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Appearance and Properties of l-Sorbose-Utilizing Mutants of *Candida albicans* Obtained on a Selective Plate

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ABSTRACT

This is the first report that adaptive mutagenesis can arise by chromosomal nondisjunction, a phenomenon previously associated exclusively with DNA alterations. We previously uncovered a novel regulatory mechanism in *Candida albicans* in which the assimilation of an alternative sugar, l-sorbose, was determined by copy number of chromosome 5, such that monosomic strains utilized l-sorbose, whereas disomic strains did not. We present evidence that this formation of monosomy of chromosome 5, which is apparently a result of nondisjunction, appeared with increased frequencies after a selective condition was applied, *i.e.*, by adaptive mutagenesis. The rate of formation of l-sorbose-utilizing mutants per viable cell per day ranged from 10^{-6} at the initial time of detection to 10^{-2} after 4 days of incubation on the selective plate.

THE ability of the microbial cells to adapt to the changing environment has been the subject of numerous studies. A number of characteristic features of microbial adaptability to selective conditions have been investigated as a result of renewed interest during the last decade, primarily due to a prominent article by Cairns *et al.* (1988). It was originally suggested that mutations arise in nondividing microorganisms subjected to selective pressure and that only the selected mutations appeared at increased frequencies in the population, a phenomenon initially denoted "directed mutation." However, subsequent studies suggested that higher mutation rates occurred in genes not selected for, and the phenomenon was denoted as "adaptive mutation" (Foster 1993). Currently, "adaptive mutations" denote mutations that are formed at higher frequencies in response to the selective conditions, even though they may not be necessarily "directed" (Foster 1998). Under the current usage, mutants resistant to and induced by ultraviolet light would be considered to arise by adaptive mutations, as would nutritionally selected mutants induced by starvation or stress conditions.

Several *Escherichia coli* and *Saccharomyces cerevisiae* model systems were used to show that adaptive and preexisting mutants differed, since only the former occurred with higher frequencies in a delayed time-dependent or cumulative fashion (Shapiro 1984; Cairns *et al.* 1988; Hall 1991, 1992). At least with the *E. coli lacZ* system, adaptive mutants and those arising without selection have different distributions of mutagenic changes and are formed by different mechanisms (reviewed in Foster 1998).

We previously demonstrated that regulation of gene expression in *Candida albicans* is achieved by changing chromosome copy number (Janbon *et al.* 1998). Using l-sorbose-utilizing mutants, we showed that the reduction of one copy of chromosome 5 (previously denoted chromosome III; Janbon *et al.* 1998) activates a structural gene for l-sorbose dehydrogenase positioned on another chromosome. We hypothesized that chromosome number may be a common means to control important functions in *C. albicans.*

Previous studies with *C. albicans* revealed that mutants utilizing l-sorbose or d-arabinose (Rustchenko *et al.* 1994), or resistant to heavy metals (Malavasic and Cihlar 1992), arose during the course of prolonged incubation in a cumulative fashion, suggestive of adaptive mutations. In this article, we quantitatively analyzed the formation of l-sorbose-utilizing mutants and demonstrated that they do indeed appear on a selective plate with increased frequencies, in agreement with the current definition of adaptive mutagenesis. Furthermore, we found that the mutants arising on day 12, which by all criteria were formed by adaptive mutagenesis, lacked one chromosome 5 homologue, identical to the karyotypic change responsible for utilization of l-sorbose as reported in our original study (Janbon *et al.* 1998). Our results are conceptually similar to adaptive mutagenesis previously reported for *E. coli* (reviewed in Cairns 1998) and *S. cerevisiae* (Hall 1992; Steele and Jinks-Robertson 1992; Heidenreich and Wintersberger 1997; Marini *et al.* 1999) that occurred by DNA alterations. This is the first report that adaptive mutations can arise by chromosomal nondisjunction.

