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Phenotypic switching in *Candida albicans* is controlled by a *SIR2* gene

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We report the cloning of a gene from the human fungal pathogen *Candida albicans* with sequence and functional similarity to the *Saccharomyces cerevisiae SIR2* gene. Deletion of the gene in *C.albicans* produces a dramatic phenotype: variant colony morphologies arise at frequencies as high as 1 in 10. The morphologies resemble those described previously as part of a phenotypic switching system proposed to contribute to pathogenesis. Deletion of *SIR2* also produces a high frequency of karyotypic changes. These and other results are consistent with a model whereby Sir2 controls phenotypic switching and chromosome stability in *C.albicans* by organizing chromatin structure.

Keywords: *Candida albicans*/heterochromatin/phenotypic switching/*SIR2* gene

Introduction

Beginning with a single cell, many microorganisms have the capacity to generate different phenotypic variants in just a few generations of growth. The resulting heterogeneous population is well-suited for sudden environmental changes; even if only a few cells in the population possess a phenotype needed for survival, these cells can proliferate and, in turn, regenerate a similarly diverse population. Such variation, which has been especially well-characterized in some bacteria, is often mediated by specialized DNA recombination events that change the pattern of gene expression (Dybvig, 1993).

Candida albicans is responsible for the most common fungal infections in humans (Edwards, 1990). It is capable of growing in a budding form (blastospore) or in various filamentous forms ranging from pseudohyphae to true hyphae (Odds, 1985; Scherer and Magee, 1990). Mass conversion of a culture from one form of growth to the other can be affected by a number of environmental conditions including temperature, pH and nutrient composition (Soll, 1986).

In addition to the bud–filament transitions, *C.albicans* is capable of undergoing a different type of morphological change that has been termed 'phenotypic switching'. This switching is most easily observed in the morphology of colonies (Soll, 1992; Soll *et al*., 1993). A single cell can divide and—in the absence of environmental signals—give rise to several distinct types of colonies. This switching occurs spontaneously at frequencies well above those produced by point mutation and has been reported to be reversible. In addition, cells isolated from each type of colony usually produce the same type of colony on replating, indicating that a variant colony morphology, once formed, is heritable. In this paper, we use the term phenotypic switching to refer to the generation of variant colony morphologies, irrespective of the underlying mechanisms.

Low doses of UV radiation (>90% survival) and limitation of zinc in the growth medium stimulate the formation of variant colonies in wild-type populations almost 100-fold (to a frequency of ~10^{-2}) (Bedell and Soll, 1979; Slutsky *et al*., 1985). Switching has been demonstrated to occur at sites of infection in the host (Soll *et al*., 1987), and it has been proposed that it contributes to pathogenesis (Soll *et al*., 1989).

Two examples of phenotypic switching have been described in *C.albicans*: the white–opaque transition and 3153A-type switching. In the white–opaque transition, first described in the strain WO-1 (Slutsky *et al*., 1987; Rikkerink *et al*., 1988), cells switch between a form that gives rise to hemispherical, creamy white colonies (white phase) and a form that produces flat, gray colonies (opaque phase). The difference between white and opaque colonies reflects a dramatic difference in the appearance of individual cells. While cells in a white colony are relatively round (comparable in shape to those of *S.cerevisiae* cells), those isolated from an opaque colony are elongated, asymmetrical and show surface pimples (Soll *et al*., 1993). The other switching system, 3153A-type switching, was described in cultures of the standard laboratory strain 3153A (Slutsky *et al*., 1985), and it has also been observed in other laboratory strains and clinical isolates (Pomes *et al*., 1985; Soll *et al*., 1987). Distinct from the white–opaque transition, this type of switching produces at least seven different colony morphologies. The predominant colony type in this phenotypic strain is 'smooth', but variant colonies arise at a frequency of ~10^{-4}. These variant colony morphologies reflect, at least in part, the combination of the three main cellular forms of *C.albicans* cells (blastospores, pseudo-hyphal and hyphal cells) found in the colony dome, although the exact relationship between the colony morphology and the cellular forms that comprise it is not known (Slutsky *et al*., 1985; Radford *et al*., 1994).

The molecular basis of phenotypic switching in *C.albicans* is not well understood, and several possible mechanisms can be considered. *Candida albicans* is diploid, and chromosomal rearrangements occur frequently and result in changes in electrophoretic karyotypes. Altered karyotypes are associated with variant colony morphologies (Rutschenko-Bulgac *et al*., 1990; Ramsey *et al*.,

1994), but it has been difficult to establish a cause and effect relationship (but see Janbon *et al*., 1998).

Although some molecular models of phenotypic switching have invoked genomic rearrangements, epigenetic mechanisms have also been considered (Soll, 1989). Heritable epigenetic changes are well characterized in *S.cerevisiae*. For example, genes controlling mating type in budding yeast are located in chromosomal domains where chromatin adopts a specific structure (analogous in some ways to heterochromatin) responsible for silencing these genes (Loo and Rine, 1994). The *SIR* (silent information regulator) genes are required to establish the silent state, and mutations in these genes can affect the efficiency with which *S.cerevisiae* cells pass on the silent state to their daughters (Laurenson and Rine, 1992). Phenotypic switching in *C.albicans* could in principle be controlled using a similar mechanism (Ramsey *et al*., 1994). According to this hypothesis, regulatory genes that control colony morphology would be located in chromosomal positions that exist in two alternative states: a silenced state and an active state. Transition between the two states would result from changes in the chromatin structure, and once established, the on- or off-state of chromatin would be inherited. Phenotypic switching would occur when the chromatin state spontaneously changes, a characteristic of silenced domains in *S.cerevisiae*.

In the present study, we sought to test whether genes that mediate chromosomal silencing in *S.cerevisiae* are involved in the regulation of switching in *C.albicans*. We cloned a *C.albicans* gene (*SIR2*) with sequence similarity to the *SIR2* gene from *S.cerevisiae*. *Candida albicans* cells in which both copies of the gene have been disrupted showed abnormally high levels of variant colony formation. These and other results presented in this study show that the frequency of *C.albicans* phenotypic switching is controlled by the *SIR2* gene.

Results

Isolation and characterization of C.albicans SIR2

In order to test the idea that phenotypic switching in *C.albicans* is related to gene silencing in *S.cerevisiae*, we cloned a *C.albicans* counterpart of the *S.cerevisiae SIR2* gene. This gene, along with others, is involved in maintaining silent chromatin in several locations in the *S.cerevisiae* genome (Laurenson and Rine, 1992). Based on sequence similarity between Sir2 proteins from *S.cerevisiae* and *Kluyveromyces lactis* (Chen and Clark-Walker, 1994; Brachmann *et al*., 1995), we designed degenerate primers which were used for PCR amplification of *C.albicans* genomic DNA (see Materials and methods for details). A single PCR product was obtained and sequenced. The conceptual translation of this nucleotide sequence gave an amino acid sequence with a high similarity to that of the Sir2 protein from *S.cerevisiae*. The nucleotide sequence derived from the PCR product was then used to isolate a longer fragment of DNA by a PCR-walking strategy (Siebert *et al*., 1995). By this method, a gene was identified which contained an open reading frame (ORF) that is highly similar to other members of the Sir2 protein family (Brachmann *et al*., 1995) (Figure 1). The ORF of the *C.albicans SIR2* gene is predicted to encode a protein of 515 amino acids which displays 58% identity across the entire amino acid sequence to the *S.cerevisiae SIR2* gene product.

To assess whether *C.albicans SIR2* is functionally equivalent to *S.cerevisiae SIR2*, we attempted to complement a *sir2* mutant strain of *S.cerevisiae* by introducing the *C.albicans SIR2* gene. The *C.albicans SIR2* ORF was placed under the control of the *S.cerevisiae GAL1* promoter and introduced into a *sir2* strain of *S.cerevisiae*. One of the phenotypes displayed by *S.cerevisiae sir2* mutant strains is the inability to mate due to the inappropriate expression of genes that are normally silenced (Rine and Herskowitz, 1987). The overexpressed *C.albicans SIR2* gene was able to partially restore mating, showing >1000-fold effect; however, the mating efficiency was 10 times lower than that produced by the *S.cerevisiae SIR2* gene expressed under similar conditions (Figure 2). No mating was observed for the *sir2* mutant strain transformed with a control plasmid. Thus, *C.albicans* Sir2 protein can provide at least one SIR2 function in *S.cerevisiae*, the partial restoration of mating in a *S.cerevisiae sir2* mutant.

Candida albicans sir2/sir2 mutants switch at high frequency

To analyze the function of the *SIR2* gene in *C.albicans* we generated a null mutation of the gene. Because *C.albicans* is a diploid organism, we used homologous recombination in two steps to construct a strain deleted for both copies of this gene (Fonzi and Irwin, 1993) (Figure 3).

