High Frequency Variation of Colony Morphology and Chromosome Reorganization in the Pathogenic Yeast *Candida albicans*

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A clinical isolate of the pathogenic yeast *Candida albicans* varied in its colony morphology from smooth (o-smooth) to semi-rough type (SRT) and concomitantly lost its virulence for mice. In terms of DNA content, the smooth parent was near triploid when *Saccharomyces cerevisiae* strains of known ploidy were used as references. The SRT variant showed several features characteristic of polyploidy. From the SRT variant, revertant-like smooth (r-smooth) variants with recovered virulence were derived at a frequency of 5×10^{-3} . The results of pulsed-field gel electrophoresis on chromosomal DNA showed changes in patterns of chromosome-sized DNA bands in the SRT variant as well as in r-smooth variants, which correlated with these variations. Correlations between colony morphology, state of ploidy and virulence of this asporogenous yeast are considered.

INTRODUCTION

Genetic switch processes with gene rearrangement have been found to alter expression of cell surface constituents in unicellular micro-organisms, including phase variation of bacterial flagella (Iino & Kutsukake, 1983; Simon *et al.*, 1980), antigenic variation in a relapsing fever *Borrelia* (Meier *et al.*, 1985), pilus expression in *Neisseria* (Meyer *et al.*, 1982) and trypanosome antigenic variation (Borst & Cross, 1982). In the dimorphic, pathogenic yeast *Candida albicans*, high-frequency switching of colony morphology has been examined, in which this asexual yeast switches heritably at a frequency of 10^{-2} to 10^{-4} between at least seven colony-morphology phenotypes (Slutsky *et al.*, 1985) or between white and opaque ones (Slutsky *et al.*, 1987). However, because systems for genetic analysis are limited in this yeast, no clear mechanism has been deduced for these switching phenomena.

Although many *C. albicans* strains are diploid (Whelan & Magee, 1981), we recently discovered the phenomenon of ploidy shift in a strain with polyploidy consisting of at least two different states of ploidy, diploid (or near diploid) and tetraploid (or near tetraploid): diploid cells in a population of the strain enter G2-delay and then bypass the M-phase, resulting in a shiftup of ploidy state; tetraploid cells engage in reductional nuclear division during shiftdown of ploidy state, producing diploid daughter cells. During the process of shiftdown, multiple spindles can be observed by electron microscopy, resembling meiosis II of sporulation in *Saccharomyces cerevisiae* (Suzuki *et al.*, 1986b). However, no functional significance of this ploidy shift in *C. albicans* was apparent. Here, we investigate the high-frequency variation (or switching) of colony morphology between smooth and semi-rough types, and the correlation of this variation with pathogenicity.

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Abbreviations: IW, irregular wrinkle; SRT, semi-rough type; DABA, diaminobenzoic acid; DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PFGE, pulsed-field gel electrophoresis.

METHODS

Organisms, media and growth conditions. A strain of *C. albicans* (NUM961) was isolated from a patient at Nagoya University Hospital; it was of serotype A (determined by using Candida Check Serum supplied by Iatron Laboratories, Tokyo, Japan), and showed production of both germ tubes and chlamydospores when tested according to McGinnis (1980). *S. cerevisiae* strains X2180-1A (*MATa*), X2180-1B (*MATα*) and X2180-2D (*MATαa*) were kindly given to us by Dr Ohya, Tokyo University, and used as standards for DNA content and chromosome banding patterns. An authentic *C. albicans* strain, STN57, a derivative of CBS5736 (Suzuki *et al.*, 1986a), was also used as a standard. Yeast cells were grown in YPD medium [containing, per litre, 10 g Bactopeptone (Difco), 10 g yeast extract and 20 g glucose] with shaking (120 strokes min⁻¹) at either 30 or 37 °C as described below. For solid medium, 2% (w/v) agar was added to YPD medium. In order to isolate colony-morphology variants from the parent, yeast cells grown on YPD plates were suspended in sterile 0.5% NaCl, plated onto YPD plates after appropriate dilutions and incubated at either one of the two temperatures. For examination of virulence for mice, cells were incubated in YCB-AS broth (Shimidzu *et al.*, 1987), composed of 11.7 g Yeast Carbon Base (Difco) and 5.0 g ammonium sulphate per litre of distilled water.