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MATERIALS AND METHODS

Nomenclature of *C. albicans* chromosomes: In the nomenclature of Wickes *et al.* (1991), which is used in this article, the penultimate largest to smallest chromosomes are designated by Arabic numerals 1–7, whereas the largest chromosome, containing the ribosomal DNA cluster, is designated R. The nomenclature used in our previous publications consists of roman numerals I–VIII to designate the smallest to the largest chromosomes, respectively, and a and b to designate two homologues of different sizes. The following are equivalencies for the two types of chromosome assignments:

Chromosome:	VIII	VII	VI	V	IV	III	II	I
	R	1	2	3	4	5	6	7

Designations of phenotypes and strains: The phenotypic symbols Sou^+ and Sou^- denote the ability and inability, respectively, to grow on l-sorbose medium. Mutants derived from the *C. albicans* strain 3153A that utilize l-sorbose are denoted as Sor, followed by an isolation number. For example, Sor52 denotes a Sou^+ mutant derived from the Sou^- parental strain 3153A, whereas Sor52-1 denotes a Sou^- revertant derived from the Sou^+ mutant Sor52. Furthermore, Sor52-1-1 designates a second round Sou^+ mutant subsequently derived from the Sou^- revertant Sor52-1. *SOU1* denotes the gene that is responsible for the assimilation of l-sorbose.

Strains: The *C. albicans* laboratory strain 3153A has been studied extensively with respect to spontaneous and selected changes in its chromosomal pattern, as well as for the associated phenotypes (reviewed in Rustchenko *et al.* 1997; Janbon *et al.* 1998). In this work, 27 independent clones, denoted clones 1–27, were derived from 3153A as part of the fluctuation studies of Sou^- (parental phenotype) → Sou^+ (mutant phenotypes). These clones were used to obtain clonally related but independently derived Sou^+ mutants. A portion of these Sou^+ mutants, Sor31 to Sor40, Sor41 to Sor50, and Sor51 to Sor60, were arbitrarily chosen from day 4, 8, and 12, respectively, as described below in *Isolation of independent clones.* A number of the Sou^- revertant strains, which lost the ability to assimilate l-sorbose, were used in additional experiments. In particular, 25 independent clones derived from the Sou^- revertant Sor52-1 were used to obtain a second round of Sou^- → Sou^+ mutants as part of the fluctuation study.

A strain of *S. cerevisiae*, no. 865 (*MAT*α *ura3-52 his3-*Δ*200*), was used as a scavenger to exhaust contaminating nutrients that may be present in agar (Mack *et al.* 1994).

Media and growth conditions: Yeast extract/peptone/dextrose (YPD), synthetic dextrose (SD, also denoted glucose medium), and l-sorbose media were previously described (Rustchenko *et al.* 1994). Uracil and histidine were added as previously indicated (Sherman 1991). *C. albicans* and *S. cerevisiae* strains were incubated at 37° and 30°, respectively.

Maintenance: All strains were maintained and stored under conditions that prevented the increase of spontaneous mutants due to chromosomal instability and the selection of variants, as previously described (Rustchenko-Bulgac 1991).

Scavenger cells: A number of controlled experiments were initially carried out to determine the requirement for scavenger cells to exhaust contaminating nutrients that may be present in agar (Mack *et al.* 1994). These initial experiments were performed with the same cell suspension of *C. albicans* prepared as follows: 3153A cells from the −70° preserved culture were streaked on YPD plates, and the plates were incubated overnight to obtain young colonies. Cells from 10 colonies were suspended in water, diluted, and plated on YPD medium, resulting in ∼30 colonies per plate. Cells from all colonies on two plates were suspended in water and washed twice, resulting in 6.4×10^7 cells, which represented the population of strain 3153A preserved as the −70° culture.

This same cell suspension was used in the following control experiments with and without the addition of 1×10^8 cells of *S. cerevisiae* strain no. 865, which acts as a scavenger: (i) comparing the appearance of Sou^+ mutants from 3153A cells on l-sorbose medium, similar to the procedure used in the fluctuation test described below; (ii) estimating the viability of Sou 3153A cells on l-sorbose medium by transferring whole agar discs to YPD medium (see below, *Viability on selective medium by transferring agar discs to glucose medium*); and (iii) estimating the viability of Sou^- 3153A cells on l-sorbose medium by washing agar blocks and replating cell suspensions (see below, *Viability on selective medium by washing cells from the surface of the plates*).

There were no significant differences between the numbers of viable *C. albicans* cells on l-sorbose medium in methods (ii) and (iii) described above, with and without scavenger cells.

In the first experiment (i), 10^6, 10^5, and 10^4 3153A cells with and without scavenger cells were each spread on two l-sorbose plates. The total number of Sou^+ mutants in this series was consistent with the number of 3153A cells plated except that ∼10 times more colonies appeared in each of the first 2 days on medium lacking the scavenger cells. Analysis of the scavenger strain by itself on l-sorbose medium revealed that the scavenger cells remained fully viable until 8 days after plating (see below). Taken together, the control experiments indicated that the scavenger cells prevent the increased appearance of Sou^+ mutants in the first days of incubation, but do not affect viability of *C. albicans* Sou^- cells.