The viability of *C.albicans* under laboratory conditions was not affected by deleting either one or both copies of *SIR2*. However, while a wild-type strain shows a uniform pattern of colony morphology, strains lacking two copies of the *SIR2* gene displayed many different colony morphologies (Figure 4A), some emerging from the same colony in the form of sectors (Figure 4B). The colony morphologies we observed are broadly similar to those described in strain 3153A by Soll and colleagues (Slutsky *et al*., 1985). In addition to the normal smooth type, we found regular and irregular wrinkled, semi-rough, 'fuzzy', 'scallop' and 'heavy mycellated scallop' types, colony morphologies which had all been reported previously in the literature (Slutsky *et al*., 1985; Soll *et al*., 1987; Radford *et al*., 1994). The unusual feature of the *sir2/sir2* mutant strain was the extremely high frequency with which variant colonies arose (see below).

Features of phenotypic switching described for *C.albicans* are the heritability of each state and the apparent reversibility of some of the transitions. To address whether the high frequency switching we observed in *C.albicans sir2/sir2* mutants exhibits these properties, single non-sectored colonies from each morphology type were disaggregated, and cells from the individual colonies were replated. In each case, we determined the fraction of new colonies (excluding mixed colony types) that resembled the parental colony morphology as well as the fraction of spontaneous variants. Figure 5 shows that the majority of the new colonies exhibited the same colony morphology seen in the parent colony, demonstrating the heritability of each form. However, each set of the new colonies also exhibited significant percentages of colonies with morphologies distinct from the parental type. As

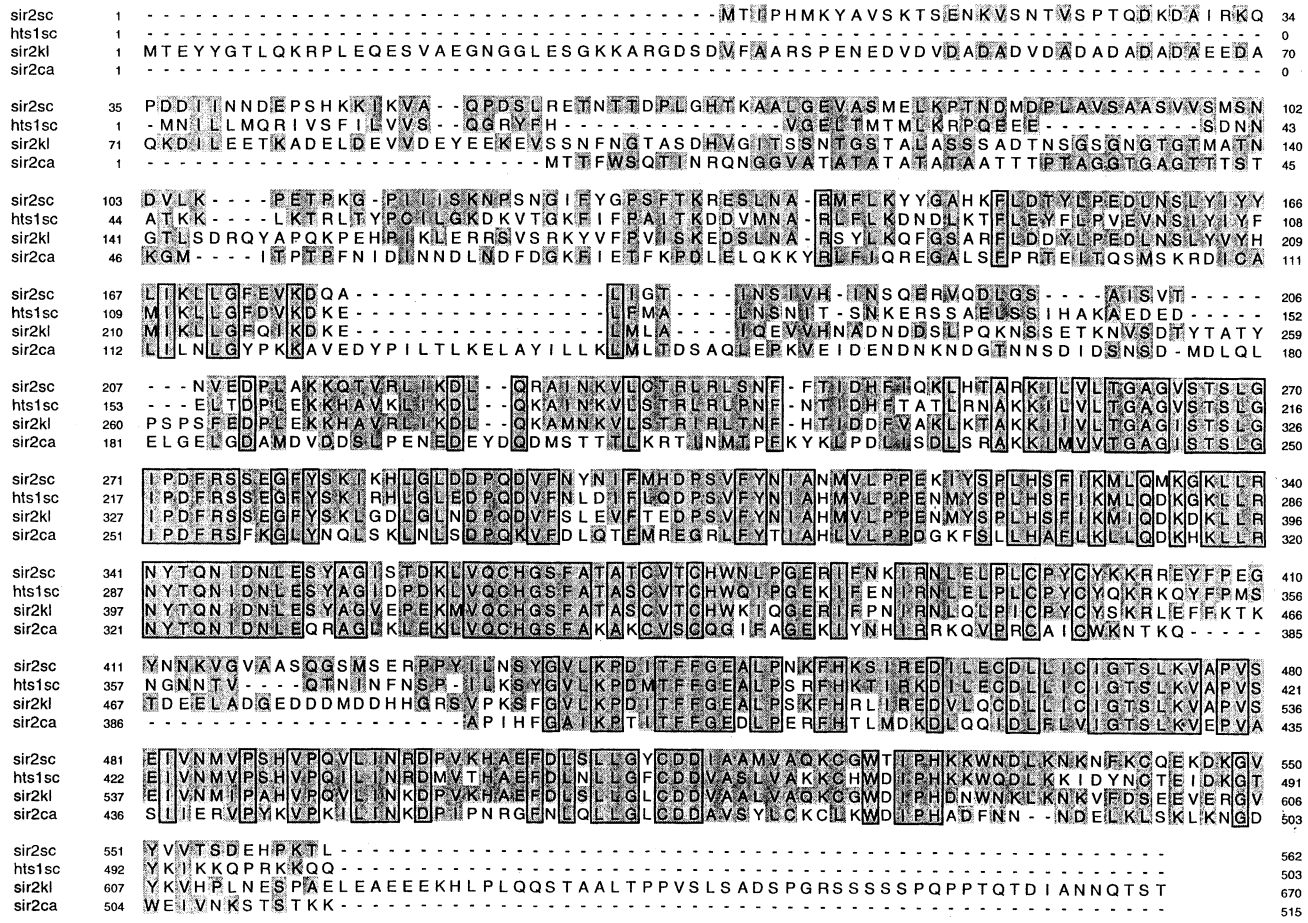


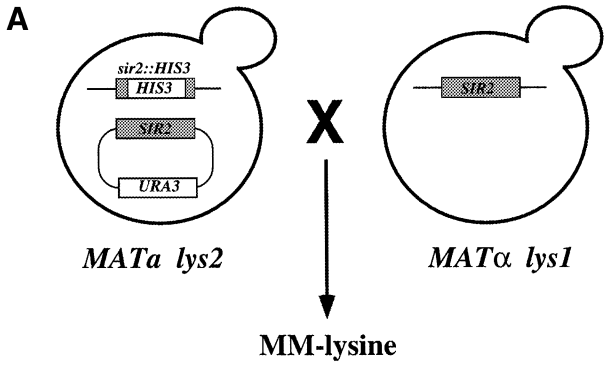
Fig. 1. Alignment of *C.albicans* Sir2 protein and related sequences from other species. Sequence comparison of *C.albicans* Sir2 (sir2ca), *S.cerevisiae* Sir2 (sir2sc), *S.cerevisiae* Hst1 (hst1sc) and *K.lactis* Sir2 (sir2kl). Alignment was performed using the program PILEUP (GCG Inc.). Identical residues are boxed and dashes represent gaps introduced to optimize alignment. Shaded residues are those that are identical or similar based on the Blosum 62 matrix.

summarized in Figure 5, each of the colony morphologies gives rise to several different colony morphologies at frequencies between 10^{-1} and 10^{-2}, with the exception of the 'heavily mycelliated scallop' colonies which give rise only to the smooth type and at a low frequency (10^{-3}). On a gross phenotypic level, some of the morphological transitions appear reversible; however, we emphasize that we have only scored for colony morphology and more subtle differences may exist between colonies that appear similar under our plating conditions. In other words, several distinct types of 'smooth' or 'fuzzy' colonies could exist but not be distinguished by our assay.

Reduced Sir2 levels are responsible for high-frequency switching

To confirm the correlation between phenotypic switching and the expression of the *SIR2* gene in *C.albicans*, we placed this gene under the transcriptional control of the *C.albicans MAL2* promoter. This promoter is repressed in the presence of dextrose but is induced in the presence of maltose (Brown *et al.*, 1996). Western blot analysis of *C.albicans* cells carrying the *MAL2–SIR2* construction, grown in non-induced (dextrose) and induced (maltose) conditions showed that levels of Sir2 protein could be modulated by adjusting the carbon source (not shown).

The inducible *MAL2–SIR2* construct (or an empty vector used as a control) was integrated into the genome of both wild-type and *sir2/sir2 C.albicans* strains. The resulting strains were plated on rich medium (YEP) containing either dextrose or maltose as the carbon source, and the resulting colony morphologies were scored (Figure 6). Wild-type strains showed no observable switching regardless of whether the control vector or the *SIR2* expression vector was present in the genome or whether maltose or dextrose was used as the carbon source. A slight growth defect was observed in wild-type cells overexpressing the *SIR2* gene. In contrast, *sir2/sir2* cells carrying the *SIR2* expression vector showed a high frequency of switching in the non-inducing conditions (dextrose) and no observable switching under inducing conditions (maltose). Of special importance was the observation that non-sectored colonies obtained from maltose plates, regardless of their parental colony morphology, did not switch provided they were continuously plated in maltose. If however, they were plated in dextrose, then other colony morphologies readily arose. These results indicate that expression of *SIR2* is sufficient to suppress high-frequency switching and further show that *SIR2* expression stably maintains the cell in whatever colony morphology it inherited from its parent, be it smooth, wrinkled, or other type.



B

	Mating efficiency
vector	$<10^{-6}$
ScSIR2	0.5
CaSIR2	0.04

Fig. 2. *Candida albicans SIR2* complements the mating defect of *S.cerevisiae sir2* mutant. (**A**) Outline of the mating assay. *Saccharomyces cerevisiae* JY3433 (MATa, *trp1*, *leu2*, *sir2*) transformed either with control plasmids (pRD53, vector; pDM112, *S.cerevisiae SIR2*) or with a *C.albicans SIR2* expression plasmid (pRD-CaSIR2, *C.albicans SIR2*) were mixed with tester strain SF15 (MATα, *lys1*, *SIR2*) and diploid formation was scored by growth in minimal medium. (**B**) Mating efficiencies determined by quantitative mating assays. The values represent the average mating efficiency of at least two independent isolates for each transformant.

