An increase in switching frequency correlates with an increase in recombination of the ribosomal chromosomes of *Candida albicans* strain 3153A

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When cells of *Candida albicans* strain 3153A are treated with a low dose of ultraviolet irradiation, they switch at high frequency (10^{-2}) between a number of switch phenotypes discriminated by colony morphology. Clones from switching lineages exhibit continuous reorganization of their ribosomal chromosomes (R chromosomes), while clones from lineages maintaining the original smooth phenotype exhibit no reorganization. R chromosome reorganization results in decreases as well as increases in size of the chromosomes, but changes are not reciprocal between R chromosome homologues.

Keywords: phenotypic switching, ribosomal chromosome reorganization, *Candida albicans*

INTRODUCTION

Candida albicans and related species are capable of switching at extremely high frequencies between a number of phenotypes distinguished by colony morphology (Pomes *et al.*, 1985; Slutsky *et al.*, 1985, 1987; Soll, 1992; Soll *et al.*, 1993a). Strains can differ in the variant phenotypes in their switching repertoire, but switching systems share several general characteristics (Soll, 1992) and, in the case of two tested switching systems, involve the regulation of the same phase-specific genes (Morrow *et al.*, 1992, 1994). Recently, it was demonstrated that strains of *C. albicans* exhibit a high degree of karyotype polymorphism which can be visualized by chromosome separation techniques such as transverse alternating field electrophoresis (TAFE), orthogonal field alternating gel electrophoresis (OFAGE) and contour clamped homogeneous electric field electrophoresis (CHEFE) (Magee & Magee, 1987; Merz *et al.*, 1988; Lasker *et al.*, 1989; Suzuki *et al.*, 1989; Rustchenko-Bulgac *et al.*, 1990; Rustchenko-Bulgac, 1991). These changes have been observed over time in single strains (Suzuki *et al.*, 1989; Rustchenko-Bulgac *et al.*, 1990; Rustchenko-Bulgac, 1991), and occur more frequently in the chromosomal homologues containing

ribosomal cistrons (R chromosomes) (Suzuki *et al.*, 1989; Rustchenko-Bulgac, 1991).

In a recent analysis of morphological variants of *C. albicans* strain 3153A, karyotype variability was observed and interpreted to reflect a relationship between high frequency phenotypic switching and chromosomal reorganization (Rustchenko-Bulgac *et al.*, 1990; Rustchenko-Bulgac, 1991; Soll, 1992). However, karyotype stability has been demonstrated for the 'white-opaque' switching system in *C. albicans* strain WO-1 (Rikkerink *et al.*, 1988; McEachern & Hicks, 1991; Soll, 1992), suggesting that if there is a relationship between changes in the size of chromosomes and switching, it is strain-specific and most likely reflects a parallel increase in the frequencies of the two processes rather than a cause-effect relationship between them (Soll, 1992). In addition, in the studies correlating phenotypic variants with karyotypic changes in strain 3153A (Rustchenko-Bulgac *et al.*, 1990; Rustchenko-Bulgac, 1991), experiments to test whether karyotypic changes occurred at high frequency in the absence of phenotypic switching were not reported.

To investigate the possible relationship between karyotypic change and switching in strain 3153A, we have compared individual switching lineages of variant phenotypes, which are in a high frequency mode of switching, with parallel control lineages of cells showing the basic original smooth (o-smooth) phenotype, which are in a low frequency mode of switching. Our results demonstrate that, in strain 3153A, control cells in a low frequency

Abbreviations: TAFE, transverse alternating field electrophoresis; o-smooth, original smooth; R chromosome, ribosomal chromosome; R1, high-*M*, R chromosome homologue; R2, low-*M*, R chromosome homologue.

mode of switching do not undergo chromosomal reorganization through clonal lineages while cells exhibiting a high frequency mode of switching undergo continuous reorganization of the R chromosomes. Reorganization results in both decreases and increases in the sizes of both R chromosomes, but the changes are not reciprocal.