Viability of *S. cerevisiae* cells on selective medium: The viability of strain no. 865 of *S. cerevisiae*, used as a scavenger, was determined after various days of incubation on l-sorbose medium. A total of 10 independent colonies were used to inoculate 100 ml of YPD medium. After 2 days of growth at 30°, the cells were recovered by centrifugation, washed twice with 100 ml of water, and resuspended in 10 ml of water. The cell concentration was then adjusted to 1×10^9 cells/ml. On the first day of the experiment, 1×10^8 cells were spread on each of six l-sorbose plates supplemented as described with the *C. albicans* experiment. Immediately after plating, two plugs of agar, having an area of 3.1 mm^2, were excised from one plate. The cells present on the agar plug were resuspended in 400 μl of water by vigorous vortexing and appropriate dilutions were spread on YPD plates. The colonies were counted after 2 days of incubation. The same procedure was repeated daily during a 14-day period, allowing the determination of the viability of the scavenger cells.

Isolation of independent clones: *C. albicans* 3153A cells were removed from a −70° preserved culture, streaked on YPD plates, and grown overnight. One young colony was suspended in water and plated on YPD medium after appropriate dilutions to produce ∼30 subclones on the following day. A total of 27 young subclones were chosen from the same plate, thus avoiding possible bias in selection, as well as spontaneous mutations that occur in old colonies.

In addition, three groups of 10 Sou^+ mutants representing days 4, 8, and 12, respectively, were isolated from 10 of the 27 Sou^- subclones described above. Thus, three groups of 10 mutants, Sor31 to Sor40 (day 4), Sor41 to Sor50 (day 8), and Sor51 to Sor60 (day 12) were obtained. All of the mutants within a group were clonally related and were derived by independent mutational events.

Fluctuation test: Experiments were carried out to estimate the number of mutations occurring before and after the cells were exposed to the selective l-sorbose plates (Luria and Delbruck 1943; Lea and Coulson 1949; Stewart *et al.*

1990). Young colonies, grown on the same YPD plate and originating from one single colony, as described above, were each carefully removed from the agar surface and suspended in 200 μl of distilled water. A 10-μl portion was removed from each sample, diluted 4×10^3-fold, and plated on two SD control plates, a condition that was expected to yield approximately 250 colony-forming units (cfu) per plate. Each remaining sample was then mixed with 200 μl of a water suspension containing 2×10^8 previously washed cells of *S. cerevisiae* no. 865. Equal portions of the cell suspensions were subsequently plated on two l-sorbose plates with appropriate supplements (see above). The plates were incubated and the appearance of colonies was noted daily over a 2-wk period using magnification.

The average number of mutations that occurred during the growth of each culture was deduced from the distribution of the number of Sou^+ mutants in the first day of their appearance, according to the method of Stewart *et al.* (1990), using the DATAFIT program (B. G. Hall and F. M. Stewart). This program allows the estimation of *m*, the number of mutations that occurred during the nonselective growth, and μ, the number of mutations that occurred after contact with the selective medium.

Isolation of revertants: Cells of Sou^+ cultures were removed from −70° storage culture, streaked on a YPD plate and incubated for 2 days to discriminate between Sou^+ mutants, which are monosomic for chromosome 5, and faster growing Sou^- revertants, which are due to duplication of the remaining homologue of chromosome 5. The smaller Sou^+ colony was restreaked on a YPD plate and incubated overnight. A large Sou^- revertant colony, in which the normal rate of the growth was recovered, was identified and restreaked again on a YPD plate to confirm the colonial phenotype, and large colonies were subsequently tested on l-sorbose and SD media to confirm the Sou^- phenotype (see Janbon *et al.* 1998 for more details and for the frequency of the reversion).

Isolation of Sou^+ mutants from Sou^- revertants: Sou^- revertant clones of independent origin were prepared and each clone was plated on l-sorbose medium to obtain Sou^+ mutants, as described above for the fluctuation test.