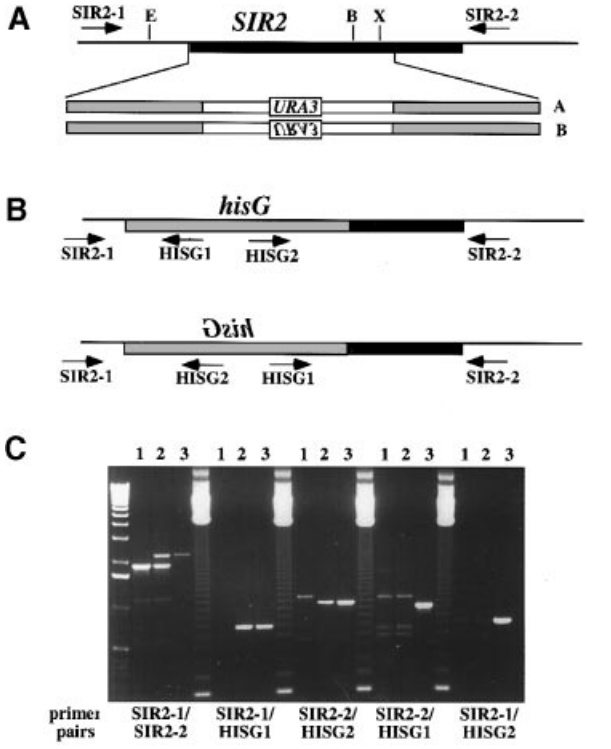


Fig. 3. Disruption of the *SIR2* gene in *C.albicans*. (**A**) The *SIR2* ORF (black bar) was replaced by the *hisG-URA3-hisG* cassette (gray-white bar) cloned in two different orientations (A and B). The SIR2-1 and SIR2-2 primers are located outside of the *sir2::hisG-URA3-hisG* transforming region. (**B**) Scheme of final disruptions after 'pop-out' the *URA3* gene. The primers used to check the presence of each disruption allele are shown. The disruption construct A is detected with the primer pairs SIR2-1/HISG1(680 bp) and SIR2-2/HISG2 (1010 bp). The disruption construct B is detected with the primer pairs SIR2-1/HISG2 (740 bp) and SIR2-2/HISG1 (950 bp).
(**C**) Identification of *C.albicans sir2* mutants by PCR analysis. Shown on the 1% agarose gel are five different sets of PCR products, each separated by a lane with a 123 bp ladder. Each set is the result of amplification with a specific primer pair, indicated at the bottom of the gel. In each set, lane 1 corresponds to CAI4 (*SIR2/SIR2*), lane 2 to JJCa5 (*SIR2/sir2::hisG*) and lane 3 to JJCa11 (*sir2::hisG/sir2::hisG*).

Candida albicans sir2/sir2 mutants exhibit spontaneous hyphal growth

The colony morphologies exhibited by the 3153A switching strain are related to the presence of particular types of cells in the colony (Radford *et al.*, 1994). Smooth colonies consist entirely of blastospores, whereas wrinkled or rough colonies consist of different proportions of true hyphae, pseudohyphae and blastospores. This correlation suggests that the different colony morphologies may result from heritable changes in the developmental pathways involved in the bud–pseudohyphae–hyphae transitions. A prediction of this idea is that a culture of the *sir2/sir2* mutant strain, because it switches so frequently, should exhibit a different spectrum of cell types than does a wild-type strain.

Consistent with this prediction, we found that inactivation of *SIR2* resulted in a stimulation of filamentous growth in liquid rich medium. A wild-type strain showed no filamentous (pseudohyphal and hyphal) forms during exponential growth in liquid YPD medium (rich medium). However, under these same conditions, the *sir2/sir2* null mutant cells assumed a variety of cell shapes, which included significant proportions of pseudohyphae and true hyphae (Figure 7A). We interpret this result as indicating that SIR2 affects the likelihood that a cell will switch from budding to filamentous growth.

We addressed this same issue for *C.albicans* colonies growing on plates. The *sir2/sir2* strain carrying the *SIR2* gene under the control of *MAL2* promoter (strain PCa10) was grown for several generations in the presence of maltose, conditions where it was phenotypically wild-type. Single cells were then spread on rich (YEP) solid medium with either dextrose or maltose present as the carbon source. After 12 h, we found (Figure 7B, left panel) that on the maltose plates, the colonies, which were all smooth, were composed exclusively of yeast-like cells (blastospores). In contrast, many of the colonies grown on dextrose showed sectors in which the cells grew in filamentous forms (Figure 7B, right panels). Under the same conditions, colonies derived from a wild-type strain (*SIR2/SIR2* with an additional copy of the *SIR2* gene under *MAL2* promoter control; strain PCa9) did not produce sectored colonies or hyphal growth (not shown). These results indicate that *SIR2* regulates the likelihood that on rich media a cell will switch from blastospore growth to filamentous growth. They further suggest that different degrees of filamentous growth are at least in part responsible for the different colony morphologies produced by the *sir2/sir2* mutant.

Genetic interactions between SIR2 and other hyphal regulatory factors

We suggested above that the *SIR2* gene affects the likelihood that a cell will switch from the budding form to the various hyphal forms. Other genes have been identified

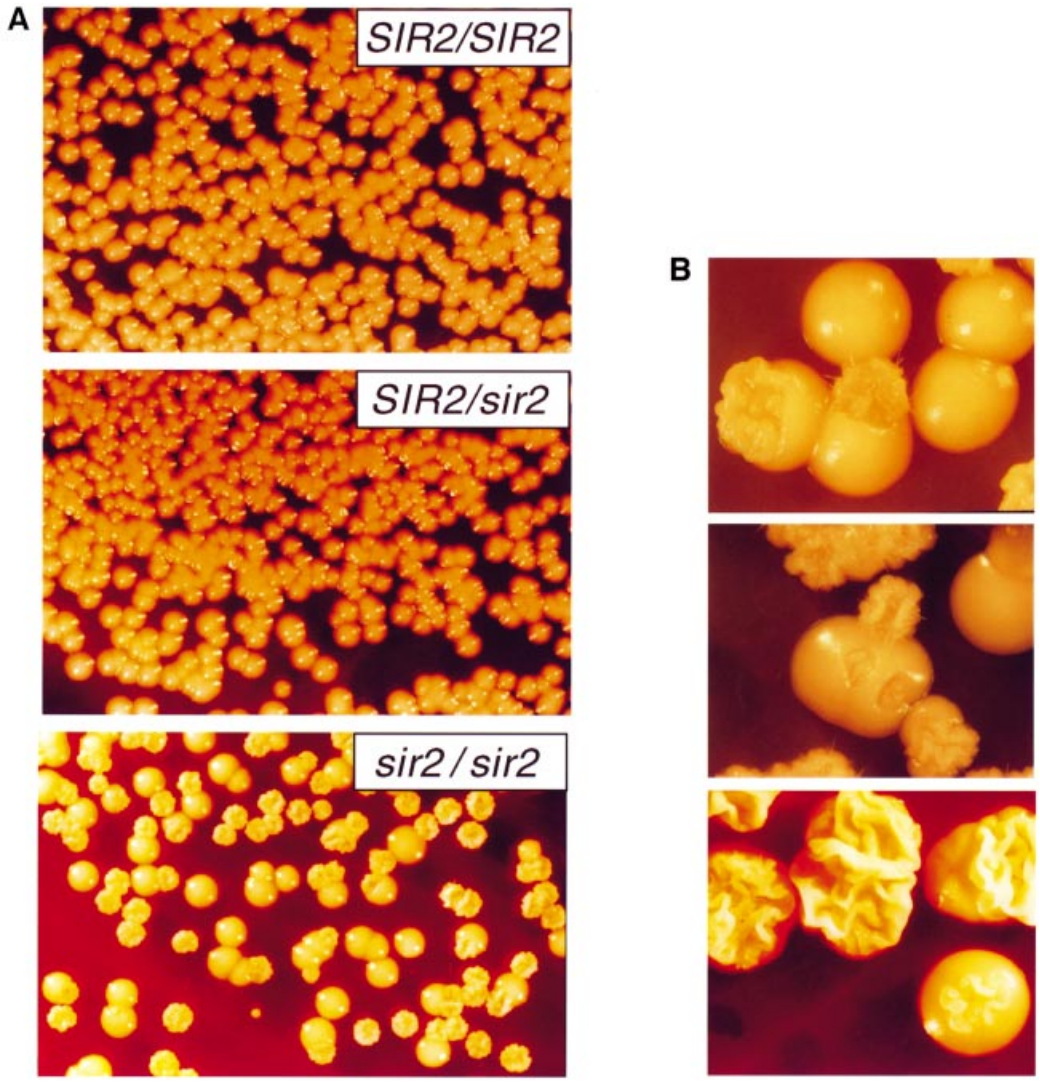


Fig. 4. Effect of *SIR2* disruption on colony morphology. (**A**) *SIR2/SIR2* cells (PCa1), heterozygous *SIR2/sir2* cells (PCa4) and homozygous *sir2/sir2* cells (PCa6) were plated in YPD solid medium and allowed to grow at 30°C for 2 days. Note the high number of variant colonies exhibited by the *sir2/sir2* strain. (**B**) Examples of sectoring in *sir2/sir2* mutants (PCa6 strain).

that affect the bud–pseudohyphae–hyphae transitions in response to external signals. The *CPH1* gene is the *C.albicans* homolog of *S.cerevisiae STE12*, and *C.albicans* cells carrying a deletion of this gene are partially defective in their ability to produce hyphal growth in response to environment signals, including starvation (Liu *et al*., 1994). The *TUP1* gene, on the other hand, is a negative regulator of hyphal growth, and deletion of this gene leads to constitutive hyphae formation under conditions (rich media) where a wild-type strain exists solely as blastospores (Braun and Johnson, 1997). We constructed the double mutants *sir2/sir2 cph1/cph1* and *sir2/sir2 tup1/tup1* and compared the switching frequency of these double mutants with strains carrying mutations in only single genes (Figure 8).

