zinc, and serum, etc. are required. The production of germ-tubes in serum has remained the method of choice for identifying *C. albicans* in clinical specimens. Although serum is excellent at promoting the yeast to mycelial conversion, unfortunately its chemical complexity makes it unsuitable for studying the molecular basis for inducing the hyphal form.

C. albicans seems to be able to regulate its own morphology under at least two circumstances: as a slow response to adverse nutrient environments, in general, and as a rapid response to certain physicochemical conditions. In the latter case, filamentous forms seem likely to develop under the conditions of temperature and pH that are found in host tissues in vivo. Several specific media have been developed for the culture of *C. albicans*. Certain defined media will support both growth forms, the determining factor being either temperature or pH. In general, the budding phenotype is prevalent at low temperatures or low pH, while the hyphal phenotype is prevalent at high temperatures and high pH. A synthetic medium (composed of a mixture of amino acids) has been described by Lee in which a switch in pH suffices to guarantee the production of either the yeast form (pH 4.5) or the hyphal form (pH 6.5), both being cultured at 37°C [115].

Approaches for the study of *C. albicans* dimorphism Different approaches have been used to gain insight into the molecular basis of dimorphism: (i) a classical genetic approach, based on obtaining morphological mutants; (ii) the "candidate gene approach", in which a gene likely to be involved in dimorphism is identified; and (iii) the use of *S. cerevisiae* as a model of filamentous growth (pseudohyphae) for cloning homologous *Candida* genes.

(i) Classical approach. Using a classical genetic strategy, three kinds of morphological mutants have been isolated and described [77]. The first corresponds to mutants that fail to give rise to mycelia or to any other kind of filamentous form (F^- phenotype), at least under the conditions that are usually used to induce hyphal morphogenesis in wild type strains [10, 25, 76]. Mutants of a second kind have usually been selected on the basis of alterations in their colony morphology. They are rough-wrinkled to different extents. The altered colonial morphology correlates with a permanent alteration in morphogenesis of the cells, which grow permanently as filamentous forms strongly resembling pseudohyphae. Budding growth is suppressed in these mutants (Y^- phenotype) [89, 90]. Finally a third type of mutants has been obtained using a careful selection of filamentous forms by successive filtering of mutagenised populations. These mutants express all types of morphogenesis in nutrient medium sequentially and do not require any type of inducer [31]. Parasexual crosses carried out with pairs of some of these mutants lead to wild type hybrids, which indicates the existence of several complementation groups in the pseudomycelial phenotype [30]. However, the diploidy and asexual life cycle of *Candida* have hindered extensive genetic characterization.

(ii) The candidate gene approach. Genes expressed differentially during morphogenesis have been identified and *Candida* strains homozygous for a null allele of the gene have been constructed. The gene is required for dimorphism if the null mutant shows a defect in hypha induction.

To isolate genes differentially expressed during dimorphism, Birse and co-workers [5] prepared a phage cDNA library from cells in the process of hypha formation. The library was plated and plaques were hybridized with labelled cDNAs prepared

from either yeast or hyphae. The following genes were cloned: *PHR1* and *ECE1*. *PHR1* is differentially regulated in response to the pH of the growth medium. It encodes a putative cell surface glycoprotein anchored to the membrane by glycosylphosphatidylinositol (GPI). Deletion of *PHR1* results in a pH-dependent defect in morphogenesis. *ECE1* (*E*xtent of *C*ell *E*longation) expression is not detected when *C. albicans* grows as a budding yeast cell but is observed within 30 min after cells have been induced to form hyphae. *ECE1* expression correlates with the extent of cell elongation. However *ECE1*-null mutants display no morphological alterations, showing that it is not essential for cell elongation or hypha formation despite the strict morphological regulation of its expression.

Other genes cloned by different research teams and that are differentially expressed during morphogenesis are: *HYR1* [2], *RBF1* [43], *CHS2* [36], *CHS3*, etc. *HYR1*-, *CHS2*- and *CHS3*-null mutants do not display any obvious morphological phenotype. However, disruption of *RBF1* causes an alteration in the cell morphology of the filamentous form and may be involved in the regulation of the yeast-hypha transition. Genetic inactivation results in stimulation of filamentous growth in both solid and liquid media, suggesting that Rbf1p would be involved in the general pathway of the yeast-to-filament transition. However, it is also possible that the filamentous growth of the mutants may be mediated indirectly, for example through some kind of disturbance in nutrient metabolism, general transcription or translation, or else through an abnormality in chromosome structure or its segregation.