Reconstruction experiments: The ability of Sou^+ mutant strains to regrow on l-sorbose plates was investigated by reconstruction experiments. Typically, a Sou^+ mutant was prepared for reconstruction experiment as follows: cells were removed from a −70° preserved culture, streaked for independent colonies on YPD medium, and incubated for 2 days to identify the majority of smaller Sou^+ colonies, which are monosomic for chromosome 5, and which have a diminished rate of growth. Subsequently, a single small Sou^+ colony was removed from the plate and suspended in water. The suspension was appropriately diluted and mixed with scavenger cells of the *S. cerevisiae* no. 865 strain, previously washed in water. The mixture, resulting in ∼400 cfu of Sou^+ cells and $\sim 2 \times 10^8$ scavenger cells was equally divided and each portion was plated on l-sorbose medium. In addition, the Sou^+ cells were also mixed and plated as described above with both scavenger cells and $\sim 2 \times 10^5$ cells of the parental strain 3153A, a condition emulating the original condition on selective plates. Similarly, both mixtures were spread on two control plates of SD with supplements.

Viability on selective medium by transferring agar discs to glucose medium: Appropriate dilutions of either the initial Sou^- strain 3153A or of a Sou^- revertant were mixed with *S. cerevisiae* no. 865 scavenger cells and plated on a number of l-sorbose plates with supplements as described above. Each day during the course of 2 wk, entire agar discs of duplicated plates were transferred with the help of a sterile spatula to the surface of two YPD plates, where all viable cells can grow. Distinct colonies of *C. albicans* appeared on the background growth of the scavenger cells because of the difference in the growth rate of the two species. The number of *C. albicans* colonies was recorded after 5 days of incubation.

Viability on selective medium by washing cells from the surface of the plates: The ability of Sou^- cells to undergo a few divisions on l-sorbose medium was investigated by the daily determination of the total number of viable cells on defined areas of the plates. These determinations were carried out during the course of only the first 4 days of incubation, because the viability was too low after this time. Three agar plugs with areas of 3.1 mm^2 were excised from an l-sorbose plate on each day with the tip of a 10-ml pipette, and the cells were suspended in 400 μl of water by vigorous vortexing. Subsequently, 150-μl portions of the suspensions were spread on YPD and SD plates that were incubated for 3 days to determine the total number of viable cells.

Pulsed-field gel electrophoresis: To prepare chromosomes of Sou^+ mutants and to avoid Sou^- revertants, cells preserved as −70° cultures were removed and a sufficient number of cells were obtained by growth on YPD plates according to the procedure of Janbon *et al.* (1998). Preparations of intact chromosomes were based on a *S. cerevisiae* protocol (Carle and Olson 1984) and improved by using agarose plugs, which were cast in plug molds (Bio-Rad Laboratories, Richmond, CA). Chromosomal separations were optimized by using two versions of pulsed-field gel electrophoresis, an orthogonal-field-alternation gel electrophoresis (OFAGE) and contour-clamped homogeneous electric fields (CHEF) system (Bio-Rad). In addition, three different OFAGE running conditions for separation of three differently sized groups of the eight chromosomes were applied singly or in combinations to obtain precise separation of an entire banding pattern, as described previously (Rustchenko-Bulgac and Howard 1993).

RESULTS

Appearance of Sou^+ mutants from 27 independent clones of strain 3153A: Our earlier results demonstrated that Sou^+ mutants arose from the parental strain 3153A after prolonged incubation on the selective l-sorbose plates in a cumulative fashion, suggestive of adaptive mutagenesis (Rustchenko *et al.* 1994). In this work, we undertook a systematic investigation of the time and frequency of appearance of Sou^+ mutants on l-sorbose plates from 27 similarly prepared Sou^- clones of strain 3153A (see materials and methods).

The time of appearance of the Sou^+ colonies was recorded as soon as they were detected under magnification (Table 1). These results with the 27 independently derived clones (Table 1, column 1) are listed according to the total number of cells plated, which ranged from 3.5×10^5 to 12×10^5 (Table 1, column 2). All 27 clones began to give rise to Sou^+ colonies on day 4 after plating, with continued daily appearance for at least 2 wk. A few of the 27 clones still gave rise to Sou^+ colonies after day 14, but these were not considered here. Also we consistently recorded a small number of microscopic colonies that appeared throughout the 2-wk period, but did not increase in size, and which were not investigated further.