The *cph1/cph1* mutant had a low switching frequency typical of wild-type cells (~10^{-4}), as observed by the frequency of different colony morphologies. The double mutant *sir2/sir2 cph1/cph1* had the same high-frequency switching as observed for the single *sir2/sir2* mutant (Figure 8B and C), indicating that *CPH1* is not required for phenotypic switching. In contrast, we were unable to detect variant colony morphologies both in *sir2/sir2 tup1/tup1* and *tup1/tup1* mutant strains (Figure 8D and F). This result is not surprising given that the *tup1/tup1* mutant is locked in the filamentous form. The observation does suggest that *TUP1* functions downstream of the switching phenomenon regulated by *SIR2*.

Candida albicans sir2/sir2 mutants show karyotypic changes

In the analysis of morphological variants of *C.albicans* strain 3153A, karyotype variability was observed and suggested to reflect a relationship between phenotypic switching and chromosomal rearrangements (Rutschenko-Bulgac *et al*., 1990; Rutschenko-Bulgac, 1991; Soll, 1992; Ramsey *et al*., 1994). To investigate the possible relationship between these two features in *sir2/sir2* mutant strains, we used pulse-field gel electrophoresis (PFGE) to compare the overall chromosome patterns from a *sir2/sir2* mutant strain that was allowed to form colonies of variant phenotypes. In parallel, control wild-type colonies (*SIR2/SIR2*, which exhibited only the basic smooth phenotype) were also analyzed. The electrophoretic karyotype of mutant

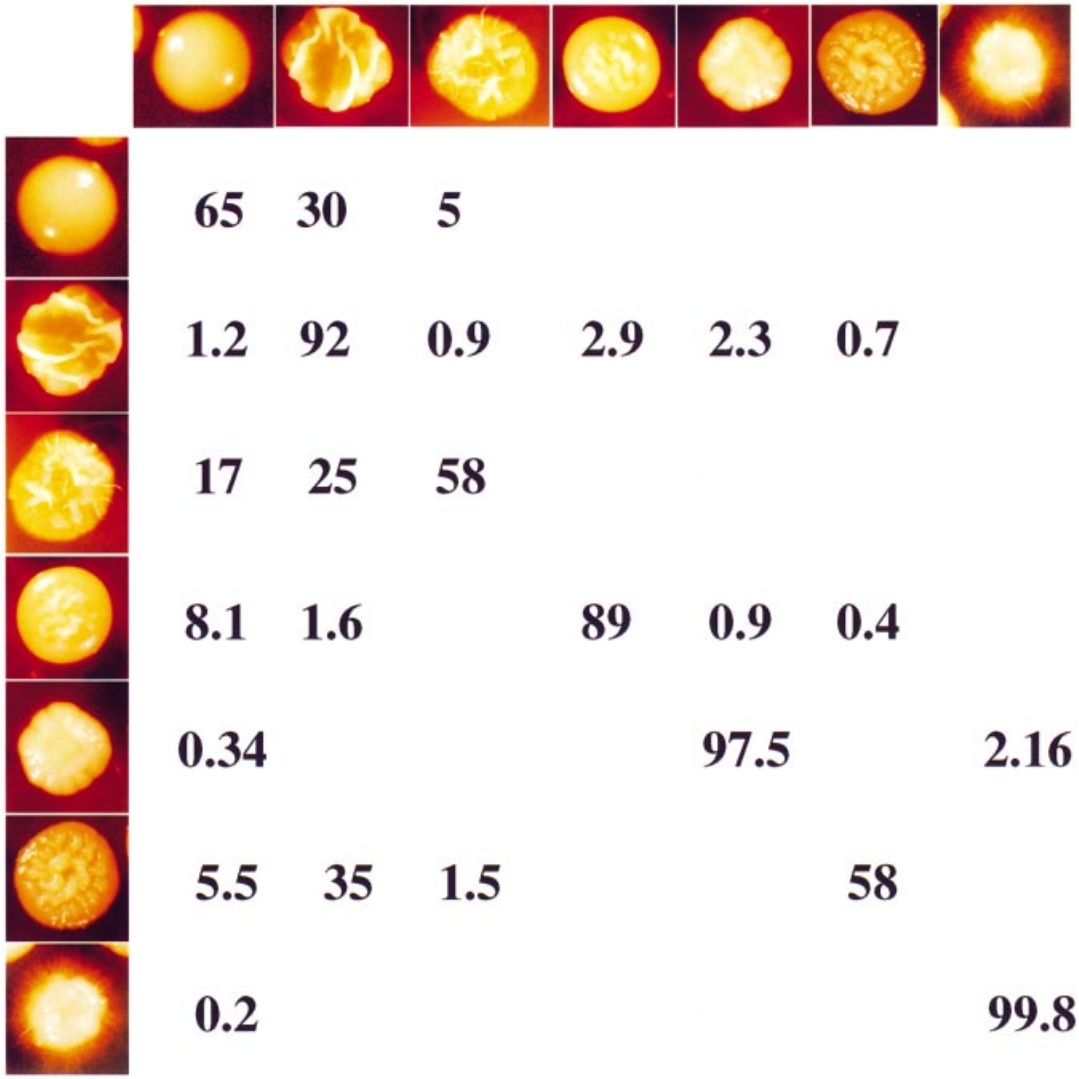


Fig. 5. Switching in *sir2/sir2* mutants is heritable. The proportion (in percentage) of colony variants emanating from a single non-sectored colony of the indicated morphology are shown in vertical columns. Colonies displaying variegated morphologies or otherwise indistinguishable morphologies (~30% of the total) were omitted from this analysis. Top bar from left to right: smooth, irregular wrinkled, fuzzy, semi-rough, scallop, regular wrinkled and heavy mycellated scallop. The results are the average of five single colonies of each parental type.

cells (Figure 9) showed changes in several chromosomes relative to the wild-type control, suggesting that Sir2 contributes to chromosome stability. However, there appeared to be no obvious correlation between a particular karyotype and a particular colony morphology; moreover, independent isolates with the same apparent colony morphologies sometimes exhibited different karyotypes (not shown). When the gels were run for a short time, all the *sir2/sir2* strains (irrespective of colony morphology) exhibited an additional DNA band whose size is between 100 and 50 kb; this band was not observed in DNA from wild-type cells. We cloned DNA fragments obtained from this band, and the sequence indicated that it is composed of ribosomal DNA (rDNA) sequences (not shown). Although we think it unlikely that the formation of these rDNA minichromosomes is directly related to the generation of variant colony morphologies, the result further suggests that Sir2 contributes to chromosome stability in *C.albicans*. Moreover, this phenotype is reminiscent of the behavior of *S.cerevisiae sir2* mutants (see Discussion).

Discussion

Control of phenotypic switching in C.albicans by a SIR2 gene

In this work, we report the cloning and sequence of a *C.albicans* gene with sequence similarity to the *S.cerevisiae SIR2* gene (Figure 1). We show that deletion of the two copies of the *SIR2* gene in *C.albicans* produces a dramatic phenotype: variant colony morphologies arise at a very high frequency (Figure 4). The variant colonies obtained from this *sir2/sir2* mutant strain resemble the colony morphologies described by Soll and collaborators as part of a phenotypic switching system (Slutsky *et al*., 1985). In contrast to the low frequency of variant colonies that appear in a wild-type strain (10^{-4}), the variant colonies appear with frequencies as high as one in every ten colonies in the *sir2/sir2* strain. In addition, expression of the *SIR2* gene under the control of a regulatable promoter suppressed this high-frequency switching phenotype, showing that this phenotype is a direct consequence of the *sir2* mutation (Figure 6). All of these considerations