DNA determination. DNA content per cell was determined using diaminobenzoic acid (DABA) by the method of Hesse *et al.* (1975). Calf thymus DNA (Sigma) was used as reference.

Fluorescent-microscope photometry. Yeast cells were harvested from YPD media, fixed in 50% (v/v) ethanol, stained with propidium iodide (10 µg ml⁻¹) in the presence of RNAase A (1 mg ml⁻¹) and examined by fluorescent-microscope photometry as described previously (Suzuki *et al.*, 1986b). A Zeiss MPM03FL photometer was used and the nuclear DNA fluorescence of individual cells was determined by measuring a random sample of 200 to 400 cells from a given culture.

Visualization of nuclear spindles by fluorescence microscopy. Exponentially growing yeast cells in YPD media were fixed with 3% (w/v) paraformaldehyde and stained with monoclonal antitubulin YOL1/24 (Sera-Lab), plus fluorescein isothiocyanate (FITC)-anti-rat IgG as described by Kilmartin & Adams (1984). Slides were mounted in *p*-phenylenediamine/90% (w/v) glycerol containing 4',6-diamidino-2-phenylindole (DAPI, 0.05 µg ml⁻¹). For phase-contrast-DAPI fluorescence, an Olympus UVFL 100PL objective was used with a combination of UG1, DM400 and L435 filters. For FITC fluorescence, a UVFL 100 objective was used with a combination of BP490, EY455, BM500 and O515 filters.

Virulence test. Cells cultured on YCA-AS broth were collected by centrifugation, washed twice and resuspended in saline (0.85% NaCl). Dilutions of the cell suspension were made for inocula by counting the cells with a haemocytometer. Viable cells of each strain were 85 to 95% of the total cells as determined by plate counting. Male general-purpose albino Swiss mice (ICR strain), 5 to 7-weeks-old, were used. Mice were injected via the lateral tail vein with 10⁷, 10⁶ or 10⁵ cells of each inoculum and observed for up to 50 d after infection.

Examination of proteinase secretion. The plate biochemical test described by Odds & Abbott (1980) was used to examine proteinase production by individual strains. Basal medium no. 2 was prepared according to their method; bovine serum albumin (BSA; fraction V, Sigma) was added, and the medium was then filter-sterilized and plated by adding agar (Noble agar, Difco). The final concentration of BSA was 0.1%.

Pulsed-field gel electrophoresis (PFGE). Sample preparation for PFGE was according to Carle & Olson (1984) and Magee & Magee (1987). After several trials, we decided to use either a 2.5 min pulse time for a 18 to 24 h run to obtain good resolution of DNA sized around 1000 kbp or a 5 min pulse time for a 45 to 48 h run to resolve the higher molecular-size DNA bands. A 0.8% agarose (Wako Chemicals) gel (20 × 20 cm) was prepared. The angle formed at the sample loading wells between the two alternative fields was adjusted to 120°. The Pulsaphor system (Pharmacia-LKB) was used with a constant voltage of 300 V and with the temperature maintained at 10 °C.

RESULTS

Spontaneous semi-rough variants derived from the smooth parent NUM961

Strain NUM961 had a smooth colony morphology on YPD plates and cells removed from the surface of such a smooth colony all had the spherical yeast form (Fig. 1a). As smooth colonies grown at both 30 and 37 °C had an unwrinkled surface, with no aerial mycelia, we will refer to the original parent strain as 'o-smooth' according to the description by Slutsky *et al.* (1985). When large numbers of cells of o-smooth were plated as colonies on YPD plates, a semi-rough variant colony appeared spontaneously at a frequency of 3×10^{-5} . Semi-rough colonies of this variant had a rough surface in the central portion of a colony with an apparently smooth periphery. When an original isolate of this semi-rough variant morphology was plated as colonies, the variant phenotype persisted in more than 99% of the offspring in successive clonal platings. We will refer to this semi-rough type as 'SRT'. The SRT did not appear to fit any of the descriptions of Slutsky *et al.* (1985, 1987) but rather seemed to share several features in common