Viability of Sou^- strains on l-sorbose medium: The

TABLE 1

The number of Sou$^+$ colonies appearing each day from the 27 clones of the original strain 3153A and the 25 clones of the revertant strain Sor52-1

	Cells plated ($\times 10^5$)	Daily appearance of colonies[a]															Total
		0 —	1 —	2 —	3 —	4 (0)	5 (1)	6 (2)	7 (3)	8 (4)	9 (5)	10 (6)	11 (7)	12 (8)	13 (9)	14 (10)	
Clones 3153A																	
1	3.5	0	0	0	0	3	2	0	2	9	2	2	4	1	2	1	29
2	4.9	0	0	0	0	5	6	3	2	76	45	21	5	5	3	2	173
3	5.1	0	0	0	0	11	13	6	2	6	5	6	2	3	3	2	59
4	5.3	0	0	0	1	24	11	3	6	6	3	3	4	2	1	1	65
5	5.9	0	0	0	0	16	6	3	1	7	5	4	4	4	3	2	55
6	6.1	0	0	0	0	21	10	6	10	18	2	5	3	5	4	2	86
7	6.4	0	0	0	0	16	23	7	5	9	6	7	4	3	4	0	84
8	6.4	0	0	0	0	7	4	2	11	17	4	2	3	4	2	1	57
9	6.8	0	0	0	1	6	4	6	8	10	8	5	6	4	2	2	62
10	7.6	0	0	0	0	7	13	12	9	26	10	9	7	2	4	3	101
11	7.9	0	0	0	1	36	25	5	7	14	8	4	4	3	2	3	112
12	8.8	0	0	0	1	30	11	10	19	21	9	12	11	4	3	1	132
13	8.9	0	0	0	0	3	12	34	16	40	14	13	6	7	5	1	147
14	9.3	0	0	0	6	25	12	13	11	19	7	8	9	8	5	3	126
15	9.4	0	0	0	0	7	10	9	10	12	6	3	3	4	2	1	67
16	9.5	0	0	0	0	9	22	7	15	29	16	10	6	3	4	2	123
17	9.6	0	0	0	0	4	10	4	8	21	1	5	5	5	2	1	66
18	9.6	0	0	0	2	23	10	6	4	19	7	2	2	3	2	4	84
19	9.7	0	0	0	4	33	5	16	11	26	17	9	7	8	3	0	139
20	9.7	0	0	0	3	19	4	15	24	38	12	7	11	2	4	1	140
21	9.7	0	0	0	1	14	8	7	6	19	5	5	4	5	1	2	77
22	9.8	0	0	0	0	6	6	8	9	16	11	7	3	6	5	0	77
23	9.8	0	0	0	0	11	8	3	4	18	4	3	3	2	4	6	66
24	9.9	0	0	0	1	67	27	8	7	24	13	8	6	8	7	4	180
25	10	0	0	0	2	51	48	15	16	26	19	10	6	3	2	1	199
26	11	0	0	0	0	20	12	18	21	28	14	10	5	4	4	1	137
27	12	0	0	0	2	19	9	11	7	15	16	7	3	5	3	2	99
Clones Sor52-1																	
1	3.9	0	0	0	1	124	281	137	53	47	28	16	8	4	3	0	702
2	7.6	0	0	0	4	75	21	19	29	33	27	19	8	7	5	2	249
3	7.6	0	0	0	0	141	256	103	62	30	27	15	6	4	0	1	645
4	8.8	0	0	0	0	108	134	144	95	40	31	14	9	4	1	0	580
5	9.5	0	0	0	0	64	221	123	83	59	67	27	19	6	2	2	673
6	11	0	0	0	0	95	195	121	90	49	43	15	7	4	3	2	624
7	12	0	0	0	1	101	120	132	110	72	60	26	6	7	4	1	640
8	5.2	0	0	0	1	139	191	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
9	5.8	0	0	0	6	201	317	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
10	6.1	0	0	0	1	112	170	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
11	6.2	0	0	0	1	133	277	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
12	6.3	0	0	0	1	131	278	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
13	6.4	0	0	0	3	301	193	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
14	6.6	0	0	0	3	179	286	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
15	6.8	0	0	0	0	89	110	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
16	6.9	0	0	0	5	155	299	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
17	8.4	0	0	0	0	73	295	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
18	8.4	0	0	0	4	232	407	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
19	9.4	0	0	0	0	105	235	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
20	11	0	0	0	4	237	352	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
21	11	0	0	0	0	64	213	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
22	12	0	0	0	0	153	284	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
23	12	0	0	0	0	74	386	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
24	13	0	0	0	0	153	225	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND
25	13	0	0	0	0	206	332	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	ND

ND, not determined.

[a] The day of formation of the Sou$^+$ mutations is presented in parentheses, assuming that 4 days are required to form visible colonies.

[b] The cumulative value was too high for determining the appearance of colonies.