METHODS

Maintenance of stock cultures of the basic o-smooth phenotype. Cells from a storage slant of *C. albicans* strain 3153A were plated on agar containing the nutrient composition of Lee's amino-acid-rich, defined medium (Lee *et al.*, 1975) supplemented with 70 µg arginine ml⁻¹ and 0.1 µM ZnSO₄ (referred to as modified Lee's medium; Bedell & Soll, 1979). After 7–9 d incubation at 25 °C, cells from a single o-smooth colony were diluted into distilled water and plated on fresh agar at a density of 50–70 colony forming units (c.f.u.) per plate. For the unirradiated o-smooth lineage, cells from a representative clone in the second set of agar plates were suspended in distilled water and plated again on agar at 50–70 c.f.u. per plate. In addition, cells from the same representative colony were inoculated into modified Lee's liquid medium, grown to late exponential phase in suspension at 25 °C, and then processed for TAFE.

Induction of high frequency switching and genesis of switching lineages. Cells from an o-smooth colony were grown in modified Lee's liquid medium at 25 °C to the mid-exponential phase of growth. Cells were then irradiated for 5 s with UV light (total energy output 31 J m⁻²) according to the methods of Morrow *et al.* (1989), then diluted and spread on agar plates containing modified Lee's medium. Cells were plated at 40–70 c.f.u. per plate. After 7 d incubation at 25 °C, an irregular wrinkle colony was selected, and cells from this colony were diluted and plated to initiate switching lineages. Several smooth colonies were also selected and plated. One colony which was still in a low frequency mode of switching was selected to generate a UV-treated, non-switching lineage.

Pulsed field electrophoresis. Cells were grown to mid-exponential phase in modified Lee's liquid medium at 25 °C and embedded in agarose plugs according to the methods of Vollrath & Davis (1987). Chromosome-sized DNA molecules were separated in a 0.65% agarose gel in buffer containing 10 mM Tris/HCl, 4.35 mM acetic acid and 0.5 mM EDTA, using TAFE (Geneline, Beckman Instruments). Gels were run at 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. This was followed by 10 min pulses at 80 V for 18 h. Gels were then stained with a 0.032% aqueous solution of ethidium bromide.

Southern blot analysis of TAFE gels. TAFE gels were blotted onto Zetabind filters (Cuno) (Morrow *et al.*, 1992). DNA was cross-linked to the filter by UV irradiation and hybridized at 65 °C with a nick-translated ribosomal DNA probe of *C. albicans*. The ribosomal probe was a 3.4 kb *Eco*RI fragment cloned from a genomic library. Nucleotide sequence analysis revealed that this fragment contains the coding sequence for both the 25S and 5.8S rRNAs (T. Srikantha, R. R. Gutell, B. Morrow & D. R. Soll, unpublished). Hybridized blots were then pressed against XAR-S film (Eastman Kodak) with a Cronex Lightning-Plus intensifying screen (DuPont) in a light-proof chamber.

DNA fingerprinting. DNA fingerprinting was performed according to the methods of Schmid *et al.* (1990) with the

moderately repetitive sequence Ca3 (Sadhu *et al.*, 1991; Anderson *et al.*, 1993; Soll, 1993).

RESULTS

Switching lineages and switching frequencies of strain 3153A

Cells plated from storage cultures of strain 3153A exhibited the basic o-smooth colony morphology (Fig. 1a) on the zinc-limiting agar containing the nutrient components of modified Lee's medium (see Methods), which has been employed to visualize the variant phenotypes in the 3153A switching repertoire (Slutsky *et al.*, 1985; Soll, 1992). Cells in the o-smooth phenotype were previously demonstrated to switch spontaneously to variant phenotypes at frequencies of roughly 10⁻⁴ (Slutsky *et al.*, 1985). In the present study, no spontaneous variant colonies were observed in 5832 colonies plated from o-smooth stock cultures. Therefore, the spontaneous

