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Chromosomal Alterations of *Candida albicans* Are Associated with the Gain and Loss of Assimilating Functions

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We have demonstrated that a normal laboratory strain of *Candida albicans* spontaneously produces mutants which acquire the ability to assimilate certain carbon sources that are not utilized by the parental strain. The examination of mutants acquiring the ability to utilize either sorbose or D-arabinose revealed a few additional phenotypic changes, including the gain and loss of the capacity to assimilate other carbon sources. The change of assimilation patterns resembled the polymorphic variation of assimilation patterns found among different wild-type strains of *C. albicans*. Most importantly, these sorbose- and D-arabinose-positive mutants were associated with chromosomal rearrangements, with each class of positive mutants having alterations of specific chromosomes. These findings demonstrated for the first time that chromosomal alterations in *C. albicans* are involved in genetic variation of fundamental functions of this asexual microorganism.

We previously reported that standard laboratory strains of *Candida albicans* spontaneously gave rise to various types of single and multiple chromosomal rearrangements at a frequency of about 1.4% (40), and we suggested that this high frequency of chromosomal aberrations provides a means for genetic variation in this asexual microorganism (38, 40). Although the chromosomal aberrations were associated with alterations of colonial and cellular morphologies, rates of growth, pseudohyphae, chlamydospore production, germ tube formation, growth at extreme temperatures, and color differences on BiGGY and phloxine B media (38–40), we were unable to discern any specific relationships between the various types of chromosomal rearrangements and phenotypic variability.

Recently, Wickes et al. (48) reported that certain *Candida stellatoidea* strains spontaneously gave rise to sucrose-positive (Suc^+) colonies that exhibited chromosomal rearrangements. In addition, we observed that all of a large number of spontaneously derived colony morphology mutants having chromosomal alterations were also associated with different patterns of carbon and nitrogen assimilation (37).

Because of the obvious selective importance of carbon and nitrogen sources, these findings prompted us to investigate possible relationships between the assimilation patterns, chromosomal rearrangements, and other phenotypic traits. Furthermore, changes in assimilation provide an opportunity to investigate discrete and easily assayable phenotypes.

In this paper, we describe the isolation and characterization of positive mutants, i.e., mutants that have gained the ability to assimilate substrates. In particular, independently derived spontaneous mutants that have acquired the ability to utilize either sorbose or D-arabinose were isolated from the normal strain 3153A. Of considerable importance, and in contrast to the previous report (39), these positive mutants contained altered electrophoretic karyotypes, with each class of positive mutants having alterations of specific chromosomes. This is the first report that *C. albicans* can spontaneously produce mutants which acquire new functions and which are specifically related to chromosomal aberrations.

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MATERIALS AND METHODS

Definitions and nomenclature. In this study, positive mutants are defined as mutants acquiring the ability to assimilate a substrate which is not utilized by the normal parental strain. The term cryptic genes refers to genes in wild-type organisms that are not functional but can be activated by mutation or recombination (15).

Phenotypic traits of having or lacking the ability to grow on, for example, sorbose medium are denoted, respectively, Sor^+ or Sor^-. Specific Sor^+ mutants are denoted by numbers, for example, Sor1 and Sor2, etc.

Strains. A commonly used laboratory strain, 3153A, has been well characterized with respect to genetic instability and electrophoretic karyotyping (38). A total of 10 Sor^+ mutants, Sor1 through Sor10, and 15 Ara^+ mutants, Ara1 through Ara15, acquiring the ability to assimilate, respectively, sorbose and D-arabinose were spontaneously derived from the 3153A strain.

Media. Synthetic media were prepared either from 0.67% (wt/vol) Bacto yeast nitrogen base without amino acids and with the addition of 2% (wt/vol) of the various carbon sources or from 0.17% (wt/vol) Bacto yeast nitrogen base without amino acids, without ammonium sulfate, with 2% glucose, and with the addition of 2% (wt/vol) of the various nitrogen sources. Solutions of the components used to prepare Bacto yeast nitrogen base media, as well as all carbon sources, were filter sterilized. YPD consisted of 1% (wt/vol) Bacto yeast extract, 2% (wt/vol) Bacto peptone, and 2% (wt/vol) glucose. LBC medium (21) was used to test the colonial morphologies of *C. albicans* subclones. A total of 2% agar was added for the preparation of solid media.

Maintenance and growth of strains. We have previously described our procedures for preserving and maintaining *C. albicans* strains in light of their instabilities (38).

Tests for assimilation of carbon and nitrogen sources. Cells from the −70°C stock cultures were transferred to YPD master

TABLE 1. Growth of numerous normal strains of *C. albicans* on different media with 21 carbon sources and 3 nitrogen sources and growth of the normal strain 3153A on different media at various temperatures

Carbon or nitrogen source	Growth				Carbon or nitrogen source	Growth			
	Numerous normal strains[a]	3153A[b]				Numerous normal strains	3153A		
		22°C	37°C	40°C			22°C	37°C	40°C
D-Glucose	+	+	+	+	Arbutin	0	0	0[c]/+	±
D-Fructose	+	+	+	+	D-Ribose	0	0	±[c]	0[c]/+
Xylitol	+	+	+	+	Dulcitol	0	0	0	±/+
D-Trehalose	+	+	+	+	Myo-inositol	0	0	0	0
N-Acetyl-D-glucosamine	+	+	+	+	α-D-Melibiose	V	0	0	0
D-Xylose	+	+	+	+	α-L-Rhamnose	0	0	0	0
Adonitol	V	+	+	+	D-Cellobiose	V	0	0	0
D-Melezitose	V	+	+	+	D-Raffinose	0	0	0	0
L-Arabinose	V	+	+	+	*i*-Erythritol	0	0	0	0
MGP	V	±[c]	+	+	L-Proline	+	+	+	+
L-Sorbose	V	±[c]	0[c]	0[c]	Glycine	+	+	+	+
D-Arabinose	0	0[c]	+	+	KNO_3	0	±	±	+

[a] Assimilation by large numbers of independent clinical isolates classified as *C. albicans*, as summarized from previously published reports (25, 26, 29, 34, 47, 49). +, positive in 99% or more of the strains; 0, negative in 99% or more of the strains; V, variable responses in 1% or more of the strains.

[b] The levels of growth are denoted as follows: +, substantial; ±, reduced; ±, poor; 0, none. Variable responses from experiment to experiment are indicated by slashes (for example, 0/+).

[c] Papillae were obtained under these conditions, suggesting the formation of positive mutants.

plates for the pregrowth at 22°C overnight. Growth was determined by transferring small droplets of uniform suspensions of cells to plates of various media with an array of inoculating rods on custom-made replicators and then incubating the plates at 22, 37, and 40°C. To guarantee exact and stable temperatures of 37 and 40°C, plates were incubated in water baths in plastic containers. The relative level of growth and the appearance of papillae were recorded daily for the first 3 days and subsequently weekly for 2 weeks. These assimilation patterns were determined three times in independent experiments.