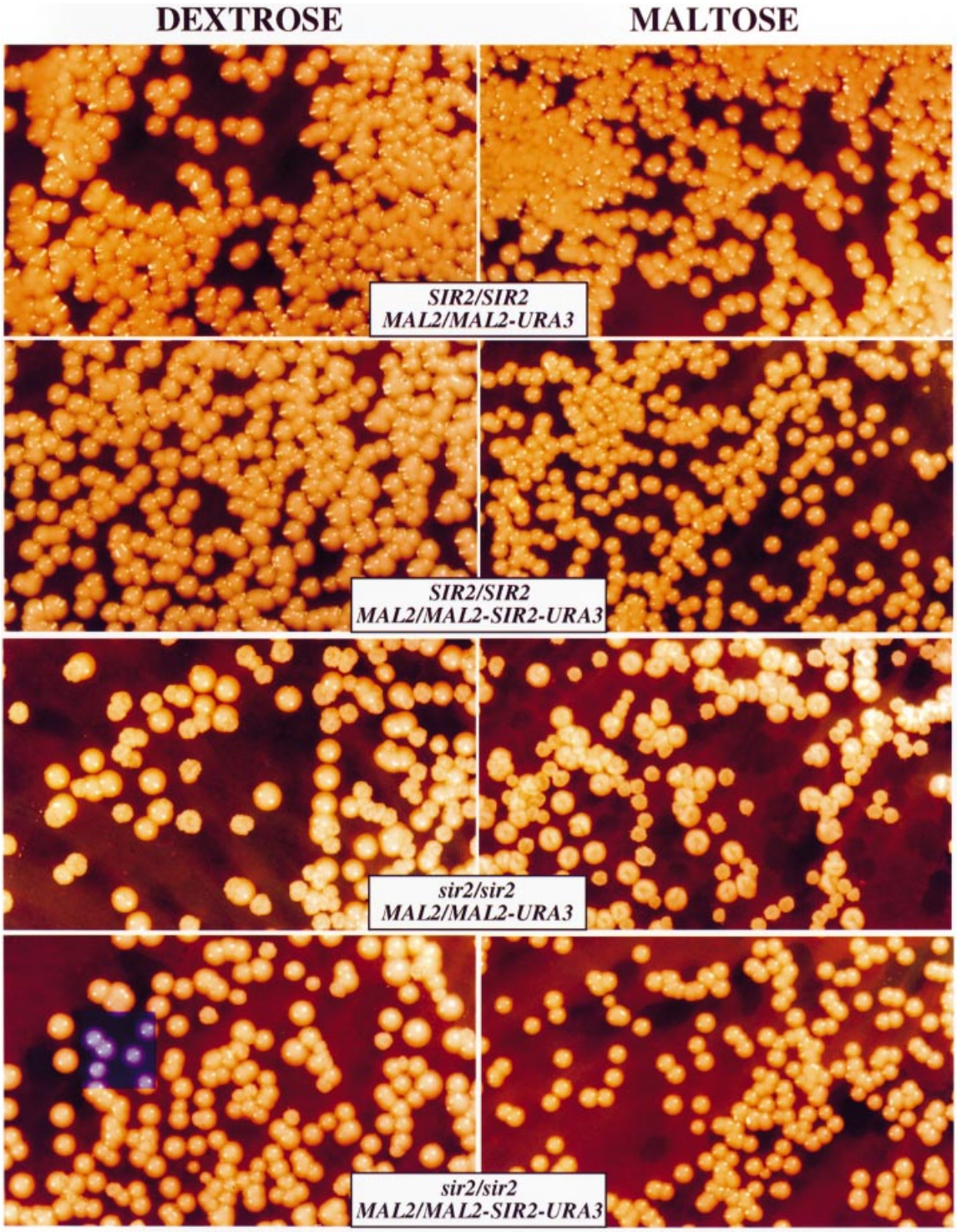


Fig. 6. Suppression of high-frequency switching by *SIR2* expression. Cells from wild-type (*SIR2/SIR2*) and *sir2* mutant (*sir2/sir2*) strains, carrying either a control vector (*MAL2–URA3*) or a *SIR2* expression vector (*MAL2–SIR2–URA3*) introduced in the *MAL2* locus, were grown in rich medium with either dextrose (non-induced conditions) or maltose (induced conditions). PCa1 (*SIR2/SIR2*, *MAL2/MAL2–URA3*), PCa9 (*SIR2/SIR2*, *MAL2/MAL2–SIR2–URA3*), PCa6 (*sir2/sir2*, *MAL2/MAL2–URA3*) and PCa10 (*sir2/sir2*, *MAL2/MAL2–SIR2–URA3*) cells were spread in YEP maltose plates, grown for 2 days at 30°C, and a single smooth non-sectored colony from each strain was removed and suspended in water. Appropriate dilutions were plated in either dextrose or maltose YEP medium plates. Photographs were taken after 2 days at 30°C. Dextrose represses the *MAL2* promoter, although low levels of Sir2 protein are still produced, which may explain the partial switching phenotype of JJCa11. Note the different colony morphologies present in strain PCa6 in dextrose and maltose plates.

support the idea that defects in the *SIR2* gene result in a high-frequency switching phenotype.

An additional phenotype of the *sir2/sir2* mutant strain is a high frequency of karyotypic changes, including the presence of an extra DNA band in the karyotype (Figure 9). Although several other karyotypic differences are apparent in Figure 6, the only change observed in all the *sir2/sir2* mutant strain isolates was the presence of this

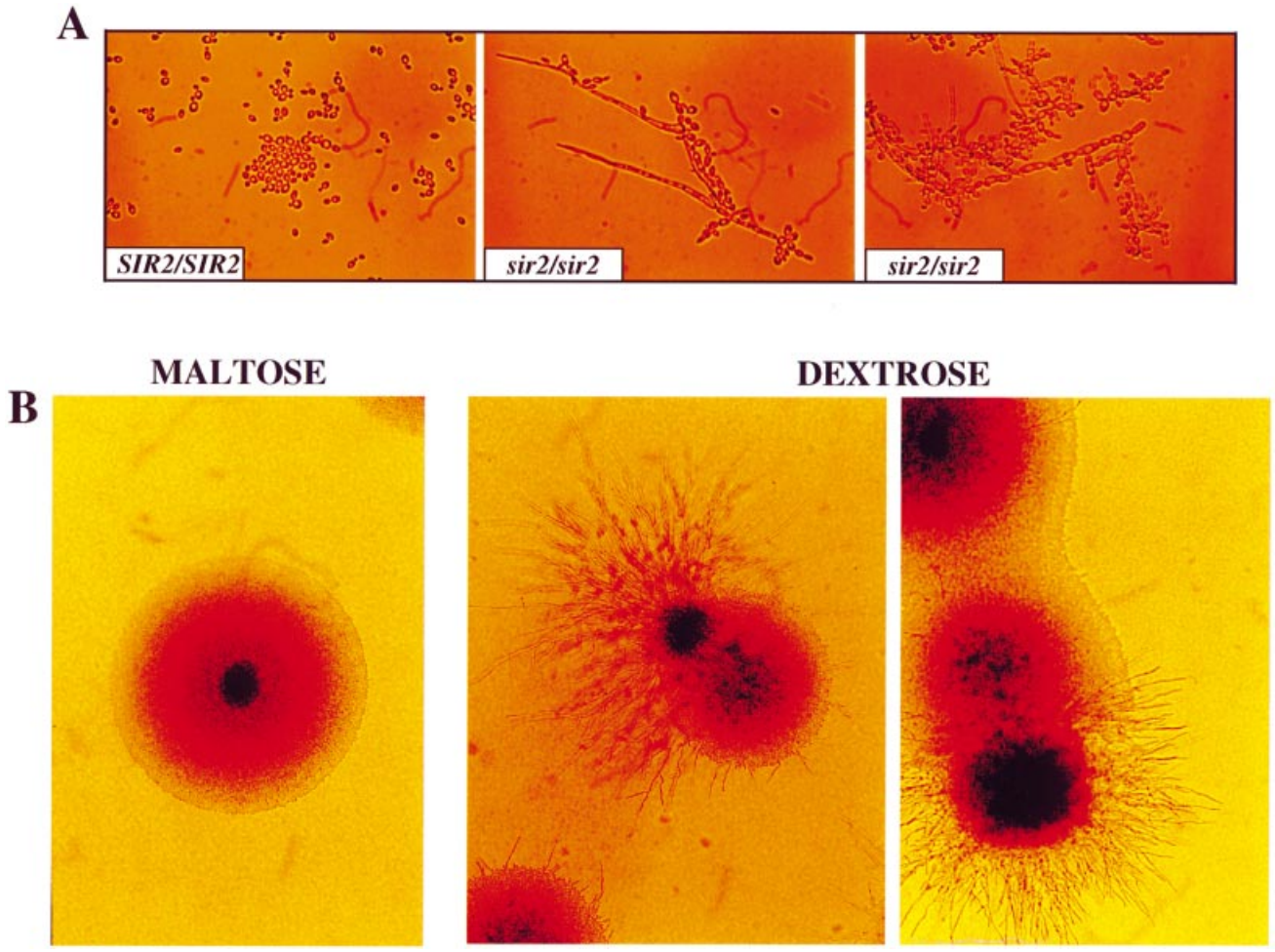


Fig. 7. Enhanced filamentous growth of *sir2/sir2* strains. (**A**) Overnight cultures from PCa1 (*SIR2/SIR2*) and PCa6 (*sir2/sir2*) were diluted 100-fold in fresh YPD liquid medium and allowed to grow for 4 h at 30°C before being photographed at 40× with phase optics. (**B**) Colony growth of strain PCa10 (*sir2/sir2*, *MAL2/MAL2-SIR2-URA3*) in YP-maltose (conditions where *SIR2* is expressed) and YPD plates (conditions where *SIR2* expression is repressed). Individual cells from an overnight culture in YPMaltose were diluted, placed under a coverslip in solid medium, and grown for 12 h at 30°C before being photographed at 25× with phase optics. Note the filamentous growth where switching has occurred.

extra band. This 'minichromosome' is composed, at least in part, of rDNA, a result that was, in part, anticipated by Soll and colleagues (Ramsey *et al*., 1994). In that work, the authors noted a correlation between an increase in switching frequency and an increase in recombination of the chromosomes that carry rDNA genes in *C.albicans* strain 3153A. They also noted that this instability of the rDNA chromosomes in *C.albicans* strain 3153A resembles the increase in intrachromosomal recombination between rRNA genes seen in a *S.cerevisiae sir2* mutant (Gottleib and Esposito, 1989). Although the level of resolution of our pulse-field gel is not sufficiently high to detect changes in the large chromosome that contains the rDNA gene cluster, we propose that the extra band seen in the *sir2/sir2* mutant strains is made up of rDNA which may have arisen through recombination between ribosomal RNA genes.