(iii) The use of *S. cerevisiae* as a model of filamentous growth. *S. cerevisiae* is also a dimorphic organism capable, under certain nutritional conditions, of switching from a budding yeast to a filamentous pseudohyphal form (and in the case of haploid cells the switch leads to invasive growth). The similarities between the dimorphic switching of *S. cerevisiae* and *C. albicans* suggest that these morphological pathways may be regulated by similar mechanisms in both organisms. In *S. cerevisiae*, the morphological transition is controlled by signalling components that are also involved in the mating response of haploid cells. *C. albicans* contains functional homologues of Ste7p (Hst7p), Ste12p (Cph1p, a transcription factor) and Ste20p (Cst20p) that act in a common regulatory pathway involved in hyphal growth in *Candida*. Null alleles of these genes have been constructed in *Candida*; showing that homozygous null mutants are defective in hyphal formation on some media, but these mutants can still be induced to form hyphae by serum, which implies that serum induction of hyphae is independent of the MAP kinase cascade [52, 59].

Recently, three important *C. albicans* genes have been cloned: *TUP1*, *EFG1*, and *CLA4*. It has been reported that all of them are important regulators for *C. albicans* morphogenesis. *TUP1*, a transcriptional repressor, controls filament formation in *C. albicans*. The *tup1* mutant strain of *C. albicans* displays constitutive filamentous growth under all conditions tested. A model for the control of filamentous growth in *C. albicans* by *TUP1* has been proposed. *TUP1* represses the genes responsible for initiating filamentous growth and this repression is abolished under inducing environmental conditions [6].

CLA4 encodes a member of the Ste20p family of serine/threonine protein kinases. Deletion of both alleles of this gene in *C. albicans* causes defects in hyphal formation and drastically reduces *C. albicans* virulence in the mouse model [60].

The product encoded by *EFG1* is a member of a conserved class of bHLH (basic helix-loop-helix) proteins that regulate morphogenetic processes in fungi. This protein plays a dual role as a transcriptional activator and repressor, whose balanced activity is essential for the yeast, pseudohyphal and hyphal morphogenetic stages of *C. albicans* [120]. Double mutants *efg1* and *cph1* fail to produce germ tubes and hyphae under any condition tested and show reduced in a mouse model [63].

Pathogenicity studies

The pathogenicity of *C. albicans* makes important understanding its relationship with the host.

Experimental animal models Animal models have been developed to study, from the point of view of the parasite, virulence determinants and, from the point of view of the host, the contribution of the different aspects of the immune response to control the infection; as well as to study the in vivo fungistatic or fungicidal capacity of different drugs.

In these models, different types of infection are induced, as will be detailed below, and the ability of *Candida* to elicit lesions in different tissues, or even the death of the animals is analyzed. Of the existing models, the most used is the murine one since mice are cheaper than rats, guinea-pigs and rabbits, and because of the similarity of the murine systemic infection to that found in humans [17, 78, 80].

Types of assays (i) Cutaneous infections. Generally, an area of the animal's skin is slightly scarred and the yeast is inoculated. The development of the skin colonization is monitored and samples are taken at different times. For more details, see the review by Ray [95].

(ii) Vaginitis. This is usually induced in ovariectomized and treated with oestrogen rats [113] and mice [100]. Previously, untreated hamsters have also been used [37].

(iii) Intestinal infection. These infections are difficult to induce in adult mice but not in six-day-old ones [24, 40], or in germ-free mice [13], which are able to retain the infection for several weeks, thereby enabling its study.

(iv) Systemic infection. Systemic infection with *C. albicans* is induced in mice, rats, guinea-pigs and rabbits. Like the above models, these are sometimes used to assess the efficiency of antifungal agents although, more recently, they have also been used to study virulence determinants.

Two types of systemic infections can be set up: lethal, in which the survival time is measured, and non-lethal, in which

the severity of the infection is measured by fungal cell counts (CFUs) in internal organs or by the lesions produced by the microorganism in them (observed by anatomical pathology studies).

(v) Other infections:

- The rat palate candidiasis model is used to study a kind of lesion frequently occurring in individuals with dentures [65].

- Models of pielonephritis have been developed in rabbits [84], as well as models of endocarditis [83] and endophthalmitis [110].

- A murine model has been developed for the study of muguet: air is injected into the backs of mice and *C. albicans* is inoculated into the subcutaneous sac thus formed [53, 88].

Systemic infection in mice We focus on this model in greater depth because, as mentioned above, it is the one generally used in virulence studies and in the assessment of antifungal agents. This model has also been used to study the main mechanisms of host defence involved in this kind of infection.

The systemic infection that can be induced in mice is very similar to that found in humans. In both mice and humans, the main focus of infection occurs in the kidneys and the brain, which are the most representative organs of the course of the disease. After intravenous inoculation (through the lateral vein of the tail), *Candida* is deposited in lungs, liver, spleen and kidney, but of all these organs, the only one in which the infection is established is the kidney, and it is there where it is possible to follow the course of the disease [78, 80]. When the number of microorganisms inoculated is lower, the greatest damage is seen in the brain [80].