Chlamydospore and pseudohypha formation. Chlamydospore formation was determined at room temperature on Bacto corn meal agar supplemented with 1% (vol/vol) Tween 80. The tests were performed by standard procedures (9). Determination of the ability to form pseudohyphae was made on the same medium.

Germ tube formation. The strains to be tested for germ tube formation were grown on glucose peptone agar at room temperature for 72 h (39). A single colony was touched with the tip of a straight needle, and the cells so harvested were emulsified in 0.5 ml of prewarmed calf serum (HyClone Laboratories, Logan, Utah). The suspensions were incubated at 37°C for 3 h. The percentage of cells with germ tubes was determined microscopically at the end of the incubation period (9).

Colony morphologies. The colony forms of positive mutants were examined by using our standard conditions, growth on LBC medium for 4 weeks at 22°C, with approximately 10 colonies per plate (40).

Electrophoretic separation of chromosomes. Separations of all chromosomes were achieved by using two different systems of pulsed-field electrophoresis: the CHEF-DRII (contour-clamped homogeneous electric field) system (Bio-Rad Laboratories) (8) and the orthogonal-field-alternation gel electrophoresis system (4). The running conditions used for both of these systems have been previously described (36, 38, 39). In order to elucidate the position of the smallest chromosome, Ia, we used a new condition with the CHEF-DRII, which consisted of a constant voltage of 7.5 V cm^{-1}, approximately 130 mA at the start of the run, a 100-s pulse time, 0.5× Tris-borate-EDTA cooling buffer at 10°C, 1% (wt/vol) agarose, and a 27-h running time.

Hybridization probes and protocols. The following chromosomes were identified with the indicated probes: chromosome I, pBL1-6 (20); chromosome II, *BEN4* (23) and pBL2-1 (20); chromosome III, Gα (41); chromosome IV, pBL4-1 (20); chromosome V, *ADE2* (19); chromosome VI, *HIS3* (35), WOL-28, WOL-29, WOL-41, and WOL-64 (24a), and *HPT1* (43a), chromosome VII, *GAL1*, *TUB2*, *MGL1*, and *TRP1* (35); and chromosome VIII, WOL-25 (24a) and *SOR9* (23).

RESULTS

Assimilation of carbon sources by the normal strain 3153A. We have chosen 20 carbon sources, in addition to glucose, and three nitrogen sources, in addition to ammonium (Table 1) for testing the growth of the normal strain 3153A. These nutrilites represent substrates that are always utilized (such as glucose and fructose, etc.), that are never utilized (such as inositol and rhamnose, etc.), or whose utilization varies among strains of *C. albicans* (such as *N*-methyl-α-L-glucopyranoside [MGP] and sorbose, etc.), according to various reports (API Laboratory Products, API 20C Clinical Yeast System) (25, 26, 29, 34, 47, 49). Unlike in standard biotyping procedures, the growth of the 3153A strain on these various media was examined at the three different incubation temperatures of 22, 37, and 40°C. Although the assimilation patterns for some carbon and nitrogen sources could be clearly assigned as positive or negative, the growth on some other media was strongly dependent on the incubation temperature. For example, D-arabinose was not utilized at 22°C but supported good growth at 37 and 40°C (Table 1). Also, the degree of growth clearly differed on different carbon sources. For example, high levels of growth occurred after 1 day on glucose, fructose, and xylose media, whereas growth occurred only after 3 days on D-arabinose and melezitose media, and the levels in the latter cases were inferior to that of the growth on, for example, glucose medium. We also noted that the degree of growth on certain media and at certain temperatures varied with the cell density of the inoculating suspension, with higher cell densities giving rise to higher degrees of growth. In addition, some responses varied

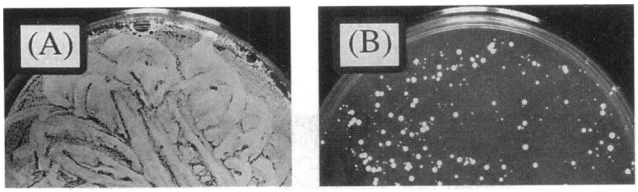

FIG. 1. Generation of positive sorbose mutants. (A) Confluent growth is observed after plating of approximately 10^6 cells of the parental strain 3153A on glucose medium and incubation of the plate at 37°C for 2 weeks. (B) Positive sorbose colonies arise after plating of cells on sorbose medium as described for panel A and incubation of the plate at 37°C for 1 week.

from experiment to experiment, as shown in Table 1 for arbutin, ribose, and dulcitol, indicating that the growth was highly sensitive to subtle environmental variations. Overall, a greater number of substrates were assimilated at 40°C than at 37°C and at 37°C than at 22°C. The dependencies on temperature and cell densities may be factors leading to the lack of interlaboratory reproducibility in biotyping (7, 30). The occurrence of large numbers of well-growing papillae on the spot of inoculation sometimes could be confused with confluent growth. Also, the same normal strain can vary after prolonged maintenance in separate laboratories (38, 39).

Isolation and frequencies of Sor$^+$ and Ara$^+$ mutants. The occurrence of colonies of various sizes and at various frequencies on certain media was clearly discernible after a few days to 1 month of incubation of the normal 3153A strain at all three temperatures, as summarized in Tables 1 to 3 and illustrated in Fig. 1 and 2 for Sor$^+$ colonies and in Fig. 3 for Ara$^+$ colonies. For example, colonies appeared after about 10^6 cells were plated on sorbose and D-arabinose media and the plates were incubated, respectively, at 37 and 22°C for prolonged times. After 1 week, the frequency of mutants appearing on sorbose plates, as shown in Fig. 1, was estimated to be approximately 1.4×10^{-3}. Because growth is generally slower on D-arabinose medium and at 22°C, the fully developed colonies were observed and counted after 1 month, and the frequency was estimated to be approximately 1.2×10^{-3}. In both cases, the estimates are only approximate because of the presence of numerous minute colonies that were barely visible. It is unclear if this continued appearance of colonies occurred by adaptive mutation, i.e., mutation induced by the specific selective medium (10), or by general catabolite derepression of spontaneous mutagenesis (22).

Ten Sor$^+$ mutants were isolated as single colonies, each from an independent subclone of 3153A to ensure that each mutant arose from a separate event. Subsequently, the mutants were further purified by subcloning twice on sorbose medium.