Fig. 1. Morphological differences between the o-smooth parent and the SRT1 variant of *C. albicans* NUM961 as shown by phase-contrast (*a, c*) and fluorescent microscopy (*b, d*). Cells were stained with propidium iodide. (*a, b*), Cells from a mature smooth colony of the o-smooth parent grown on YPD agar; (*c, d*), cells from a mature SRT1 colony. An Olympus BH2-RFK was used with combination of BP545, DM580 and O590 filters. Bar, 10 μ m.

with the colony types classified as 'A' by Brown-Thomsen (1968). Characteristics of the SRT colony morphology became more distinct when the variant cells were cultured at 37 °C rather than at 30 °C or lower temperatures. No difference was detected in the antigenic determinants of the cells of SRT colonies compared to o-smooth cells using commercial Candida Check Serum. In contrast to o-smooth cells, those removed from the surface of SRT colonies consisted of both budding yeasts and pseudohyphal cells (Fig. 1 *c*). The size of yeast cells of SRT colonies was heterogeneous: small cells, 3 to 4 μ m long, similar to those of o-smooth; and large cells, ovoid, pyriform or clavate in shape. The large cells were 3.5 to 7 μ m wide and 4 to 20 μ m long. These different-sized cells of the SRT variant seemed to us to be in a different ploidy state, as described previously (Suzuki *et al.*, 1986*b*). In addition to the SRT colonies, irregular-wrinkle (IW) colonies appeared spontaneously at a frequency of 1×10^{-4} . These IW colonies were different from the SRT in that the whole of an IW colony had a wrinkled surface. One representative spontaneous SRT variant, designated SRT1, was characterized further.

DNA content and chromosomal banding pattern of SRT1

In order to examine the ploidy of this variant, DNA content determinations were done on both the o-smooth parent and SRT1 as well as IW variants (Table 1). The mean total DNA content per cell was 52.8 ± 0.6 fg in the o-smooth parent, 76.0 ± 5.1 fg in the SRT1 and

Fig. 2. Histograms comparing nuclear DNA content of the o-smooth parent (a), SRT1 (b), RSK10 (a representative RSK variant similar to o-smooth) (c), RSK20 (d) and RSK18 (a polyploid RSK variant) (e). Cells were grown to stationary phase in YPD and stained with propidium iodide. Fluorescence intensity of nuclear DNA was measured using a Zeiss fluorescent microscope-photometer MPM03. In each experiment, data were obtained from more than 200 nuclei.

53.4 ± 0.5 fg in an IW variant. The o-smooth parent thus had a much higher DNA content than the authentic *C. albicans* strain STN57 (39.2 ± 1.6 fg) and many other strains of this organism which have a DNA content close to that of *S. cerevisiae* diploid strains (Riggsby *et al.*, 1982). Also, the SRT1 variant had approximately one and a half times the DNA content of its parent. Fluorescent microscopic observations showed that cells of the o-smooth parent and those of the IW variant had a nucleus and that small cells of SRT1 variant had one nucleus but large yeast cells of the same SRT1 strain had either one large nucleus or more than two (up to eight) nuclei. Fig. 2 shows the wider distribution of DNA content in the SRT1 variant. Attempts were made to visualize the nuclear spindles of both strains, by using anti-yeast-tubulin antibodies. Small mitotic yeast cells of SRT1 had either one thicker spindle or two spindles (Fig. 3b, c). Cells from the IW variant showed similar features to the o-smooth parent. Hence, we refer to the nature of ploidy of SRT1 as 'polyploidy.'