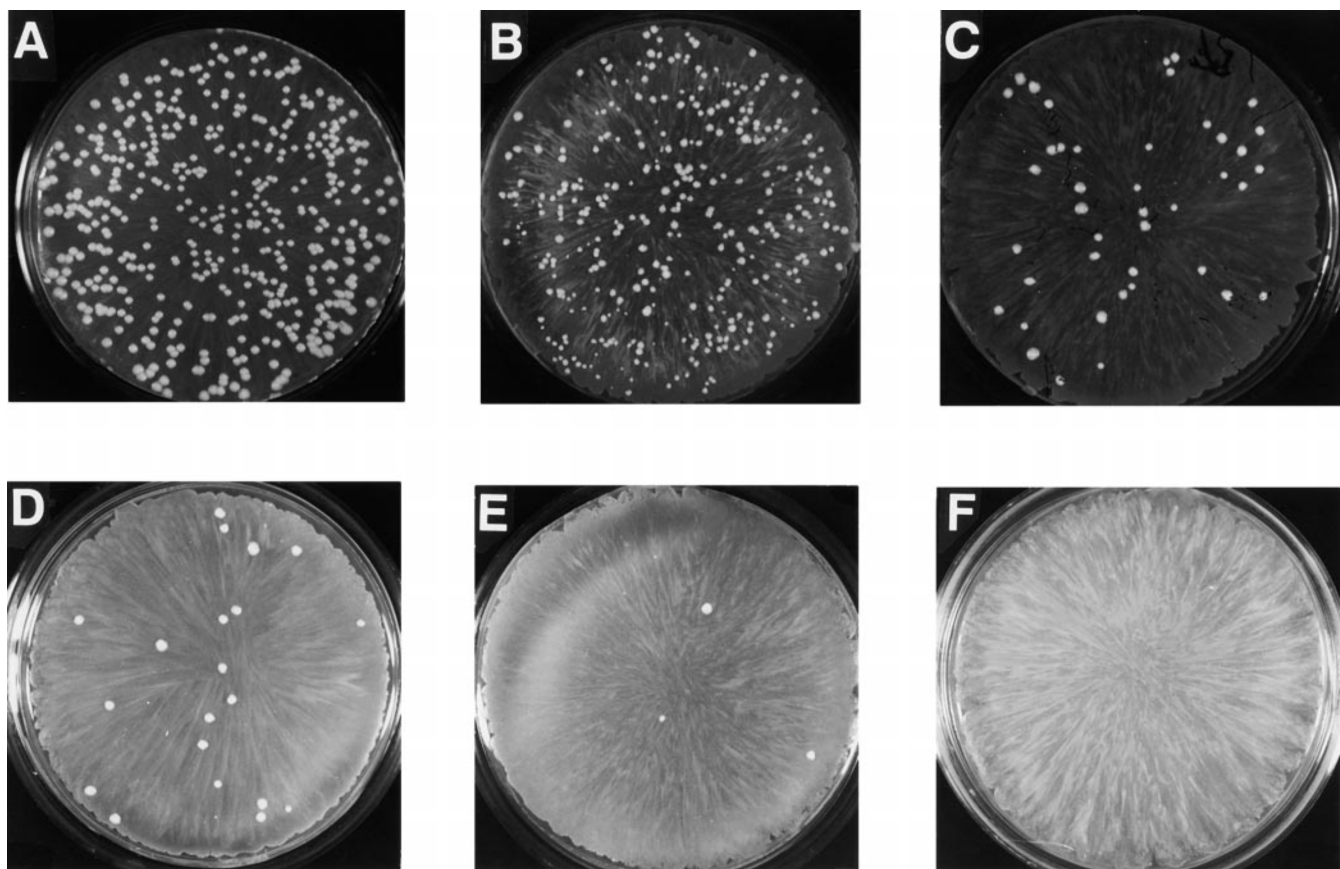

Figure 1.—The death of Sou$^-$ cells of strain 3153A on l-sorbose medium. Cells were spread on l-sorbose plates that were either not incubated (B), or incubated for 1 (C), 2 (D), 3 (E), and 4 (F) days, and the viability was determined by transferring the l-sorbose agar discs to YPD medium. No loss of cells was observed on a synthetic medium lacking a carbon source, after incubating the plates for 5 days and transferring the agar discs to YPD medium (A). Approximately 450 colony-forming units were spread on each plate. Residual background growth of the *S. cerevisiae* scavenger cells appeared after transferring the agar discs to YPD medium.

viability of 3153A cells on l-sorbose medium was determined by the daily transfer of entire agar blocks to YPD plates, as described in materials and methods. As presented in Figures 1 and 2, massive death of the cells occurs during incubation on l-sorbose plates. After 3 days, <1% of the cells originally plated were viable. By 9 days, the number of viable cells corresponded approximately to the number of Sou$^+$ mutants. The toxicity of l-sorbose was also observed previously with *Neurospora crassa* (Brockman and De Serres 1963).