Initially, five independent subclones of 3153A were plated on D-arabinose medium. All plates contained mixtures of different-sized colonies after 1 month of incubation at 22°C. Two colony forms were easily distinguished: normal-sized creamy colonies and small green colonies. Both types of colonies were isolated from each plate and subcloned twice under same conditions. Mutants derived from the normal-sized creamy colonies were denoted Ara1 to Ara5, whereas the ones derived from small green colonies were denoted Ara6 to Ara10. After being subcloned, Ara1 to Ara5 gave rise to colonies with various sizes, and Ara6 to Ara10 gave rise to the two types of colonies seen with the original plating of the parent strain 3153A, i.e., small green colonies and normal-sized creamy colonies. After being subcloned on selective medium, Ara3, which was initially identified as a normal-sized creamy colony, arose as small green colonies. Thus, Ara3 appeared to be a very unstable mutant, similar to other unstable mutants previously isolated spontaneously from 3153A (38–40). Five normal-sized creamy colonies, similar to Ara1 to Ara5, were chosen from the Ara6 to Ara10 platings, subsequently subcloned, and denoted Ara11 to Ara15.

The nature of the green pigment in Ara$^+$ mutants was not investigated, but the pigmentation appeared more intense on D-arabinose medium and yellow or cream-colored on many other media.

Assimilation patterns of positive mutants. The growth of all 25 mutants, Sor1 to Sor10 and Ara1 to Ara15, was tested at the three temperatures and on media with 21 different carbon sources and 3 different nitrogen sources instead of ammonium, as listed in Table 1. In addition, the growth of the strains was tested at these three temperatures on YPD medium. All tests were repeated three times, and the results were generally reproducible. Similar to the case with the parental strain 3153A (Table 1), all 25 mutants did not grow at all on ribose, dulcitol, inositol, melibiose, rhamnose, cellobiose, raffinose, and erythritol media.

The results indicated that the Sor1 to Sor10 mutants, denoted group 1 in Table 2, grew substantially and equally at all temperatures on media with sorbose, as well as with glucose, fructose, xylitol, trehalose, xylose, melezitose, and three alternative nitrogen sources, i.e., proline, glycine, and KNO_3, as illustrated in Fig. 2 for the test medium containing sorbose. In contrast, growth of the group 1 mutants on

TABLE 2. Selected examples of the assimilation of carbon sources by the Sor$^+$ and Ara$^+$ mutants at various temperatures with ammonium as the nitrogen source and the corresponding altered chromosomes and colony forms on the selection plates

Group	Strain(s)	Growth[a] with:												Altered chromosome	Colony form on original selection plate
		Glucose			Sorbose			D-Arabinose			MGP				
		22°C	37°C	40°C	22°C	37°C	40°C	22°C	37°C	40°C	22°C	37°C	40°C		
	3153A	+	+	+	±[b]	0[b]	0[b]	0	+	+	±[b]	+	+		
1	Sor1–Sor10	+	+	+	+	+	+	±	±	±	±[b] | +[b]	+	0[b] | +[b]	III	Normal
2a	Ara1, Ara4, Ara5[c]	+	+	+	0[b]	0[b]	0[b]	+	+	±	+	+	+	II	Normal sized, creamy
2b	Ara2	+	+	+	±	0[c]	+	+	+	±	+	+	+	II	Normal sized, creamy
3	Ara3[d], Ara6–Ara15	+	±	0 | ±	0[b] | +[b]	0[b] | +	0 | ±	0[b] | +	± | +	0	0[b]	±	0	VI	Small, green

[a] The levels of growth are denoted as follows: +, substantial; ±, reduced; ±, poor; 0, none. The range of levels for different members within the same group is indicated by a vertical line. The growth on YPD was similar to the growth on ammonium-glucose medium.

[b] Formation of colonies or papillae. Because a high frequency of papillae can be confused with substantial growth, some results are presented as, for example, 0[b] | +.

[c] Ara1, Ara4, and Ara5 were deficient in germ tube formation.

[d] Ara3 contains normal chromosome VI (see text).

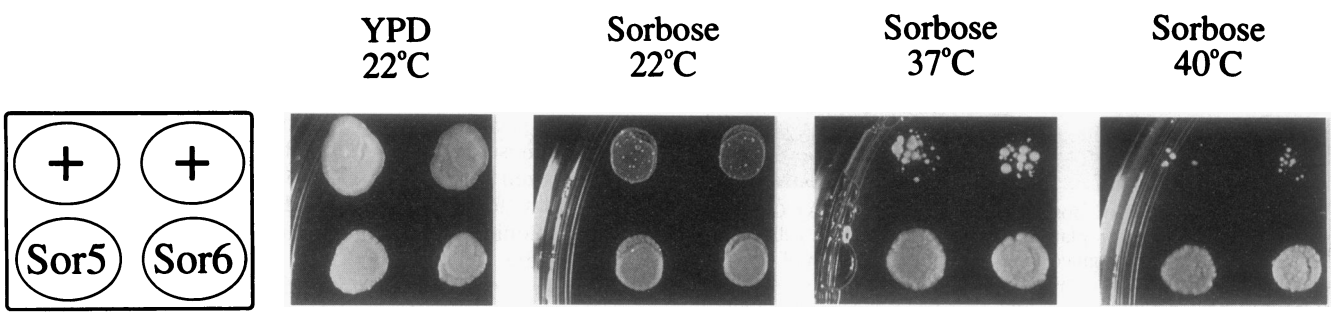

FIG. 2. Comparative growth of the parental strain 3153A (+) and representative positive sorbose mutants (Sor5 and Sor6) after 1 week of incubation on YPD medium at 22°C and on sorbose medium at 22, 37, and 40°C.

D-arabinose at the two elevated temperatures was diminished in comparison with that of the parental strain 3153A. Furthermore, these mutants grew poorly at 22°C but better than 3153A, as summarized in Table 2. Only the single Sor10 mutant grew less on adonitol and *N*-acetyl-D-glucosamine, two media not included in Table 2. Also, the growth of individual members of group 1 varied considerably, although reproducibly, on MGP media at some temperatures (Table 2). Because the group 1 mutants gave identical responses with most of the conditions, we considered this group to be functionally uniform.

Clear and distinct responses were observed with two sets of Ara^+ mutants, one set consisting of Ara1, Ara2, Ara4, and Ara5 and the other consisting of Ara3, and Ara6 to Ara15, whose phenotypes differed on the selection medium and which were subsequently shown to differ in their electrophoretic karyotypes. The mutants which originated from normal-sized cream-colored colonies grew well and were assigned to functional group 2. These group 2 mutants differed from 3153A on L-arabinose, MGP, D-arabinose, and arbutin media, as partially presented in Fig. 3 and Table 2 and 3. Group 2 was further subdivided into two subgroups, 2a (Ara1, Ara4, and Ara5) and 2b (Ara2). The single representative of 2b, Ara2, was separated from 2a solely to emphasize the difference in sorbose assimilation. Also, as described below, all members of subgroup 2a, but not subgroup 2b, were defective in germ tube formation.