Fig. 1. Switching in *Candida albicans* strain 3153A. To initiate high frequency switching, a stock culture was treated with a low dose of UV irradiation, killing less than 8% of the cell population. (a) Original smooth (o-smooth) colony morphology exhibited by cells from the stock culture. (b) Irregular wrinkle colony morphology. (c) Star colony morphology. (d) Revertant smooth (r-smooth) colony morphology (revertant smooth is slightly less smooth than o-smooth and still in a high frequency mode of switching). (e) Example of cells plated from an r-smooth colony in which one colony (wr) has switched to irregular wrinkle phenotype. (f) Example of cells plated from a star colony in which one colony (s) has switched to a smooth phenotype. In all cases, colonies were photographed after growth at 25 °C for 7–9 d.

Table 1. Frequency of variant colony formation in the switching and nonswitching lineages analysed

Switching lineage	No. of colonies plated	% Original phenotype	Frequency of switching
(a) Lineage 1			
O-smooth	1500	100	$< 6.7 \times 10^{-4}$ * ← UV†
Irr. wrinkle	1568	11	MC‡
Rev. smooth§	3727	99.8	2.1×10^{-3}
Irr. wrinkle	9543	0.1	MC‡
Star	2601	99.6	4.2×10^{-3}
Rev. smooth	2057	99.8	1.9×10^{-3}
Star	1149	86.1	1.6×10^{-1}
Rev. smooth	—	—	—
(b) Lineage 2			
O-smooth	1500	100	$< 6.7 \times 10^{-4}$ * ← UV†
Irr. wrinkle	1568	11	MC‡
Star	3140	58	4.2×10^{-1}
Rev. smooth	1431	99.7	2.7×10^{-3}
Star	1516	99.5	5.3×10^{-3}
Rev. smooth	—	—	—
(c) Lineage 3			
O-smooth	1813	100	$< 5.5 \times 10^{-4}$
O-smooth	635	100	$< 1.5 \times 10^{-3}$
O-smooth	1003	100	$< 9.9 \times 10^{-4}$
O-smooth	881	100	$< 1.1 \times 10^{-3}$
(d) Lineage 4			
O-smooth	1500	100	$< 6.7 \times 10^{-4}$ * ← UV†
O-smooth	1438	100	$< 7.0 \times 10^{-4}$
O-smooth	1137	100	$< 8.8 \times 10^{-4}$
O-smooth	1125	100	$< 8.9 \times 10^{-4}$
O-smooth	687	100	$< 1.5 \times 10^{-3}$

* Since no colonies out of 1500 were variant, the estimated frequency had to be below 1 in 1500 or less than 6.7×10^{-4} .

† UV treatment (see Methods).

‡ In two cases, cells plated from irregular wrinkle colonies in a lineage formed predominantly smooth phenotypes (89% or more) suggesting mass conversion (MC).

§ The plug of revertant smooth was inadvertently frozen and, therefore, was not karyotyped.

|| The last switch phenotype in each lineage was not plated.

switching frequency of the stock culture of o-smooth cells was below 1.7×10^{-4} .

It was previously demonstrated that low doses of UV irradiation, which kill less than 15% of the cell population, stimulate the frequency of switching in strains 3153A (Slutsky *et al.*, 1985), WO-1 (Morrow *et al.*, 1989) and 1001 (Pomes *et al.*, 1985). We therefore irradiated a stock culture of strain 3153A with a low dose of UV, killing 8% of the cell population. Variant colonies in the surviving population appeared at a frequency of 10^{-2} ,