The technique of PFGE has allowed the visualization of *S. cerevisiae* chromosomes (Carle & Olson, 1985; Schwartz & Cantor, 1982) as well as those of *C. albicans* (Magee & Magee, 1987). Using this technique we observed at least 10 bands in the o-smooth parent and two additional bands in SRT1 (Fig. 4, lanes 2 and 3). Five were common to the o-smooth parent, SRT1 and STN57 (Fig. 4, lanes 21 and 22). On the other hand, no difference was detected between the banding patterns of the o-smooth parent and the IW variant (data not shown).

Fig. 3. Fluorescent micrographs of polyploid SRT1 cells double-labelled for DNA (DAPI-fluorescence, top) and tubulin (FITC-fluorescence, bottom). A mitotic small cell with a thin spindle (a), a mitotic large cell with a thick spindle (b), and a mitotic large cell with two spindles (c) of SRT1 and the o-smooth parent (d). Bar, 10 μ m.

Loss of virulence of the SRT1 variant

Another interesting finding was the loss of virulence of SRT1 for mice. Even when 10^7 cells of SRT1 (more than ten times the dosage level generally used for virulence tests) were intravenously inoculated into the caudal vein of mice, no lethal effect was observed even after 40 to 50 d, whereas the same dosage of the o-smooth parent variant killed all mice tested within 2 weeks (Table 2). (The IW variant also killed all mice tested within 2 weeks: data not shown.) Secretion of acid proteinase, which has been considered to be a virulence factor of *C. albicans* (MacDonald & Odds, 1983; Kwon-Chung *et al.*, 1985), was detected in the o-smooth parent and the SRT1 variant as well as in the IW variant. Therefore, loss of virulence in the SRT1 variant may be due to a deficiency of some unknown virulence factor(s) other than the production and secretion of proteinase.

High-frequency variation of colony morphology of the SRT1 variant

When cells of SRT1 were plated as clones on VPD plates, apparently smooth colony morphology appeared at a high frequency (5×10^{-3}). We will refer to this revertant morphology as 'r-smooth'; the r-smooth variant isolated here was designated RSK. Sixteen RSK variants were obtained spontaneously (RSK5 to RSK20). DNA determinations and virulence tests were done on each of the variants (Tables 1 and 2). The mean DNA content of RSK20 was twice that of the o-smooth strain (total DNA content 110.5 ± 13.6 fg per cell). The other RSK variants had a DNA content 0.6 to 1.3 times that of their o-smooth grandparent. However, all these RSK variants had more DNA than the diploid *S. cerevisiae* strain X2180-2D. In all RSK variants, polyploid large yeast cells were found as either a very minor fraction (12 RSK variants,

Fig. 4. (a) PFGE banding patterns of chromosomal DNA in variants of *C. albicans* NUM961. A 0.8% agarose gel (20 × 20 cm) was run at a 5 min pulse rate at 10 V cm⁻¹ for 45 h. Gels were stained with ethidium bromide (1 µg ml⁻¹) for 30 min. Lane 1, *S. cerevisiae* X2180-2D (diploid); lane 2, *C. albicans* NUM961 o-smooth parent; lane 3, semi-rough SRT1 variant; lanes 4 to 11, revertant smooth variants RSK5 to RSK20, respectively. (b) RSK13 to RSK16 (lanes 12 to 15, respectively) and *S. cerevisiae* X2180-2D (lane 16) in a different run (conditions as above). (c) RSK17 to RSK20 (lanes 17 to 20, respectively) in a different run (conditions as above). (d) *C. albicans* STN57 (lane 21) and NUM961 o-smooth parent (lane 22) in a different run (conditions as above).

Table 1. Mean DNA content and ploidy state of the o-smooth parent NUM961, SRT1 and r-smooth RSK variants