The other Ara^+ mutants, those in group 3, totally differed from group 2 by growing less on all media, including YPD, at all or some of the three temperatures but predominantly at 37 and 40°C (Fig. 3 and Tables 2 and 3). Surprisingly, the Ara5 to Ara10 mutants, which characteristically grew slowly on the selection plates and produced small pigmented colonies, appeared mostly as papillated growth after 2 weeks on the selection D-arabinose medium at 22°C, as illustrated in Fig. 4 and presented in Tables 2 and 3. This implies that they are highly unstable for D-arabinose assimilation, a phenomenon reminiscent of chromosomal and colonial-form instability in spontaneous morphological mutants (38–40). Because we observed that the group 3 mutants grew significantly better at 37 and 40°C on media with alternative sources of nitrogen, like proline and glycine, the assimilation of the various carbon sources was also tested with media in which glycine was used instead of ammonium sulfate (Table 3).

The lack of growth or poor growth at 40°C, and diminished growth at 37°C, of the members of group 3 on all media, including YPD, indicates that these mutants are generally temperature sensitive. The enhanced growth on glycine and proline media suggests that ammonium repression may play a role in the temperature sensitivity.

Although members of groups 1 and 3 could have been further divided on the basis of their assimilation patterns as indicated by the ranges of responses in Tables 2 and 3, we believe that the groups define the major phenotypes. While some members within the same group truly differed in certain growth responses, the large number of papillae of other members of the groups could have been confused with confluent growth.

The assimilation patterns of the positive mutants can be summarized as follows. Approximately one-half of the Sor^+ mutants exhibited diminished or substantially diminished growth on L-arabinose at 40°C, and all of them exhibited diminished growth on D-arabinose at 37 and 40°C (Table 2). On the other hand, they all exhibited good growth on sorbose medium at all temperatures (Fig. 2 and Table 2), and approximately one-half of them exhibited enhanced growth on MGP at 22°C. The following additional changes were observed with the group 2 Ara^+ mutants, which generally had clear and well-defined growth responses that were not complicated by multiple temperature sensitivities or papillated growth: they all grew poorly at 40°C on arbutin medium, and they all grew well at 22°C on MGP medium with both ammonium and glycine (Tables 2 and 3). Also, one mutant, Ara2 (subgroup 2b), grew well on sorbose medium at 22 and 40°C (Tables 2 and 3). Thus, there were only a few differences between the normal strain and the group 1 and 2 mutants, in contrast to the many differences between the normal strain and the group 3 mutants.

Despite the diminished and papillated growth of the remaining Ara^+ mutants (group 3), on many types of media, the majority of them nevertheless clearly acquired a better or a good growth response on sorbose medium at 22 and 37°C and a poor growth response at 40°C.

Cellular morphogenesis. The normal strain 3153A and all 25 mutants were examined for their ability to form germ tubes, chlamydospores, and pseudohyphae, as described in Materials and Methods. The results, summarized in Table 4, indicated that the three mutants, Ara1, Ara4, and Ara5, of subgroup 2a, which originated from normal-sized creamy colonies, totally lost the ability to germinate. In addition, one of them, Ara5, did not form chlamydospores. The other mutants germinated and formed chlamydospores and pseudohyphae in the normal fashion.

Colonial morphologies. Colonial morphologies, manifested by a large number of shapes and textures, previously proved to be a reliable and sensitive means for detecting unspecific chromosomal aberrations (36, 38–40). We have used this approach and examined the types and homogenity of the colony forms of all positive mutants with our standard protocol (see Materials and Methods). As summarized in Table 4, two of the group 1 Sor^+ mutants and all of the group 2 Ara^+ mutants had individual morphologies that differed from that of

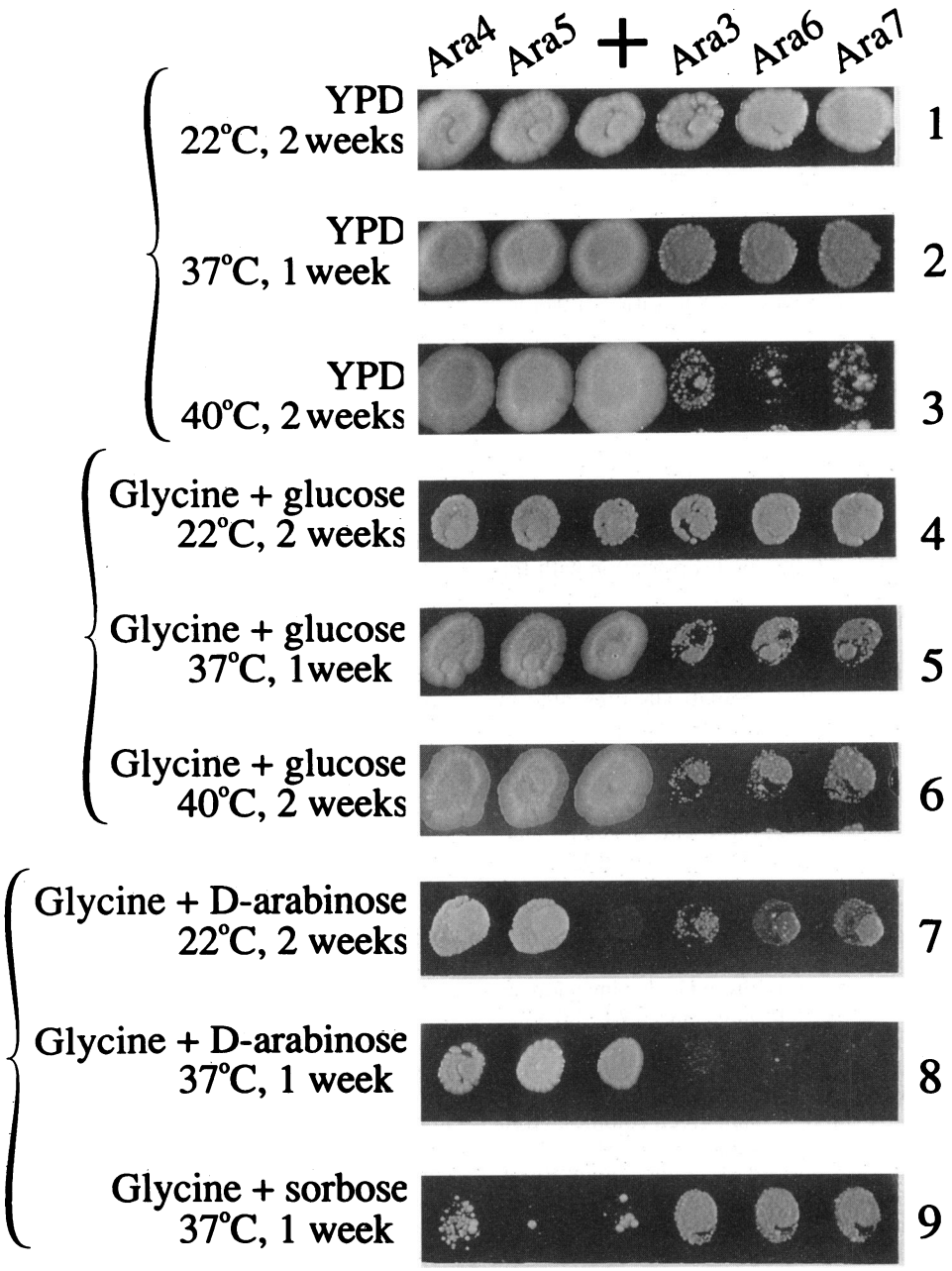

FIG. 3. Comparative growth of the parental strain 3153A (+) and positive arabinose mutants after 1 or 2 weeks of incubation on the media and at the temperatures indicated.

the normal strain, but the populations of colonies were uniform in appearance. However, most of the Sor^+ mutants and the group 3 Ara^+ mutants gave rise to mixed forms, which is indicative of chromosomal instability (38).