This massive decline in living cells was not observed when cells were incubated on a synthetic medium lacking a carbon source (Figures 1 and 2), or when cells were maintained in suspension in water (data not presented). In addition, we did not observe cell death or any obvious difference in the rate of growth on media containing just glucose or a mixture of l-sorbose and glucose. However, the cells died on medium containing a mixture of l-sorbose and ethanol.

We also tested the viability on l-sorbose medium of two Sou$^-$ revertants, Sor33-1 and Sor52-1, that were isolated from the day 4 Sou$^+$ mutant, Sor33, and the day 12 Sou$^+$ mutant, Sor52, respectively. The Sor52-1 survival was similar to that of strain 3153A, whereas the Sor33-1 survival was even less, with ~0.1% viability after 2 days of incubation.

The results of the reconstruction experiment: To determine whether the late appearance of Sou$^+$ mutants was due to slower growth or to subsequent formation on the selective plate, reconstruction experiments were performed as described in materials and methods (Hall 1990; Steele and Jinks-Robertson 1992). As presented in Table 2, all 10 independent Sou$^+$ mutants from day 4, Sor31 to Sor40, regrew on l-sorbose medium between the third and fourth day after plating, which is the approximate time of the original visualization of the respective colonies on a selective plate. Furthermore, 9 out of 10 Sou$^+$ mutants that originally arose on day 12, the Sor51 to Sor53 and Sor55 to Sor60 mutants, also regrew between the third and fourth day after plating, similar to the day 4 mutants, thus dismissing the possibility that the late appearance of the mutants on the original plate was due to slow growth. The sole exception, Sor54, grew in 6 days, which was still

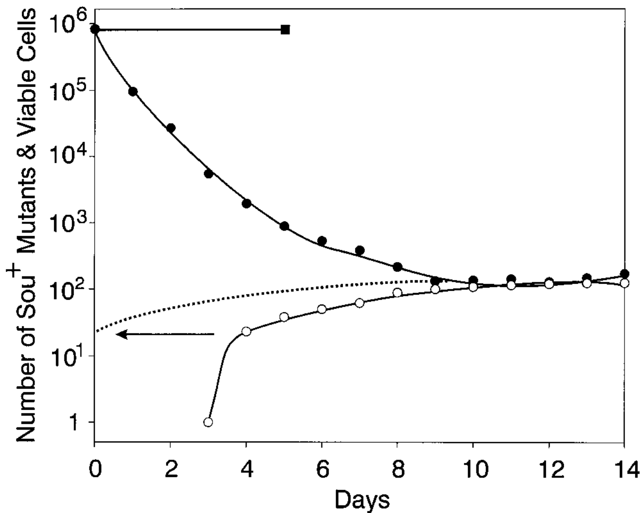

Figure 2.—The survival of 3153A Sou$^-$ cells on l-sorbose medium from the results of an experiment presented in Figure 1 (●) compared with the daily accumulation of Sou$^+$ colonies averaged from 27 independent clones of strain 3153A, calculated from the results in Table 1 (○). Also shown is the deduced curve representing the cumulative formation of the Sou$^+$ mutants 4 days before appearance of the colonies (the dotted line displaced by 4 days as indicated by the arrow). The stable number of colonies on a control synthetic medium lacking a carbon source is also indicated (■).

one-half of the time in which it originally appeared. The regrowth of the group of Sou$^+$ mutants, Sor41 to Sor50, that originally appeared on day 8 was heterogeneous. Four of the 10 mutants in this group regrew on l-sorbose medium between the third and fourth day, similar to the majority of day 4 and day 12 mutants. On the other hand, the remaining 6 mutants required an extended period of time for regrowth. In addition, these mutants appeared unstable and gave rise to greatly diminished numbers of colonies on l-sorbose, <10%, in comparison with SD control medium, indicating that the Sou$^+$ mutants were producing high rates of Sou$^-$ cells. One mutant, Sor44, did not grow at all on l-sorbose plates, whereas another, Sor45, grew slowly, having a delayed appearance that was similar to the delayed appearance originally observed.

Consistent results were observed in reconstruction experiments that were repeated twice more with representative Sou$^+$ mutants, including the day 4 mutants, Sor31 and Sor32, and the day 12 mutants, Sor51, Sor52, and Sor53. In addition, similar results were observed in an independent experiment with three more early- and late-appearing mutants, obtained from strain 3153A (data not presented).