As mentioned above, two types of systemic infections can be set up:

- Lethal. High doses (1×10^7–1×10^6 blastospores per mouse) are used to induce it. This kind of study normally elicits an acute infection, and it is usually used to observe the main differences in mortality among different *Candida* strains. Variables usually measured are: median survival time (MST), the LD_{50} (number of blastospores able to kill half the mice) and the rate of dead mice vs. total (D/T).

- Sublethal. Lower doses (1×10^5–5×10^4 blastospores per mouse) are used to establish a chronic infection. The course of the infection is usually monitored by the degree of tissue damage and the fungal load in the most representative organs; namely brain and kidneys.

Mouse strains Two types of mouse are usually employed. These are *outbred strains* and *inbred strains*. The former are strains with a broad genetic variability among the members of the same litter [80]. This type offers the advantage of being cheaper since they reproduce well, and they can also be considered to be more representative of a human population. An example is the CD1 strain (Charles River Breeding Laboratories). This strain has been reported to show medium susceptibility to *Candida* infections.

C. albicans activates complement in vitro and, as it has been shown by immunofluorescence experiments, also in vivo [114]. *C. albicans* can activate the classic route of the complement, but if this is inhibited, it is able to activate the alternative one [50]. Generally, complement factor C5-deficient strains—for example DBA/2 and A/J (Charles River Breeding Laboratories)—are characterized by an increased susceptibility to disseminated and cutaneous candidiasis, and a predominance of the non-protective Th2 response, and therefore higher levels of tissue colonisation. By contrast, strains with a functional complement system, such as Balb/c, CBA/CaH or DBA/1 (Charles River Breeding Laboratories) develop Th1 immune response (which is protective) and are more resistant to this disease [1].

Mutant strains These are mice strains with specific mutations that confer them certain disfunctions of varying degrees, which allows independent, detailed study of the different factors involved in the protection against a given microorganism. However, the immune response functions as a whole. So, studies with mutant strains can only provide hints of the relative importance of the different mechanisms of defence. Despite this, when these murine strains are used to compare differences in virulence among different *Candida* strains, the conclusions are much more reliable.

Examples of such mutants are:

(i) *nu/nu*: these are athymic mice unable to generate functional T lymphocytes.

(ii) *scid/scid*: these strains have a severe deficiency in T and B lymphocytes and are therefore unable to orchestrate specific immune responses.

(iii) *transgenic mice*: these are mice in which some of the genes whose products are involved in the immune response, such as cytokines, have been mutated, thus enabling the study of the role of these factors in an infection: IL-4(–), γIFN(–) mice [49] or animals deficient for tumor necrosis factor receptor (TNFR-p55 or TNFR-p75) are available [119].

Resistance to systemic candidiasis As mentioned above, one of the objectives of animal studies was to analyze the immune response of the host, because knowledge of the main factors involved in the response against the yeast could open new perspectives in therapy against candidiasis, above all in immunocompromised hosts. Some reviews have appeared recently [1, 122]. Here, we shall only summarize some of the most important aspects.

Innate response Many in vivo studies suggest that the resistance mechanism could be independent of T-cell mediated immunity. This innate immunity, probably due to neutrophils and macrophages, is critical for the clearance of acute systemic infections. It has been demonstrated that neutropenia is a major risk factor that increases susceptibility to systemic candidiasis [96, 98] and that depletion of neutrophils in mice (by injecting them anti-neutropenic monoclonal antibodies) increases susceptibility to both systemic and vaginal challenges [27]. However, macrophage activation, which seems to be necessary for the complete expression of resistance against fungal infections [97], needs T-cell mediated immunity to limit or eradicate fungal infection.

TNF also plays a major role in non-specific resistance against *C. albicans*. Steinshamn et al. have suggested that granulocytes would contribute to the regulation of TNF activity during infection with *C. albicans* [119].

Adaptative response Resistance to systemic candidiasis in immune hosts seems to be associated with a predominant Th_1 response, with the generation of DTH lymphocytes specific for *C. albicans* antigens, and with an increase in the fungicidal activity of macrophages. Th_1 responses are characterized by γIFN and IL-2 production. Romani et al. have shown the association of Th_1 responses with the production of IL-12, with the stimulation of inducible nitric oxide synthase (iNOS), and with increases in the fungicidal capacity of macrophages [99]. In contrast, susceptible mice undergo a typical Th_2 response, characterized by the predominant production of IL-4 and IL-10 [15], both of which cause a significant decrease in nitric oxide (NO) production, as well as in the anti-*Candida* activity of macrophages. Neutralization (using monoclonal antibodies) of IL-10 in immunized mice increases their resistance to *C. albicans* reinfection [15]. By contrast, IL-4 or IL-10 administration exacerbates candidiasis in infected mice [121]. Later experiments by the same group, in which the role of IL-2 was studied [116], revealed that its production exerts different effects depending on whether it takes place at the beginning of the infection or when the infection is installed. These results and others reviewed in [1, 122] indicate that, despite the existence of general trends, about which most investigators agree, the immune response against *C. albicans* still affords controversial results. Furthermore, when considering studies in which cytokine production has been addressed in situ, in different tissues, it should be taken into account that the pattern of cytokine production depends on factors such as the route of administration, the dose, the mouse strain used, the stage of infection, and the tissue analyzed.