Electrophoretic karyotypes of Sor^+ and Ara^+ mutants. Electrophoretic karyotypes of positive mutants were obtained by using six different conditions of pulsed-field gel electrophoresis (see Materials and Methods). In this way, eight pairs of *C. albicans* 3153A chromosomes (38, 40) can be successfully separated as three short chromosomes, represented by five bands on the gel (or bottom group), as shown in Fig. 4A for the Sor^+ mutants, or as three short and two middle-sized chromosomes, represented by three bands on the gel (bottom and middle groups), as shown in Fig. 4C for the Ara^+ mutants. Separations of the three longest chromosomes (top group) are not presented, although they showed the typical pattern of high variability of chromosome VIII (36), as summarized schematically in Fig. 5. Also, we have not presented the CHEF-DRII separations of chromosome Ia, which were longer in the four Ara^+ mutants Ara2 to Ara5 (Fig. 5).

The electrophoretic karyotypes of all 25 positive mutants differed from that of the parental strain 3153A. Most importantly, alterations were clearly related to the selected positive phenotypes within the same phenotypic group. They were either identical or similar, even though they were derived independently. Specifically, chromosome III was altered in all

TABLE 3. Selected examples of the assimilation of carbon sources at various temperatures by the Ara$^+$ mutants with glycine as the nitrogen source

Group	Strain(s)	Growth[a] with:											
		Glucose			Sorbose			D-Arabinose			MGP		
		22°C	37°C	40°C	22°C	37°C	40°C	22°C	37°C	40°C	22°C	37°C	40°C
	3153A	+	+	+	±[b]	0[b]	0[b]	0	+	+	+[b]	+	+
2a	Ara1, Ara4, Ara5[c]	+	+	+	0[b]	0[b]	0[b]	+	+	+	+	+	+
2b	Ara2	+	+	+	±[b]	0[b]	+	+	+	+	+	+	+
3	Ara3[d], Ara6–Ara15	+	±	± | ±	±[b] | +[b]	0[b] | +	0	0[b] | +	0[b]	0	+[b]	± | +	0

[a] The levels of growth are denoted as follows: +, substantial; ±, reduced; ±, poor; 0, none. The range of levels for different members within the same group is indicated by a vertical line.

[b] Formation of colonies or papillae. Because a high frequency of papillae can be confused with substantial growth, some results are presented as, for example, 0[b] | +.

[c] Ara1, Ara4, and Ara5 were deficient in germ tube formation.

[d] Ara3 contains normal chromosome VI (see text).

10 Sor$^+$ spontaneous mutants. Except for those of Sor5, both chromosomes IIIa and IIIb comigrated at one or another of the original positions (Fig. 4A and 5). The identity of chromosome III alterations was established by hybridization with the Gα probe, as shown in Fig. 4B.

All four Ara$^+$ mutants from group 2, which were isolated as normal-sized creamy colonies, contained altered chromosomes IIb, which were shortened and which in subgroup 2a mutants comigrated with chromosome Ib (schematically presented in Fig. 5). Also, three of four Ara$^+$ mutants contained longer-than-normal chromosomes Ia. In some but not all preparations of members of this subgroup, chromosome IIIa or IIIb appeared at amounts between one and two copies. Apparently, this variability or unstable condition represents mixed populations of cells and gives rise to composite electrophoretic karyotypes (39).

The remaining mutant, Ara3, from the phenotypic set of colonies identified from selection plates as normal sized and creamy, had an electrophoretic karyotype that did not correspond to either group 2 or 3 and was altered by having a longer chromosome Ia and duplicated chromosomes IVa and IVb. However, its assimilation patterns were similar to those of group 3 mutants derived from small green colonies.

The last major group of mutants having similar electrophoretic karyotypes was isolated as small green colonies: Ara6 to Ara10, which were derived from the parent strain, and Ara11 to Ara15, which were derived as larger colony forms from Ara6 to Ara10, as described in Materials and Methods. Thus, the Ara11 to Ara15 mutants probably acquired compensatory mutations which enhanced their growth but did not affect either the karyotype or the assimilation patterns. The Ara6 to Ara15 mutants all contained an abnormal third chromosome VIc, each having an approximate 1-Mb deletion that varied slightly in size among different members of the set (Fig. 4C). These abnormal chromosomes VIc were identified by hybridization to the *HIS3* probe (Fig. 4D) after a full range of probes for chromosomes I to VIII was tested (data not presented). The lack of hybridization to five other chromosome VI probes, WOL-28, WOL-29, WOL-41, WOL-64, and *HPT1*, confirms the substantial size of the deletions and establishes the loss of common sequences among the Ara$^+$ mutants (Fig. 4D). Because the sizes of the homologs VIc were similar but not identical, the deletions apparently were generated with at least one different endpoint. It is also possible that chromosomes VIc were formed by translocations which were composed from a portion of chromosome VI containing the *HIS3* region and a portion of another, unidentified, chromosome lacking the region of the other tester markers. However, formation of these translocations would require the initial trisomy of both chromosome VI and the unidentified chromosome and would require the subsequent loss of the other products of the translocation.

DISCUSSION

Phenotypes and chromosomal aberrations of positive mutants. In this study, we have shown that the growth of strain 3153A of *C. albicans* on media containing one of a number of carbon sources or containing KNO_3 as a nitrogen source is temperature dependent (Table 1). On some media, including MGP, sorbose, D-arabinose, arbutin, and D-ribose media, papillae arose instead of confluent growth or over a background of weak confluent growth (Table 1 and Fig. 1 to 3). These two findings, papillated growth and temperature-dependent assimilations of substrates, reflect genetic plasticity of *C. albicans* and cast doubt on the current classification protocol for the genus *Candida*, which recommends testing assimilation at just one temperature, either 25 or 28°C (18).