Hyphal growth and switching

The blastospore–filament transitions in *C.albicans* are affected by many environmental conditions including temperature, pH and the composition of the supporting medium (Odds, 1988). Under the appropriate conditions, an entire population of cells can be converted from blastospores to filaments and back again. Phenotypic switching (monitored by observing colony morphologies), on the other hand, occurs spontaneously on a single type of medium and at frequencies far lower than that seen in bud–filament transitions (Soll, 1992). More importantly, regardless of the type of variant colony from which they are isolated, *C.albicans* cells are still capable of undergoing the blastospore–filament transition in response to environmental stimuli (Anderson *et al*., 1989). However, the blastospore–filament transition and phenotypic switching are not entirely independent, as there is a relationship between particular colony morphology and the types of cells that make it up. Radford *et al*. (1994) reported that smooth colonies are composed entirely of blastospores, wrinkled colonies are composed almost entirely of branched hyphal cells with very few blastospores, and semi-rough colonies consist of both blastospores and true hyphae, but in a proportion different from that seen in wrinkled colonies. Fuzzy colonies consist of blastospores, pseudohyphae and true hyphae, with aerial hyphae in discrete areas. Finally, scallop colonies are composed entirely of pseudohyphal cells. For at least some of the colony variants obtained from the *sir2/sir2* mutant strains, we found similar correlations between the different colony

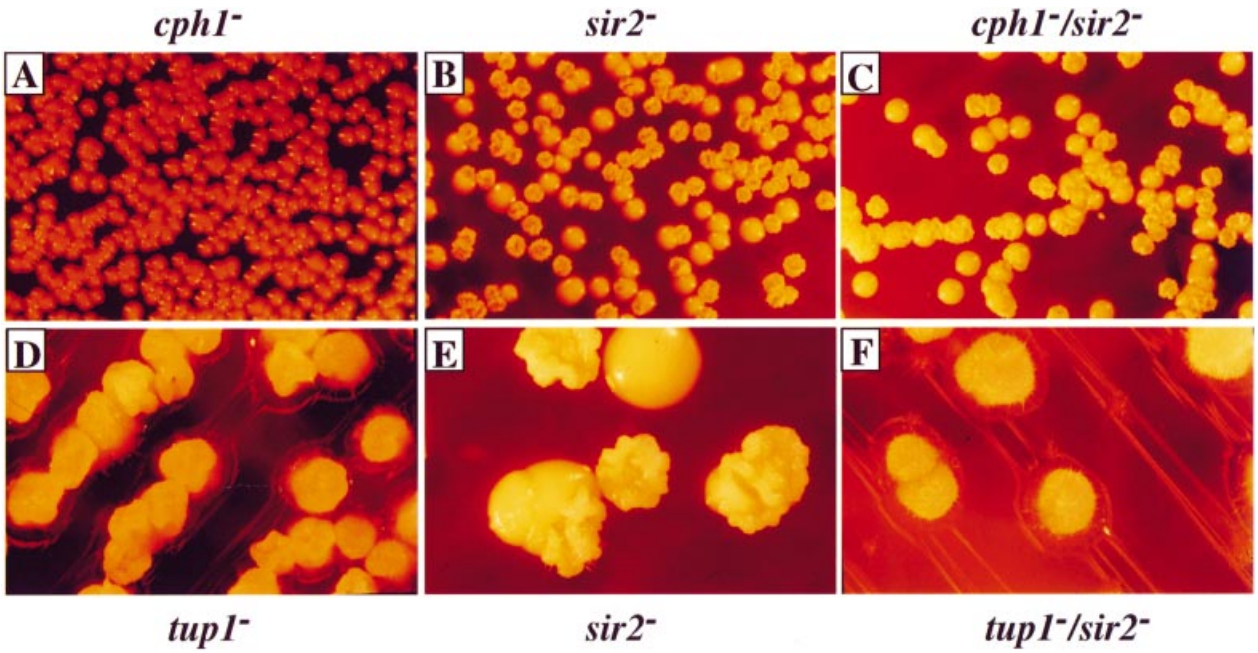


Fig. 8. Phenotypic switching in *cph1/cph1* and *tup1/tup1* strains. Colony morphologies of a *cph1/cph1* strain (JKC19) (**A**), a *sir2/sir2* strain (JJCa8) (**B**, **E**), a *cph1/cph1 sir2/sir2* strain (JJCa10) (**C**), a *tup1/tup1* strain (BCa2-10) (**D**), and a *tup1/tup1 sir2/sir2* strain (JJCa14) (**F**) are shown.

morphologies and the cellular types that comprised them (not shown). Consistent with this view, a *Candida tup1/tup1* strain, which is locked in the filamentous forms, does not show variant colony morphologies in response to a *sir2* deletion (Figure 8).

Since the *sir2/sir2* mutant strains switched colony morphologies with such high frequency, we thought it likely that we could detect an effect of *SIR2* in the frequency with which individual cells made the spontaneous blastospore–filament transition. We found that disruption of the *SIR2* gene increased the frequency of this transition. For example, *sir2/sir2* cells spontaneously underwent hyphal growth under conditions where the wild-type grew only as blastospores. This effect was observed in rich liquid medium (Figure 7A) as well as on rich solid medium (Figure 7B). In addition, the *sir2/sir2* mutant made the blastospore–filament transition more quickly than did the wild-type parent when exposed to filament inducing media such as 20% calf serum, corn meal medium or Spider medium (data not shown).

A variety of environmental signals can affect the blastospore–filament transition, and a large body of evidence suggests the existence of several parallel signaling pathways, any one of which can trigger the blastospore–filament transition (Odds, 1988; Liu *et al*., 1994; Braun and Johnson, 1997; Lo *et al*., 1997; Stoldt *et al*., 1997). It is possible that the phenotypic switching of colony types reflects a heritable change in one or more of these pathways. According to this idea, the proportion of blastospores, pseudohyphae and hyphae, and the timing of their appearance would be heritable, and differences in these parameters would lead to the different types of colony morphology. How might a change in a signaling pathway be inherited, and how might the *SIR2* gene control it? The simplest type of model proposes the existence of several genes in *C.albicans* (provisionally called RCM, regulators of colony morphology) whose expression activates or represses one or more of these signaling pathways. These RCMs could include known genes such as *TUP1* (described above) or *EFG1* (see Stoldt *et al*., 1997) which regulate the blastospore–filament transition. Normally, the expression patterns of these genes would change only rarely, accounting for the low frequency of colony switching seen in wild-type strains. The effect of the *SIR2* disruption, according to this model, would be to allow heritable changes in the expression pattern of these genes to occur at much higher frequencies. In the next section, we discuss possible mechanisms that could account for how the pattern of expression of these genes might be inherited.

Is the phenotypic switching of C.albicans related to gene silencing in S.cerevisiae?

The involvement of a *SIR2*-like gene in the control of phenotypic switching in *C.albicans* and the fact that this gene can, at least in part, substitute for the *S.cerevisiae SIR2* gene suggests two different mechanisms for phenotypic switching. Both models are based on known functions of the *SIR2* gene in *S.cerevisiae*.