Strain	DNA (fg per cell)*	Relative ploidy†		Presence of polyploid cells‡
		A	B	
o-smooth	52.8 ± 0.6	3.0	1.0	—
SRT1	76.0 ± 5.1	4.3	1.4	++
RSK5	45.6 ± 1.7	2.6	0.9	+
RSK6	44.5 ± 0.3	2.5	0.8	+
RSK7	42.1 ± 3.8	2.4	0.8	+
RSK8	46.5 ± 1.0	2.6	0.9	+
RSK9	48.3 ± 1.8	2.7	0.9	+
RSK10	53.7 ± 1.3	3.1	1.0	+
RSK11	49.6 ± 2.0	2.8	0.9	+
RSK12	40.1 ± 2.1	2.3	0.8	+
RSK13	62.0 ± 5.4	3.5	1.2	++
RSK14	32.0 ± 2.0	1.8	0.6	++
RSK15	48.6 ± 4.0	2.8	0.9	+
RSK16	57.6 ± 4.2	3.3	1.1	+
RSK17	37.4 ± 0.6	2.1	0.7	++
RSK18	70.1 ± 4.1	4.0	1.3	++
RSK19	49.6 ± 0.3	2.8	0.9	+
RSK20	110.5 ± 13.6	6.3	2.1	+
X2180-1A	17.6 ± 1.5	1.0	0.3	—
X2180-2D	36.5 ± 1.8	2.1	0.7	—
STN57	39.2 ± 1.6	2.2	0.7	—

* Determined using DABA.

† Relative ploidy was determined after normalization of the haploid *S. cerevisiae* X2180-1A (A) or of the o-smooth *C. albicans* parent (B).

‡ Cell of exponential-phase cultures of each strain were examined by fluorescent-microscope photometry. —, Percentage of polyploid cells (which includes multinucleate cells) less than 0.1%; +, percentage 0.1%–5%; ++, percentage more than 5%.

Table 2. Virulence test for mice of the o-smooth parent, semi-rough SRT1 and r-smooth RSK variants

Strain	Mortality after 40 d	Mortality rate (%)	Median death time (d)
o-smooth	20/20	100	3
SRT1	0/20	0	—
RSK5	3/10	30	9
RSK6	5/10	50	7
RSK7	3/10	30	16
RSK8	4/10	40	15
RSK9	4/5	80	17
RSK10	6/10	60	27
RSK11	6/10	60	24
RSK12	8/10	80	10
RSK13	4/5	80	19
RSK14	8/10	80	14
RSK15	2/5	40	15
RSK16	3/5	60	14
RSK17	4/5	80	23
RSK18	1/5	20	16
RSK19	4/5	80	17
RSK20	3/5	60	23

indicated as + in Table 1) or a few percent or more of their population (four RSK variants, indicated ++ in the Table). In RSK13 and RSK18, ploidy state and DNA content remained similar to that of their parent SRT1, but in the other RSK variants, DNA content showed variation. Only in the four r-smooth variants RSK9, RSK10, RSK11 and RSK16 had the DNA

content returned to the level found in their o-smooth grandparent. This seemed to us to suggest the occurrence of ploidy-shift (in the case of RSK20) or transition to an aneuploid state (in most other cases). Any direct correlation between the type of colony morphology and ploidy state or between ploidy state and virulence (Tables 1 and 2) in these RSK variants was difficult to find. However, recovery of virulence was correlated with the colony transition from semi-rough to r-smooth. These reversion phenotypes, especially found on variation of ploidy states among r-smooth variants, suggested that the r-smooth variation was not so much a true reversion but a second site reversion. PFGE banding patterns of the RSK variants were different from each other. Various numbers of bands, from 9 to 14, were detected. However, at least 5 common bands were observed among the o-smooth parent, SRT1 and RSK variants as well as STN57 (Fig. 4). This indicated that the variation from semi-rough to r-smooth was not simply due to a reversion process but was to some event accompanied by chromosome reorganization. No direct correlation was found between either a particular PFGE banding pattern and virulence or between the number of bands and ploidy state (11 bands in RSK20, which had the highest DNA content, and 14 bands in RSK6, which had a DNA content less than its o-smooth grandparent). There appeared to be no specific correlation between banding pattern and virulence.

DISCUSSION

C. albicans is an opportunistic pathogen and the high-frequency variation of virulence described here appears to be a potential model system for use in the study of its pathogenicity. From our work, one can assume the existence of virulence factor(s) other than acid proteinase in this organism. The avirulence of SRT1 does not seem to be due to differences in growth rate nor in ability to produce acid proteinase compared to the o-smooth parent. It is also possible that SRT1 cells may produce factor(s) that repress activity of the proteinase in mice.