Twenty-five mutants of strain 3153A, Sor1 to Sor10 and Ara1 to Ara15, were isolated on sorbose and D-arabinose media, respectively, and designated positive mutants (Fig. 1). All of them had altered electrophoretic karyotypes, and most importantly, the types of altered chromosomes were clearly related to the selected positive phenotypes. Alterations of chromosome III occurred with Sor$^+$ mutants, and alterations of chromosomes II occurred with the three Ara$^+$ mutants Ara1, Ara4, and Ara5. The remaining Ara$^+$ mutants were associated with what we believe are alterations of chromosome VI, as described in Results (Fig. 4 and 5; Tables 2 to 4). Thus, despite the independent origin of each mutant, the Sor$^+$ mutants and two phenotypic groups of Ara$^+$ mutants each contained specific chromosomal changes. Because Ara1 contained a normal chromosome Ia, we believe that alterations of chromosome Ia in other members of group 2 mutants arose secondarily to the main alterations of chromosome IIb. Ara3, which was initially identified as a normal-sized creamy colony but subsequently gave rise to small green colonies, was an exception, since its electrophoretic karyotype did not correspond to that of either the group 2 or 3 mutants. Nevertheless, the Ara3 electrophoretic karyotype abnormality, which consisted of aneuploidy of chromosome IV, is more closely related to the abnormalities of group 3 mutants, which Ara3 phenotypically resembles. This exceptional case was probably the result of sequential events due to genetic instability, as previously observed with certain other mutants.

Chromosome VIII was also altered in many mutants, but

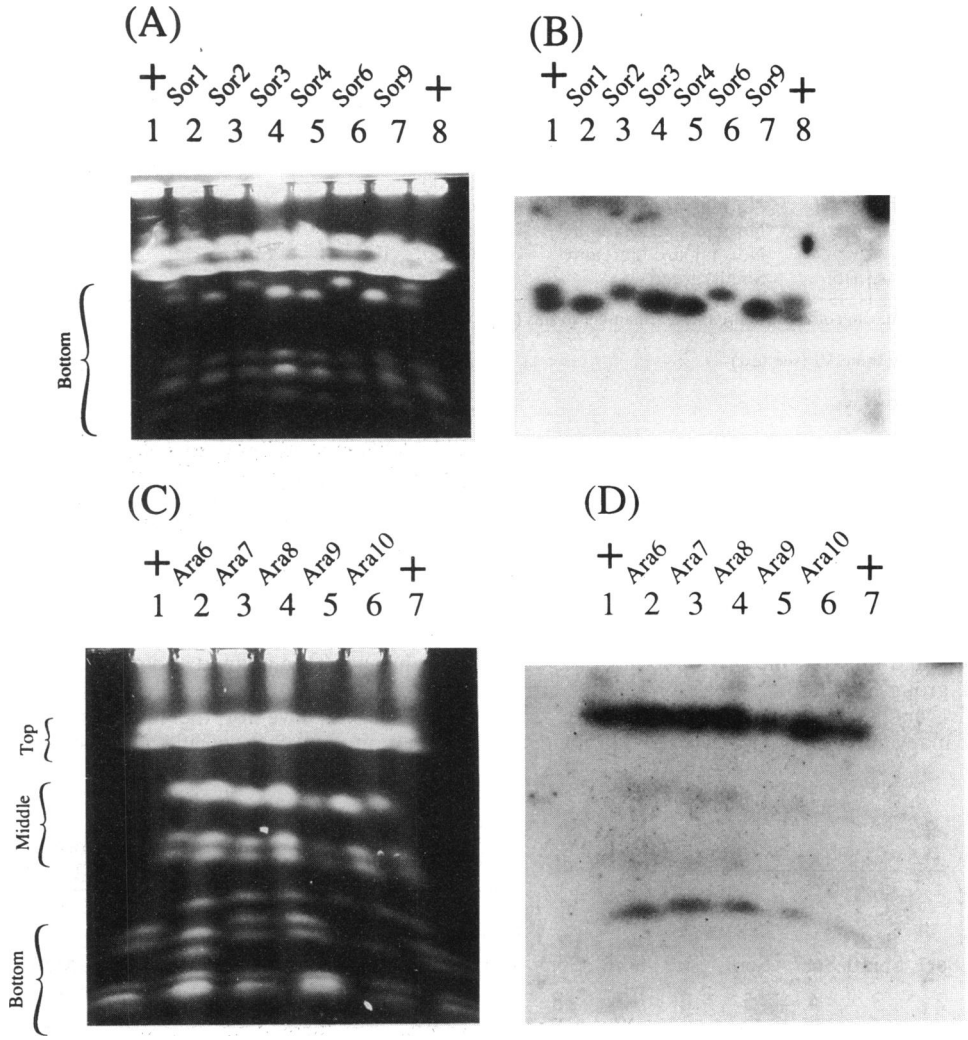

FIG. 4. Electrophoretic karyotypes of positive Sor$^+$ and Ara$^+$ mutants and the normal strain 3153A (+). (A) Orthogonal-field-alternation gel electrophoresis separation of chromosomes of the normal strain 3153A and six representative Sor$^+$ mutants. The condition was selected to accentuate the separation of the bottom group of chromosomes. (B) Hybridization to the Gα probe, revealing the loss of one homolog III and the duplication of the other. (C) Orthogonal-field-alternation gel electrophoresis separation of chromosomes of the normal strain 3153A and five representative Ara$^+$ mutants. The condition was selected to accentuate the separation of the middle and bottom groups. Chromosomes VIa and VIb in the top group were not separated with this running condition. (D) Hybridization to the *HIS3* probe, revealing the formation of an abnormal chromosome VI (homolog VIc).

these alterations are due mainly to variations in the number of rDNA units (36) and are not considered significant because they occur with an approximate frequency of 87% among spontaneous morphological mutants (36) and are also observed among normal subclones (36). In addition, chromosome VIII varies extensively among natural isolates (36, 38). Thus, by overlooking the aberrations in chromosome VIII, the group of Sor$^+$ mutants shown in lanes 2 to 5 of Fig. 5 may be considered to have the same electrophoretic karyotype. Similarly, the groups of mutants within lanes 6 and 7, and lanes 15 and 16, respectively, are believed to have equivalent electrophoretic karyotypes.

We have further investigated the phenotypes of positive mutants and found that they also were basically associated with groups defined by chromosomal alterations, as summarized in Tables 2 to 4. For example, members of subgroup 2a were identical to each other with respect to their patterns of utilization of those carbon and nitrogen sources (Tables 2 and 3) used to test the parental strain 3153A (Table 1). Furthermore, all the members of subgroup 2a lost the ability to germinate (Table 4) and displayed identical colonial forms (data not shown). These results were not unexpected because members of subgroup 2a share one major aberration, i.e., comigration of homolog IIb with its homolog IIa and with homolog Ib, as well as displaying a mixed banding pattern of chromosome III (Fig. 5), a fact that reflects mixed populations of cells due to genetic instability (39).

The assimilation patterns among the mutants of groups 1 and 3 were also specific (Tables 2 and 3). An important result of the assimilation tests was that positive mutants had a few additional limited acquisitions and losses of assimilation functions not directly selected (Tables 2 and 3). These additional attributes created distinct patterns within each of the groups 1 to 3.