The first model is based on the role of the *S.cerevisiae SIR2* gene in telomere position effect. According to this model, the RCM genes would be located in regions of silent chromatin, and their expression would change when the chromatin state in their proximity expands to repress them or contracts to activate them. In a wild-type strain, such changes would happen infrequently, but according to this model, the *SIR2* disruption would destabilize the chromatin structure allowing its contraction and expansion to occur at much higher frequency. A destabilization of this type naturally occurs at telomeres and is also seen in the silent mating-type cassettes in a *sir1* mutant strain (Pillus and Rine, 1989; Gottschling *et al*., 1990; for review see Rivier and Rine, 1992). A key feature of this model, based on the situation in *S.cerevisiae*, is that a particular

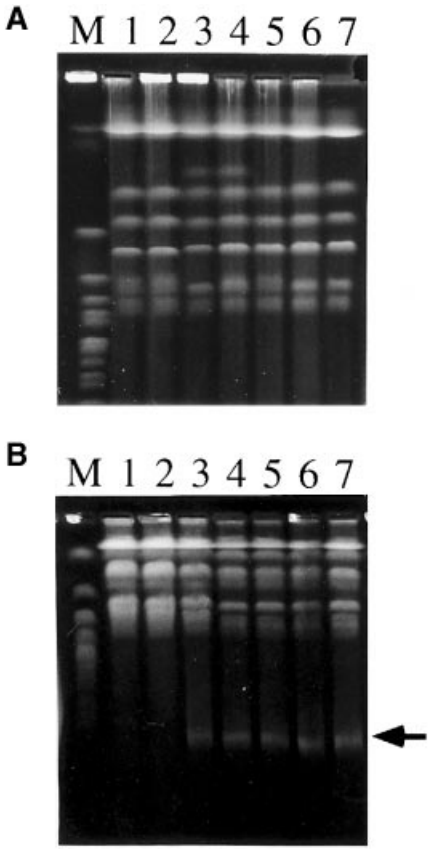


Fig. 9. Electrophoretic karyotypes of wild-type and *sir2/sir2* mutant cells. CHEF gel electrophoresis separation of chromosomes of wild-type strain 3153A (two independent colonies, lanes 1 and 2) and five independent *sir2/sir2* mutant colonies with different morphologies (fuzzy, lane 3; scallop, lane 4; semi-rough, lane 5; irregular wrinkled, lane 6; and smooth, lane 7). *S.cerevisiae* chromosomes are used as DNA size markers. (**A**) Running conditions are adequate for separation of *C.albicans* chromosomes (100 V with the following pulse regime: 1 min pulses for 6 h, 2 min pulses for 12 h, 4 min pulses for 16 h, and 7 min pulses for 20 h). (**B**) Running conditions are adequate for separation of *S.cerevisiae* chromosomes (100 V with 1 min pulses for 24 h). The arrow indicates the extrachromosomal band present in *sir2/sir2* mutant cells.

chromatin state, once established, is usually inherited by the two daughter cells following cell division.

According to the second model, the variant colony morphologies would arise from DNA rearrangements, and the role of *Sir2* would be to suppress such chromosome alterations through the formation of specialized chromatin. These rearrangements could include chromosomal translocations, other recombinational events, and even chromosome loss and duplication (see Rustchenko *et al*., 1997; Chibana *et al*., 1998; Janbon *et al*., 1998). According to this model, the role of *SIR2* would be to negatively regulate chromosomal alterations through the formation of specialized chromatin that would render parts of the genome less accessible to the DNA rearrangement machinery. A possible substrate for the *SIR2* product might be the major repeat sequences found on a number of *C.albicans* chromosomes (Chibana *et al*., 1998). Precedents for a role of *SIR2* in suppressing chromosome alterations exist from studies in *S.cerevisiae*. For example, cleavage of a specific DNA site by the HO endonuclease (an early step in the programmed DNA rearrangement leading to mating type interconversion) is blocked in the silent mating-type cassettes by a *Sir2*-dependent mechanism (Klar *et al*., 1981). Moreover, *Sir2* also suppresses recombination in the rDNA repeats of *S.cerevisiae* (Gottlieb and Esposito, 1989). According to this model, the *C.albicans sir2/sir2* mutant would undergo a much higher than normal level of chromosomal alteration and therefore exhibit a higher frequency of colony variants. Although the karyotypic analysis (Figure 9) supports this view, it is more difficult to account for the apparent reversibility of switch between some of the different colony forms. However, a single colony morphology could, in principle, result from several different types of chromosomal alteration. Alternatively, some of the chromosome alterations themselves could be reversible.

Candida albicans has no known sexual cycle, and the ability to switch between different phenotypes is an alternative way to obtain the variability required to survive an uncertain environment. This idea raises the possibility that some types of external stress could inactivate the

Table 1. *Candida albicans* strains

Strain	Genotype	Sources
SC5314	Wild type	(Gillum et al., 1984)
CAI4	*ura3::1 imm434/ura3::1 imm434*	(Fonzi and Irwin, 1993)
JKC18	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG*	(Liu et al., 1994)
JKC19	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG-URA3-hisG*	(Liu et al., 1994)
BCa2-9	*ura3::1 imm434/ura3::1 imm434 tup1::hisG/tup1::hisG*	(Braun and Johnson, 1997)
BCa2-10	*ura3::1 imm434/ura3::1 imm434 tup1::hisG/tup1::hisG-URA3-hisG*	(Braun and Johnson, 1997)
JJCa2-3	*ura3::1 imm434/ura3::1 imm434 SIR2/sir2::hisG-URA3-hisG*	This study
JJCa4-2	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG SIR2/sir2::hisG-URA3-hisG*	This study
JJCa5	*ura3::1 imm434/ura3::1 imm434 SIR2/sir2::hisG*	This study
JJCa6-1	*ura3::1 imm434/ura3::1 imm434 tup1::hisG/tup1::hisG SIR2/sir2::hisG-URA3-hisG*	This study
JJCa7	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG SIR2/sir2::hisG*	This study
JJCa8	*ura3::1 imm434/ura3::1 imm434 sir2::hisG/sir2::hisG-URA3-hisG*	This study
JJCa9	*ura3::1 imm434/ura3::1 imm434 tup1::hisG/tup1::hisG SIR2/sir2::hisG*	This study
JJCa10	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG sir2::hisG/sir2::hisG-URA3-hisG*	This study
JJCa11	*ura3::1 imm434/ura3::1 imm434 sir2::hisG/sir2::hisG*	This study
JJCa14	*ura3::1 imm434/ura3::1 imm434 tup1::hisG/tup1::hisG sir2::hisG/sir2::hisG-URA3-hisG*	This study
PCa1	*ura3::1 imm434/ura3::1 imm434 MAL2/MAL2-URA3*	This study
PCa4	*ura3::1 imm434/ura3::1 imm434 SIR2/sir2::hisG MAL2/MAL2-URA3*	This study
PCa6	*ura3::1 imm434/ura3::1 imm434 sir2::hisG/sir2::hisG MAL2/MAL2-URA3*	This study
PCa9	*ura3::1 imm434/ura3::1 imm434 MAL2/MAL2-SIR2-URA3*	This study
PCa11	*ura3::1 imm434/ura3::1 imm434 sir2::hisG/sir2::hisG MAL2/MAL2-SIR2-URA3*	This study

SIR2 gene in wild-type *C.albicans* and thereby activate phenotypic switching when it might be most beneficial. This idea has some support from the recent discovery that gene silencing in *S.cerevisiae* (which requires the *SIR2* gene) is inactivated as cells become older (Sinclair *et al*., 1998). Perhaps a different type of stress would produce a similar lifting of silencing in *C.albicans*.

Materials and methods

Strains and media

Saccharomyces cerevisiae strains used in this work are JRY2334 (*MATa ade2 trp1 can1 leu2 his3 lys2*), JRY3433 (JRY2334 *sir2::HIS3*) and SF15 (*MATα lys1*). *Candida albicans* strains are listed in Table I. *S.cerevisiae* standard media were prepared as described in Sherman *et al*. (1986). For culturing *C.albicans* YPD or YPM media (1% Yeast Extract, 2% Bacto-peptone and either 2% glucose or maltose) were used as rich medium. For minimal medium, CM or CM-uri (CM lacking uridine and uracil) was used (Ausubel *et al*., 1989).

Cloning of SIR2

By aligning *S.cerevisiae* Sir2, *S.cerevisiae* Hst1 and *K.lactis* Sir2, we defined two regions of conserved motifs: SLGIPDFRS and YTQNIDNL. We synthesized the following degenerate oligonucleotides: S1: 5′-TCWTTRGGKATCCCKGAYTTCAGATC-3′ and S2: 5′-CAAATTAT-CAATRTTYTGZGTATA-3′ (K:T/G, R:A/G, W:A/T, Y:T/C, Z:C/A). These oligos were used for amplification with 100 ng of *C.albicans* SC5314 DNA as template in a volume of 50 µl. PCRs contained 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 30 pmol of primers and 5 U of *Taq* polymerase (Boehringer Mannheim). After 30 cycles of amplification (1 min 94°C, 1 min 50°C and 2 min 72°C), reactions were incubated for 10 min at 72°C and the amplification products were separated on an agarose gel. A single fragment of 250 bp was isolated, treated with T4 DNA polymerase and cloned into pUC19 for sequencing. Sequences flanking this fragment were obtained with a PCR-walking strategy (Siebert *et al*., 1995) using the GenomeWalker system (Clontech) as directed by the manufacturer. A 4 kb fragment was assembled and sequenced. The analysis of the sequence revealed the presence of a 1.5 kb ORF with high sequence similarity to *S.cerevisiae* Sir2. Oligonucleotides flanking this ORF were synthesized and used to amplify in two independent reactions a fragment of 2.5 kb using *Pfu* polymerase (Stratagene). These two reaction products were cloned independently and sequenced on both strands to determine the precise sequence of the cloned fragment. Sequence comparison with the databases was carried out using the program BLAST (Altschul *et al*., 1990). Alignment, kindly performed by Burk Braun, utilized the program PILEUP (GCG, Inc.).