which was at least two orders of magnitude higher than the frequency of switching of an unirradiated cell population. An irregular wrinkle colony (Fig. 1b, e) was selected and the cells in the colony plated. Of 1568 colonies, 1398 exhibited a smooth phenotype, 169 (11%) exhibited the irregular wrinkle phenotype of the parent colony, and 1 exhibited the star phenotype (Fig. 1c, Table 1a, b). Two independent lineages were initiated from this plating (lineages 1 and 2 in Table 1a and b, respectively). Lineage 1 included the following sequence of phenotypes, beginning with the original smooth culture: o-smooth, irregular wrinkle, revertant smooth (Fig. 1d; see description of this phenotype in legend to Fig. 1d), irregular wrinkle, star, revertant smooth, star and revertant smooth. Lineage 2 included the following sequence of phenotypes: o-smooth, irregular wrinkle, star, revertant smooth, star and revertant smooth. The frequencies of phenotypes other than the one plated at each step in the two respective switching lineages are presented in Table 1(a) and (b). All phenotypes following o-smooth in the switching lineages exhibited high frequencies of switching. In the two cases of irregular wrinkle, there was mass conversion to a smooth phenotype (Table 1a, b). An example of a switch from revertant smooth to irregular wrinkle is shown in Fig. 1e, and a switch from star to revertant smooth in Fig. 1f.

Karyotypic changes associated with switching lineages

TAFE separated the chromosomes of cells in the o-smooth phenotype into nine bands (o sm, Fig. 2a). A Southern blot of the TAFE gel was hybridized with a nick-translated ribosomal probe. The two highest M_r bands hybridized with the ribosomal probe. Since *C. albicans* is diploid, the two bands were considered to contain unequal-sized chromosomal homologues carrying ribosomal cistrons (o sm, Fig. 2b). The high- and low- M_r homologues will be referred to as R1 and R2, respectively. TAFE separation of the chromosomes of the switch phenotypes in lineage 1 (Table 1a) demonstrated size changes in both R1 and R2 (Fig. 2a). In the gel pattern of the first irregular wrinkle phenotype in the lineage, a tenth band appeared between the two original, highest M_r bands (i wr, Fig. 2a). Hybridization with the ribosomal probe demonstrated that the size of R1 had decreased dramatically (Fig. 2b), and that R1 represented the new band in the gel pattern, while the size of R2 remained relatively constant (Fig. 2b). Therefore, the decrease in the size of R1 accompanying the switch from o-smooth to irregular wrinkle did not involve a reciprocal increase in the size of R2. Since the highest M_r band in the ethidium-bromide-stained o-smooth gel pattern was also present in the irregular wrinkle gel pattern (Fig. 2a), but no longer hybridized to the ribosomal probe (Fig. 2b), we can conclude that this band contained non-homologous chromosomes of roughly equal size, one of which was R1, and that only R1 changed size in the switch to irregular wrinkle in the first step in this lineage.

Fig. 2. Changes in the size of R chromosomes through a switching lineage. O-smooth cells were treated with a low dose of UV irradiation, and a sequence of variant phenotypes were clonally isolated and replated. This represents switching lineage 1 in Table 1(a). Cells from each phenotype in the switching lineage were grown in liquid medium and then prepared for chromosome separation by TAFE. The phenotypes are in the lineage connected by arrows at the top of each panel. (a) Ethidium bromide-stained TAFE gel of separated chromosomes. (b) Southern blot hybridization of the TAFE gel with a labelled ribosomal probe. In this particular lineage, the third isolate, r-smooth, was not fingerprinted. o sm, original smooth; i wr, irregular wrinkle; r sm, revertant smooth; st, star. 1 through 9 to the left of (a) represent band numbers; R1 and R2 represent the larger and smaller chromosomal homologues containing ribosomal cistrons. Note that band 1 contains two chromosomes, one of which is an R chromosome. Band 2 contains only an R chromosome.

In the next three steps of switching in lineage 1 (irregular wrinkle to revertant smooth to irregular wrinkle to star), R1 continued to decrease in size, approaching the size of R2 (Fig. 2a, b). However, in the next three switches (star to revertant smooth to star to revertant smooth), R1 increased in size (Fig. 2a, b). At the end of lineage 1, one of the homologues, presumably R1, had returned to its original size. After the first switch, the size of R2 increased steadily. This is evident when the gap between R2 and the third highest M_r band is followed through the lineage in the ethidium-bromide-stained gel pattern of switching lineage 1 (Fig. 2a).