We can suggest that the specific alterations of either chromosome III or II in the group 1 and 2 mutants activated genes

TABLE 4. Summary of the properties of the positive mutants

Phenotypic group	Strain(s)	Colony form on original selective plate	Germ tube formation (%)	Chlamydospore formation	Altered chromosome	Colonial morphology (LBC medium)
	3153A		85–96	+		Uniform
1	Sor1–Sor10	Normal	96–100	+	III	Uniform[a]
2a	Ara1, Ara4	Normal sized, creamy	0	+	II	Uniform
	Ara5	Normal sized, creamy	0	0	II	Uniform
2b	Ara2	Normal sized, creamy	80–100	+	II	Uniform
3	Ara3, Ara6–Ara15	Small, green	80–100	+	VI	Mixed

[a] Sor5 and Sor9 gave rise to uniform colonies, whereas the remaining strains (Sor1 to Sor4, Sor6 to Sor8, and Sor10) gave rise to a mixture of colonies with different colonial forms.
[b] Ara3 contains normal chromosome VI (see text).

directly involved in sorbose or D-arabinose assimilation. We wish to emphasize that the types of alterations in these two chromosomes were the same. Such aberrations are most plausibly explained by mitotic crossing over. Nevertheless, we have ruled out the possibility that positive mutants can arise by expression of recessive alleles that were originally heterozygous in the parental strain on chromosome III, because either chromosome IIIa or IIIb became homozygous and the recessive allele can be situated only on one or the other homolog. On the other hand, group 3 mutants, which were consistently temperature sensitive and exhibited poor, diminished, or papillated growth on many media and which contained a distinctly different chromosomal aberration consisting of a greatly shortened third chromosome VI homolog, may have altered regulatory genes affecting a wide range of properties. It is of interest that the same phenotype, in this case assimilation of D-arabinose at 22°C, can be achieved in cells by different means, using different types of alteration on different chromosomes.

Even though the exact nature of the chromosomal aberrations has not been defined and even though the mechanism by which these aberrations produce phenotypic changes is still unknown, we have for the first time demonstrated a clear association between specific chromosomal aberrations and

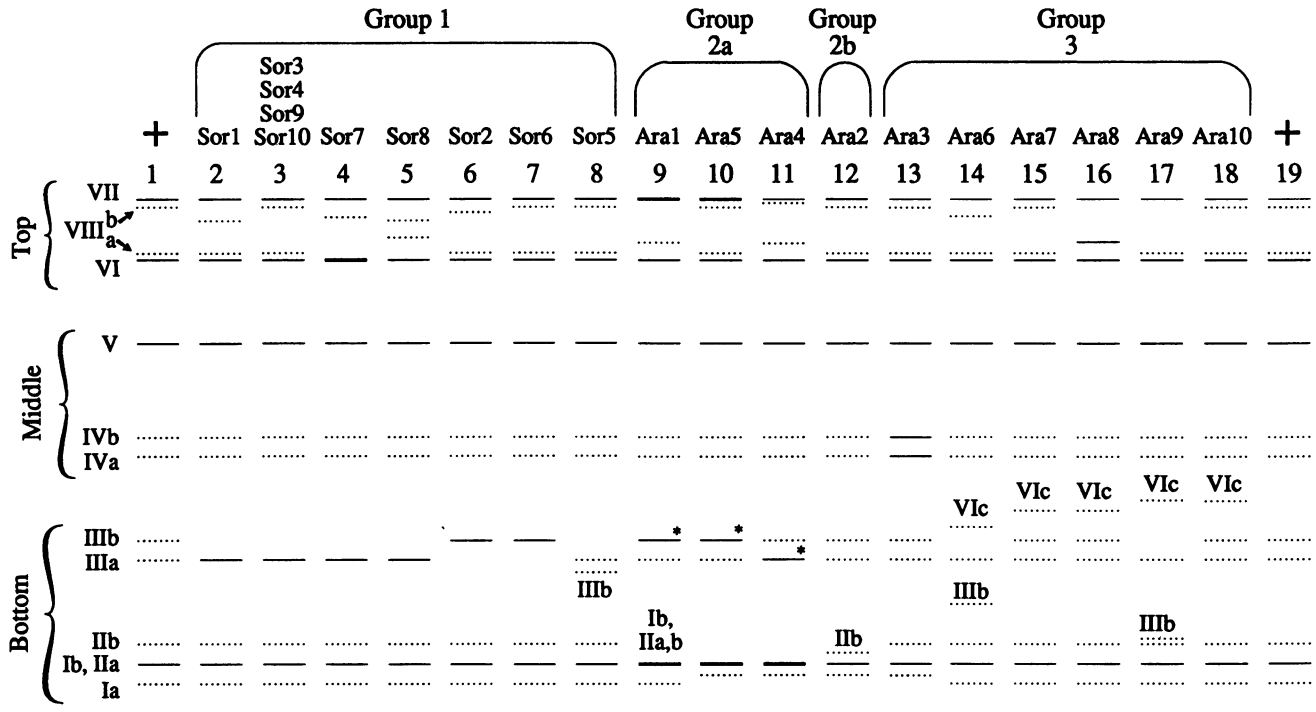

FIG. 5. Schematic representation of electrophoretic karyotypes of the 10 Sor$^+$ and 10 Ara$^+$ mutants. The experimental results showing same of these karyotypes are presented in Fig. 4. The parental strain 3153A (+) is in lanes 1 and 19. The assignment of chromosomes I to VIII and the homologous pairs, a and b, are indicated on the left, as previously reported (38, 40) and confirmed in this study with 18 hybridization probes. Dotted, thin, and thick lines correspond, respectively, to one, two, and more than two chromosomes. The major functional groups of mutants (Tables 2 and 3) are indicated by brackets at the top. The bands marked by asterisks, corresponding to chromosomes III in lanes 9 to 11, denote levels between one and two copies, as determined by ethidium bromide staining. The Ara6 to Ara10 karyotypes (lanes 14 to 18, respectively), as well as the Ara11 to Ara15 karyotypes (not presented), reveal abnormal chromosome VIc homologs. Also, the two karyotypes in lanes 14 and 17 reveal dramatic shortening of chromosome IIIb homologs.

specific phenotypes. In contrast, no specific chromosomal aberrations were previously observed with various phenotypes associated with spontaneous morphological mutants (39), probably because these were complex features that could be formed by many different types of genetic alterations. In contrast, each of the positive mutants, Sor^+ or Ara^+, is formed from a more restricted number of genetic alterations.

Cryptic genes. We wish to emphasize that the positive mutants described in this paper are unusual from the point of view of mutagenesis. While mutations normally destroy function, in this study we are dealing with both the loss and acquisition of functions. These positive mutations resemble the well-studied cases of cryptic genes in *Escherichia coli* and other bacteria that can be activated by mutation (12, 14, 15). Natural isolates of *E. coli* contain numerous sets of cryptic genes involved in diverse pathways, including amino acid biosynthesis and the assimilation and fermentation of carbon sources. These cryptic genes can be activated by different types of mutations, including single and multiple base pair changes and insertions of insertion sequence or other mobile genetic elements.