Complementation of mating defect in a sir2 S.cerevisiae mutant

The *C.albicans SIR2* ORF was amplified with *Pfu* polymerase (Stratagene) and the primers: GSIR1 (5′-CCGGAATTCATGACAAC-TTTTTGGTCACAA-3′) and GSIR2 (5′-CGCGGATCCTCATTTTT-TTGTCGAAGTTGA-3′). The product was digested with *Eco*RI–*Bam*HI and cloned into pUC19 digested with the same restriction enzymes. The resulting vector (pUC-SIR2) was used to verify the sequence and a 1.6 kb *Eco*RI–*Sal*I fragment derived from this plasmid, which contains the *SIR2* ORF, was inserted in the *CEN/ARS/URA3/*P_{GAL1} expression vector pRD53 (provided by R.Deshaies, California Institute of Technology) to form the *S.cerevisiae* expression plasmid pRD-CaSIR2. The control vectors pRD53 and pDM112 (pRD53 with the *S.cerevisiae SIR2* ORF cloned under the control of P_{GAL1}, a gift of D.Moazed, UCSF) as well as pRD-CaSIR2 were used to transform both JRY2334 (*SIR2*) and JRY3433 (*sir2*). Quantitative mating assays were performed in YPG essentially as described by Sprague (1991). SF15 was used as a mating tester strain.

SIR2 disruption construct

The disruption construct was made by first synthesizing short fragments from the 5′ (5′*SIR2*) and 3′ (3′*SIR2*) ends of *SIR2* gene and inserting the *hisG-URA3-hisG* cassette (obtained from pMB7; Fonzi and Irwin, 1993) between these fragments in two different orientations. Oligonucleotides 5′*SIR2*A (5′-CGGGGTACCCACGACAAAAACGCGTAAGTC-3′) complementary to positions –348 to –328 (A of the translation initiation codon ATG is designated +1) of the *SIR2* gene and oligo 5′*SIR2*B (5′-CGCGGATCCCTCGAGTACTAGAGGATTTCTCTCA-AA-3′) complementary to the positions –21 to –1 of the *SIR2* gene, were used to amplify the fragment 5′*SIR2*. This 0.34 kb fragment is flanked by a *Kpn*I site at its 5′ end and a *Xho*I and *Bam*HI sites at its 3′ end. Oligo 3′*SIR2*A (5′-CGCGGATCCAGGTCGACGTA-GAAATACCTGCACCAGTAACTACCATG-3′) complementary to the positions +888 to +917 in the *SIR2* coding region and the oligo 3′*SIR2*B (5′-CCCAAGCTTTGTAAGTTGAAACTTCTATTAGGAATAGGA-3′) complementary to the positions +1357 to +1387 in the *SIR2* coding region, were used to generate the 3′*SIR2* fragment. This 0.5 kb fragment was flanked by *Bam*HI and *Pst*I sites at its 5′ end and a *Hin*dIII site at its 3′ end. The 5′*SIR2* and 3′*SIR2* fragments were cloned in a tripartite ligation as *Kpn*I–*Bam*HI (fragment 5′*SIR2*) and *Bam*HI–*Hin*dIII (fragment 3′*SIR2*) in pUC19 digested with *Kpn*I and *Hin*dIII. This vector, pUCKO, was used to introduce the disruption cassette. An ~4 kb *Bgl*II–*Sal*I fragment was inserted into pUCKO digested with *Bam*HI–*Sal*I to generate pSIR2KO-A. In a similar strategy but with the cassette element obtained as a *Bgl*II–*Pst*I fragment containing the *hisG-URA3-hisG* cassette from pMB7 (Fonzi and Irwin, 1993) was inserted into pUCKO digested with *Bam*HI–*Pst*I to generate pSIR2KO-B. These *SIR2* disruption plasmids contain 5′*SIR2-hisG-URA3-hisG-*3′*SIR2* but with the disruption cassette in inverted directions. In both cases the disruption fragment was released when the plasmid was digested with *Kpn*I and *Hin*dIII.

Disruption of the SIR2 gene

Sequential disruption of both alleles of the *SIR2* gene was performed using the strategy developed by Fonzi and Irwin (1993), with the difference that we used two different versions of the disrupting plasmid, placing the *hisG-URA3-hisG* cassette in both orientations relative to the flanking genomic DNA. In this way, the PCR assay gives unique products for successive disruption events using the different constructs. The *C.albicans* strains CAI4 (Fonzi and Irwin, 1993), JKC18 (Liu *et al*., 1994) and BCa2-9 (Braun and Johnson, 1997) were transformed with the *SIR2* disruption construct obtained from pSIR2KO-A. Ura^+ transformants were screened for the *SIR2*/*sir2::hisG-URA3-hisG* genotype by PCR using oligo SIR2-1 (5′-AAAGAGGGAGGAGCATCATAT-3′) complementary to the positions –391 to –371 of the *SIR2* gene, oligo SIR2-2 (5′-TCATTTTTTTGTCGAAGTTGA-3′) complementary to the positions +1517 to +1547 in the *SIR2* coding region, and two oligonucleotides complementary to the *hisG* sequence: HISG1 (5′-GCGCGTGGCGATGCACATGGTCAG-3′), which is 290 nt from the *Bgl*II site used to clone and HISG2 (5′-GCGCGGCGGTTGAGTA-GCTCT-3′) which is 350 nt from the *Pst*I or *Sal*I sites used to clone. PCR amplifications with the primer pairs SIR2-1/HISG1 and SIR2-2/HISG2 produce fragments of 680 and 1010 bp, respectively, in the positive strains. JJCa2-3 is *ura3/ura3, SIR2/sir2::hisG-URA3-hisG*; JJCa4-2 is *ura3/ura3, cph1::hisG/cph1::hisG, SIR2/sir2::hisG-URA3-hisG*; JJCa6-1 is *ura3/ura3, tup1::hisG/tup1::hisG, SIR2/sir2::hisG-URA3-hisG*. To disrupt the second chromosomal copy of *SIR2*, we derived *ura3/ura3 SIR2/sir2::hisG* strains by streaking JJCa2-3, JJCa4-2 and JJCa6-1 on complete medium containing 5-FOA (which selects for recombinants that have lost *URA3*). The resulting strains JJCa5, JJCa7 and JJCa9, respectively, were transformed with the *SIR2* disruption construct obtained from pSIR2KO-B. We repeated the screening process with the primer pairs SIR2-1/HISG2 and SIR2-2/HISG1, which produced fragments of 740 and 950 bp, respectively. Clones which showed the presence of the two different insertions were selected producing the strains JJCa8, JJCa10 and JJCa14, respectively. The strain JJCa8 was streaked in 5-FOA to produce ura^- strains (JJCa11). CAI4 (*ura3/ura3*), JJCa5 (*ura3/ura3, SIR2/sir2::hisG*) and JJCa11 (*ura3/ura3, sir2::hisG/sir2::hisG*) were used for an additional round of transformation with the plasmid pAU15 (see below) digested with *Xcm*I, in order to integrate a *URA3* gene near the *MAL2* gene and produce isogenic ura^+ strains (PCa1, PCa4 and PCa6, respectively).

Phenotypic characterization of C.albicans sir2 mutants

To assess the switching frequencies of primary and subsequent colonies, cells were removed from individual clonal colonies of 2 day-old-cultures, suspended in water, counted, and plated at ~200 c.f.u. per plate. These plates were incubated for 3 days at 30°C and the different kinds of colonies present were counted and classified (Figure 5).

Construction of a strain with maltose-dependent expression of SIR2

To construct a maltose-regulated *SIR2* gene, we amplified the *SIR2* ORF with *Pfu* polymerase and the following primers: SIRFLAG1

(5′-CCGCTCGAGATGGATTACAAAGATGATGATGATAAAACATC-ATTTTGGTCACAAACAATCAATCGCCAAAAT-3′) and SIRFLAG2 (5′-GGACTAGTGGGTGGTTTCTTGGATTTCAA-3′). This amplification product gives an N-terminal fusion between the FLAG epitope and the SIR2 protein. The 2 kb fragment was cloned as *Xho*I–*Bam*HI into pAU15 [a derivative from pDBV52 (Brown *et al*., 1996), gift of A.Uhl, UCSF]. In this construction the *FLAG–SIR2* gene is transcribed by the *C.albicans MAL2* promoter with *URA3* as a selectable marker. The plasmid pMAL-SIR2 was digested with *Xcm*I and integrated in the *MAL2* locus of the strains CAI4 or JJCa11 to produce the ura$^+$ strains PCa9 and PCa10, respectively. Induction of the promoter requires growth in 2% maltose as the only carbon source. Addition of 2% glucose represses this promoter (Geber *et al*., 1992). Expression of the *FLAG–SIR2* gene was assessed by Western blot using the M2 anti-FLAG antibody.

Other methods

Chromosomal DNA from *C.albicans* was obtained as described in Scherer and Stevens (1987). Transformations of *S.cerevisiae* cells were performed by a modified lithium acetate technique (Gietz *et al*., 1993). Transformations of *C.albicans* were as described in Braun and Johnson (1997). PFGE was performed according to Ramsey *et al*. (1994) using a CHEF-DRII system (Bio-Rad).

DDBJ/EMBL/GenBank accession number

The accession number for the SIR2 sequence reported in this paper is AF045774.

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