In a second switching lineage (lineage 2 in Table 1) emanating from the same UV-induced irregular wrinkle colony as lineage 1, the sizes of both R1 and R2 changed, but the changes did not follow the scenario of lineage 1, and the changes in R1 and R2 were again not reciprocal. Through five sequential switches (o-smooth to irregular wrinkle to star to revertant smooth to star to revertant smooth), the size of R1 continued to decrease (Fig. 3). As

in the first lineage, the size of R2 remained constant through the first switch, but then decreased continuously through the four subsequent switches (Fig. 3). This contrasts with the scenario of lineage 1, in which the size of R1 decreased and then increased, and the size of R2 increased continuously after the first switch.

Karyotype stability in the absence of switching

To determine whether the high frequency of reorganization of R chromosomes is an exclusive characteristic of cells in a high frequency mode of switching, or a characteristic of strain 3153A regardless of switching frequency, sequential clones were karyotyped in two lineages (lineages 3 and 4 in Table 1) in which cells remained in the o-smooth phenotype in a low frequency mode of switching. The two nonswitching lineages differed. In the first (lineage 3 in Table 1), o-smooth clones were sequentially isolated without initial UV irradiation. In the second (lineage 4 in Table 1), o-smooth cells were

Fig. 3. Changes in the sizes of the R homologues in a second switching lineage. Cells from each step in the lineage, described at the top of the gel, were grown in liquid medium. Chromosomes were then separated by TAFE, and the Southern blot was hybridized with a radiolabelled ribosomal probe. O sm, o-smooth; i wr, irregular wrinkle, st, star; r sm, revertant smooth. Note that in this lineage, R1 continually decreased in size; R2 remained constant through the first star phenotype, then decreased in size.

treated with UV, and an o-smooth colony was selected from the post-irradiated culture which remained in a low frequency mode of switching throughout the subsequent o-smooth lineage. The frequency of spontaneous switching was below 1.1×10^{-3} for the sequential clones in lineage 3 (Table 1c), and below 1.5×10^{-3} for sequential clones in lineage 4 (Table 1d).

TAFE separation of chromosomes was performed on sequential isolates in the same manner as that for switching lineages 1 and 2. In both nonswitching lineages, the ethidium-bromide-stained gel patterns remained constant (Fig. 4a, b). No change was observed in the size of R1, R2 or any other chromosomal band.

Variants in the switching lineages are not storage contaminants

In the isolation of colonies with variant morphologies 7–10 d after plating, the possibility exists that rare variants may actually represent unrelated contaminants. To demonstrate that the changes observed in switching lineages had really occurred in sequential clones of the same strain, the o-smooth isolate prior to UV irradiation, the first irregular wrinkle isolate after UV irradiation, the last star isolate and the last revertant smooth isolate from lineage 1 (Table 1a) were fingerprinted with the moderately repetitive DNA sequence Ca3, which discriminates between more than 99% of independently isolated strains of

Fig. 4. The constancy of R chromosome size in nonswitching lineages. In lineage 3 (see Table 1c), sequential clones of o-smooth were grown in liquid medium and then prepared for chromosome separation by TAFE. In lineage 4 (Table 1d), an o-smooth colony, a UV-treated o-smooth progeny clone, and two sequential o-smooth clones emanating from the UV-treated clone were grown in liquid medium and prepared for TAFE. The TAFE gel was stained with ethidium bromide. Note that there are no detectable changes in the size of the R chromosomes through these lineages. S.c., *Saccharomyces cerevisiae*. Bands are numbered to the left of the gel.

C. albicans (Schmid *et al.*, 1990). The fingerprints of the four isolates were identical (data not shown), demonstrating that variants in the lineage were derived from strain 3153A.