Clinical investigations have revealed the importance of antibodies against *C. albicans* for preventing disseminated candidiasis. From animal studies it has been reported the protection conferred by immune serum against candidiasis, mainly when systemic candidiasis were established by in vitro inoculation of a lethal dose of *C. albicans* [66]. Nevertheless, some results are not so optimistic: rabbits treated with *C. albicans* hyperimmune serum were not more resistant to in vitro inoculation than the corresponding controls. Furthermore, in some cases, SCID mice were as resistant to systemic candidiasis as immunocompetent mice strains were [122].

It has been postulated that at least some of these protective antibodies are directed against both mannoproteins [9, 14] and the heat shock protein hsp90 [67].

Virulence studies with mutant strains In the latest published studies on virulence, attempts have been made to attribute responsibility for the pathogenic capacity of *Candida* to different genes.

In these studies, mutant yeast strains have been used, generated either by classic methods or by gene disruption. Their virulence has been assessed comparing it with the virulence of the wild type strain. There are many examples of such a study, which have addressed the virulence either of auxotrophic mutants [18, 48, 112, 125, 126] or of other mutants resistant to different antifungal agents [4, 54]. However, current studies tend to use mutants in which certain genes assumed to be involved in pathogenesis have been disrupted. These studies afford insight into the involvement of a given gene in virulence and, in some cases, its putative role as a new target for antifungal development. Nevertheless, regarding this latter aspect, the trend in pharmaceutical companies is to select molecular targets that are essential for viability and not only for virulence. This is so because antifungal agents are above all necessary in patients with severe immunodeficiencies. In them, fungicidal action is crucial, and not merely a fungistatic one, with which patients usually relapse when treatment is discontinued. Despite this, the knowledge of virulence factors is still important, as it is their putative application in the development of antifungals [20].

Several genes have been studied: *PHR1*, regulated by the pH of the medium and involved in morphogenesis [29]; genes involved in chitin synthesis [8], *MKC1*, a MAP kinase involved in morphogenesis and cellular integrity [23], and a mutant in which a triple deletion of the secreted aspartyl proteinase genes *SAP4*, *SAP5*, and *SAP6* has been induced [103]. In all these cases, the mutants were less virulent than the wild strains.

Three papers have shown that the deletion of some given genes lead to major losses of virulence: (i) Zhao et al. have reported that the *FAS2* gene, which is involved in fatty acid synthesis, is necessary to establish an infection, both by oral inoculation in rats [125] and by in vitro inoculation in mice [126]. (ii) Leberer et al. [60] have shown that deletion of a gene C*aCLA4*, besides preventing the dimorphic transition (yeast-to-hypha), causes a great loss in virulence in systemic infections. This of great importance because it is the first time that a gene has been shown to be involved in virulence and filamentation, processes whose interrelationship had hitherto been controversial [101]. Afterwards, in Fink's laboratory a mutant strain has been built up in which the simultaneous deletion of two genes elicits the absence of filamentation and a significant loss of virulence [63].

Although all three mutants mentioned above lost a considerable amount of virulence, according to our own criterion, they cannot be considered avirulent because mouse strains that orchestrate Th_1 responses (protective) were used. Moreover, only one study used a dose of 1×10^7 blastospores, in this case the mortality rate was 50% of the mice at day 15 after inoculation [63].

At our laboratory, we have generated, using classical mutagenesis, a non-filamentous mutant strain of *C. albicans* called 92' [77]. In studies of pathogenesis, this mutant proved to be completely avirulent: doses of 1×10^7 blastospores killed neither resistant mouse strains (Th_1 inducers) nor sensitive ones (Th_2 inducers). Additionally, nor did this *Candida* mutant kill an additional athymic mouse strain used (nu/nu). Note that, in our hands, this mutant shows a virulence similar to that of the yeast *S. cerevisiae*, it being the first avirulent *Candida* strain

described. One of the uses of this mutant will be the cloning of virulence factors. The generation of an efficient dominant marker in *Candida* would considerably facilitate this task and would be of great help in the experimental handling of clinical isolates.

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