The mutational events required for activation have been systematically investigated with the *bgl* (32, 33), *cel* (31), and *asc* (14) operons, which are the most throughly investigated systems. These operons can be activated either by insertion of insertion sequence elements in the promoter or other regions or by base pair substitution at various sites. Most importantly, the activation can be readily reversed in at least some cases by precise excision of insertion sequence elements. These and other results support the notion that expression of cryptic genes arises repeatedly in response to environmental changes. Hall and Xu (14) have suggested that silencing and activation of cryptic genes occur for those genes whose products are normally detrimental but occasionally useful.

Less-extensive studies also suggest the existence of cryptic genes in yeasts. Carlson et al. (5) have demonstrated that *Saccharomyces cerevisiae* strains often contain silent genes for sucrose fermentation and that these alleles can be activated by mutation. The wild-type *ADH4* gene in *S. cerevisiae* is not sufficiently expressed to produce a phenotype unless it is activated by insertion of a Ty retrotransposon (50). Generally, Ty insertions can affect the expression of neighboring genes in diverse ways, including activation, mating type regulation, deregulation, inactivation, and cold sensitivity, either separately or in various combinations (1). Several of the clusters of three *MAL* genes at either the *MAL1* or *MAL3* locus in naturally occurring *Saccharomyces* species were shown to differ from fully functional alleles by mutations and rearrangements, including deletions and base pair substitutions (6, 27, 29). It is unclear at this time if nonfunctional forms are revertible by mutation, although complementation occurs among certain pairwise crosses of Mal^- strains. It should be noted that completely inactive pseudogenes are believed to be rare in yeasts (17).

It remains to be seen if mutational events activating cryptic genes in *E. coli* are in any way similar to the events producing positive mutants in *C. albicans*. Chromosomal rearrangements either could activate the expression of a structural or regulatory gene or could inactivate the expression of a negative repressor. In any case, the gross chromosomal rearrangements uncovered in this and other studies are unlikely to be reversible, as are the mutational events activating and silencing cryptic genes in *E. coli*. It has not been excluded that the chromosomal aberrations, although specific for each phenotypic class of positive mutants, may be a secondary event of a more simple mutation activating cryptic genes. Also, chromosomal duplications or aneuploidy could produce genetic variability by producing phenotypic alterations due to gene dosage or gene imbalance.

Although it is premature for any definitive conclusions, we wish to propose a working and testable hypothesis concerning the role of chromosomal rearrangements in the genetic variability of *C. albicans*. We suggest that positive mutants are generated by the formation of still-undefined genetic alterations that occur spontaneously at high frequencies. These alterations may occur at or near cryptic genes, which have their coding capacities intact or nearly intact but are not expressed because of an inhibitory element or the lack of a critical promoter-like element that is corrected in the positive mutant. Thus, chromosomal aberrations could arise as secondary consequences of some general genomic perturbation.

On the other hand, the loss or destruction of functions could occur by numerous pathways and therefore could be highly variable in nature. Also, quantitative features, like colonial morphologies and other properties described above, potentially could be altered by any one of a large number of different genetic lesions. Nevertheless, we believe that a complete analysis of the consistent chromosomal alterations associated with the formation of positive mutants and the determination of the corresponding DNA sequence modifications may throw light on the underlying mechanism of genetic variability in *C. albicans*. We have designed experiments to further characterize positive mutants and to address the questions discussed above.

Adaptive mutations. A distinctive feature of positive mutants is their delayed and continued appearance during incubation. This phenomenon clearly resembles adaptive mutations, i.e., the production of spontaneous mutants from nondividing populations of microorganisms in apparent response to selective conditions (10). Study of the occurrence and properties of adaptive mutagenesis has been carried out mainly with defined systems in *E. coli*, as summarized by Foster (10), although significant experiments have been also performed with the yeast *S. cerevisiae* (13, 44, 45). In addition, mutants resistant to heavy metals have been reported to occur after 5 to 10 days and to continue to appear for 1 to 2 weeks in stationary-phase cultures of *C. albicans* (24).

In spite of persuasive experiments and a preponderance of evidence, the phenomenon of adaptive mutation has not been universially accepted and critics have postulated a variety of artifacts to explain the observations (10, 22). Nevertheless, it should be pointed out that the activation of cryptic genes in *E. coli* occurs at high frequencies, even though some of them require more than one mutation in order to be activated (3, 11). Further experiments with defined conditions may reveal whether positive mutants are formed from nondividing cells in response to the selective conditions.

Polymorphic variation, taxonomic classification, and positive mutants. The classification of approximately 196 species within the genus *Candida* and the assignment of strains to *C. albicans* are primarily based on a few morphological features, i.e., formation of germ tubes, chlamydospores, and pseudohyphae, and on the fermentation and assimilation of carbon sources (26). These same tests are also used for biotyping *C. albicans*. For example, tests of the assimilation of 19 different carbon sources by 130 isolates of *C. albicans* revealed variability in the response to 11 substrates and subsequently assignment to 33 biotypes (49). Because tests are routinely carried out at one temperature, the variations observed among *Candida* strains could be due in part to the variations in temperature dependence, as shown for strain 3153A in Table 1. Although the high degree of polymorphic variations in assim-

ilation of carbon and nitrogen sources by *C. albicans* is well established, this phenomenon has not been investigated from a genetic or mechanistic point of view. The relationships between related species and between biotypes of the same species can be evaluated from the results of phenotypic variations of spontaneous mutants derived under defined laboratory conditions.

Because the differences in the assimilation patterns are a prominant and distinguishing feature among different *Candida* species and because the electrophoretic karyotypes of many of them vary within a limited range (16, 46), similar to the variability observed among spontaneous mutants (this study and references 36 through 40), we suggest that many so-called *Candida* species are merely chromosomal and phenotypic variants of the same species. For example, spontaneous mutants losing the ability to form chlamydospores may account for such synonyms of *C. albicans* as *Candida longeronii*, a chlamydospore-negative variant (26). Also, the more frequent spontaneous occurrence of nongerminating mutants readily explains the establishment of taxa such as *Candida claussenii*, now regarded as a nongerminative synonym of *C. albicans* (26). In addition, the results reported in this study strongly indicate that the polymorphic variations observed among strains of *C. albicans* arise by the formation of positive mutants, as observed for the normal strain 3153A. In fact, over 25 years ago, Brown-Thomsen (2) pointed out the possible derivation of a *Candida tropicalis* strain from *C. albicans*. Also, Sarachek et al. (43) discussed the formation of *Candida stellatoidea* from *C. albicans* and *C. tropicalis*. Thus, even though *C. albicans* is asexual and lacks the ability to generate diversity by mating and recombination, it has the capacity to spontaneously produce a wide range of positive phenotypes. Although the molecular mechanisms for producing these mutants are still unknown, the phenomenon is indeed impressive.

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