DISCUSSION

When Rustchenko-Bulgac and co-workers (Rustchenko-Bulgac *et al.*, 1990; Rustchenko-Bulgac, 1991) reported that morphological variants of strain 3153A exhibited chromosomal variations, we assumed that the high frequency of chromosomal variability was either a trait of the strain in general and had nothing to do with switching, or that it was one more phenotypic characteristic of switching, which has been demonstrated to be extremely pleiotropic (Soll, 1990, 1992; Soll *et al.*, 1993a,b). Our conclusion that a change in chromosome size was not the mechanism of switching (Soll, 1992; Soll *et al.*, 1993b) was based on a number of observations. First, three independent laboratories working on strain WO-1 found no variation in the electrophoretic karyotype of cells switching back and forth between the white and opaque phenotypes (Rikkerink *et al.*, 1988; McEachern & Hicks, 1991; Morrow *et al.*, 1992; Soll, 1992). Second, specific

changes in karyotype were not demonstrated to correlate with specific variant phenotypes in the 3153A switching system (Rustchenko-Bulgac *et al.*, 1990; Rustchenko-Bulgac, 1991). Third, the corresponding stability of the electrophoretic karyotype in sequentially isolated non-switching clones of strain 3153A was never demonstrated (Rustchenko-Bulgac *et al.*, 1990; Rustchenko-Bulgac, 1991). We therefore re-examined the possible relationship between electrophoretic karyotype instability and switching in strain 3153A by monitoring the karyotypes of sequential clones in switching lineages, and compared karyotype stability in these lineages with that of sequential clones of nonswitching lineages in the o-smooth phenotype. Our results demonstrate that cells in a high frequency mode of switching continuously undergo changes in the size of both the R homologues, but cells in a low frequency mode of switching and expressing the basic o-smooth phenotype do not undergo similar changes.

We have found that the size of the R chromosomes can increase as well as decrease in cells undergoing high frequency phenotypic switching and that changes from large to small or small to large are progressive. Since *C. albicans* is thought to contain between 50 and 130 rDNA repeats per cell (Iwaguchi *et al.*, 1992), the alterations in size of the R chromosomes could be explained by unequal crossing-over between tandemly arrayed rDNA units. Unequal sister chromatid exchange during mitotic growth results in both deletions and duplications of rDNA repeat units in *Saccharomyces cerevisiae* (Szostak & Wu, 1980). Alterations in the electrophoretic karyotypes of clinical isolates of *C. albicans* have been observed in recurrence patients, where isolates were obtained from the same patient in a longitudinal study (Asakura *et al.*, 1991). With one exception, all band changes occurred within the chromosomes carrying the rDNA units. In another study, Iwaguchi *et al.* (1992) demonstrated that clonal variation of the R chromosomes occurred at frequencies as high as 10% of progeny clones and that these variations were directly attributed to the number of rDNA repeats per homologue. Therefore, alterations in the size of the R chromosomes appear to be common to *C. albicans*. However, it is important to note that in no study has there been a direct correlation between a colony morphology, a specific change in phenotype, and a specific chromosomal rearrangement (Suzuki *et al.*, 1989; Rustchenko-Bulgac *et al.*, 1990; Rustchenko-Bulgac, 1991; Iwaguchi *et al.*, 1992; Rustchenko-Bulgac & Howard, 1993).

The correlation between high frequency switching and the instability of the R chromosomes in *C. albicans* strain 3153A shares an interesting similarity with the phenotype of particular mutations of the silent information regulator (*SIR*) genes of *S. cerevisiae* (Laurenson & Rine, 1992). A mutation in the *SIR2* gene not only results in expression of the normally silent mating type loci *HML* and *HMR*, but also in a corresponding increase in intrachromosomal recombination between ribosomal cistrons (Gottlieb & Esposito, 1989). We are, therefore, pursuing the idea that the transition from a low to high frequency mode of

switching in strain 3153A involves a change in the gene silencing machinery and chromatin structure.

ACKNOWLEDGEMENTS

The authors are indebted to Dr T. Srikantha for providing the rDNA probe. The study was supported by Public Health Service Grants AI 2392 and DE 10758 from the National Institutes of Health.

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Received 20 September 1993; revised 10 January 1994; accepted 27 January 1994.