Electron microscopic observations on the fine structure of the cell wall of both strains revealed no distinct difference (unpublished data). In terms of the serotypes, both strains showed no difference. However, since the serotype examined here was a reflection of structure of the main polysaccharide chains in the yeast cell wall (Suzuki, 1981), differences in side-chains of the cell wall polysaccharides cannot be excluded. In the case of white-opaque colony variation in this organism (Slutsky *et al.*, 1987), Anderson & Soll (1987) found that a specific antigen discriminated between these two types of colony morphology. Further investigations on the differences between the o-smooth parent and SRT variants are in progress.

The polyploid nature of the SRT1 variant does not seem to relate directly to its loss of virulence because four polyploid strains (RSK13, 14, 17 and 18) were virulent for mice. More plausible is its relatedness to the high efficiency of variation. The frequency of variation of the SRT1 strain to r-smooth was several orders of magnitude greater than that of the o-smooth parent to SRT variants. Greater virulence of o-smooth may be correlated with less frequent variation to avirulent SRT variants.

It appears reasonable to assume that the chromosome reorganization described here is coupled to the process of ploidy shift. Cytological features of SRT1, i.e. that two or more spindles were formed in a nucleus as shown in Fig. 3, might be the structural basis for the phenomenon of high-frequency variation. Our unpublished data on the treatment of thiabendazole in the o-smooth parent (this drug binds microtubules and inhibits microtubule-mediated cellular functions) indicated a hundred-fold increase in the frequency of occurrence of SRT variants; the fact that after treatment the culture accumulated cells with multiple spindles (unpublished data) also seems to support this idea.

In terms of DNA content, one cannot discriminate between the two possible cases simply from the data on mean DNA content per cell. For example, in the polyploid state in which there is only a limited fraction of polyploid cells there is a slightly higher DNA content than expected. The other case is in the aneuploid state in which the DNA content is near the diploid or triploid level and the presence of aneuploid chromosomes gives a slight increase in the DNA content. In our study, both of these cases appeared. The r-smooth RSK20 strain had twice the DNA content of the o-smooth strain, which seemed to suggest that the process of ploidy-shift had occurred,

whereas the other RSK variants appeared to be in the aneuploid state. In either case, dosage effects of some gene(s) may be correlated with the changes of phenotypes including colony morphology, virulence and ploidy-shift in these variations.

Examination of chromosome banding patterns indicated that the variation of both o-smooth to SRT and SRT to r-smooth were accompanied by addition and/or deletion of chromosome-sized bands. Except for five common bands, the pattern of addition or deletion of bands depended on the strain. No correlation was found between individual bands and the observed phenotypes. Further investigations including Southern hybridization experiments are required to examine homology between and derivation of individual chromosomal bands. Our work strongly supports the view that *C. albicans* has polymorphisms in chromosome band-size as proposed by Magee & Magee (1987), and suggests that the chromosome reorganization described here may play a role in producing such polymorphism in this asporogenous yeast.

In *Drosophila*, a substantial case can be made for the causal association of transposable elements and mutation, especially in the case of spontaneous mutation (Rubin, 1983). Recently, Scherer & Stevens (1988) showed a dispersed, repeated DNA segment (or a gene family) isolated from *C. albicans* DNA. They also found high frequencies of DNA polymorphism among clinical isolates using this segment as a probe. The gene family was shown to have many of the features common to a variety of eukaryotic transposable elements. The chromosomal reorganization in our study has features from which one can infer the occurrence of unequal crossing-over (defined as production of a tandem duplication and a reciprocal deletion). In *S. cerevisiae*, the phenomenon of unequal crossing-over has been demonstrated to occur via the Tyl transposable element (Roeder, 1983). Therefore, further studies are needed to clarify whether such mobile elements participate in the production of polymorphisms of chromosome-sized DNA coupled with the phenomenon of high frequency variation described here.

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