All Sou$^+$ mutants exhibited some degree of instability giving rise to Sou$^-$ cells, as indicated by the diminished level of colonies on l-sorbose plates compared to those on SD plates. Also, for unknown reasons, the Sou$^+$ mutants produced fewer colonies on SD plates than on YPD plates (data not presented).

Overall, the Sou$^+$ colonies appeared sooner on retesting with l-sorbose medium than they originally appeared during isolation (Table 2), thus excluding the trivial explanation that the late appearance of Sou$^+$ mutants was due to slow growth.

The reconstruction experiment also disclosed a diversity in the growth of the Sou$^+$ mutants. Some mutants were relatively stable and grew well, some were relatively stable and grew poorly, whereas others were highly unstable. A prominent feature of some mutants, for example, those of day 8, is an excessive instability that is probably a result of high Sou$^+$ to Sou$^-$ reversion.

Rates of formation of Sou$^+$ mutants: The reconstruction experiments (Table 2) allowed us to relate two phenomena, the time of detection of the colony and the deduced time of formation of the corresponding mutation. These results with testing the Sou$^+$ mutants on l-sorbose medium indicated that the time of formation of the majority of Sou$^+$ mutations was ~4 days prior to the first detection of the small colony. The daily adjusted rates of formation of Sou$^+$ mutations (mutants per viable cell at the time of mutant formation per day) for each clone were calculated and their dynamics are presented in Figure 3A. The daily rates averaged from 27 clones are presented in Figure 3B.

There was an underlying similarity in the dynamics of colony appearances (Figure 3A). The adjusted rates of Sou$^+$ mutant formation per viable cell per day increased daily from $\sim 10^{-6}$ to $\sim 10^{-2}$ on day 4, a difference that constitutes four orders of magnitude. It is also important to note that the great increase in rates of Sou$^+$ mutants formation within the first 4 days after plating approximately corresponds to the time of the most dramatic death of cells on a plate, which is presented in Figure 2.

There is no residual growth of Sou$^-$ cells on l-sorbose medium: Experiments were carried out to determine whether Sou$^-$ cells underwent residual growth on l-sorbose medium, resulting in microcolonies. The number of viable cells at each day was compared by considering the results from two methods: first, by transferring the entire agar discs to YPD plates, and second, by washing and replating cells from defined areas of the l-sorbose plates and subsequently deducing the value for the entire surface of the plate. The presence of microcolonies would be revealed by a higher number of viable cells washed from the surface of the plates.

The average results from several plates, presented in Table 3, demonstrated that the two procedures yielded approximately equivalent numbers of viable cells, which implies that viable Sou$^-$ cells do not form microcolonies on l-sorbose plates. These results established that the increased rates of formation of Sou$^+$ mutants (Figure 3) cannot be attributed to an increased number of Sou$^-$ cells on the l-sorbose plates.

Increased rates of formation of Sou$^+$ mutants from Sou$^-$ revertants: To analyze the ability of Sou$^-$ re-

TABLE 2

The number of colonies of Sou⁺ mutants from different days on l-sorbose medium after replating and the total number of colonies on l-sorbose and glucose (SD) medium

Day of appearance	Sou^+ mutant number	Days					Total colonies	
		3–4	5–6	7–8	9–10	11–12	l-Sorbose	Glucose
4	Sor31	303	9	0	0	0	312	452
	Sor32	550	11	0	0	0	561	643
	Sor33	322	5	0	0	0	327	415
	Sor34	272	5	0	0	0	277	396
	Sor35	395	3	0	0	0	398	545
	Sor36	225	3	0	0	0	228	314
	Sor37	223	4	0	0	0	227	412
	Sor38	373	20	0	0	0	393	465
	Sor39	363	47	0	0	0	410	487
	Sor40	389	8	0	0	0	397	528
8	Sor41	165	25	0	0	0	190	205
	Sor42	0	2	6	1	12	21	291
	Sor43	3	3	2	2	3	13	318
	Sor44	0	0	0	0	0	0	213
	Sor45	1	20	251	24	0	296	519
	Sor46	379	18	0	0	0	397	425
	Sor47	269	11	0	0	0	280	378
	Sor48	0	12	24	42	16	94	392
	Sor49	149	27	0	0	0	176	302
	Sor50	0	7	7	1	0	15	250
12	Sor51	276	20	0	0	0	296	359
	Sor52	205	8	0	0	0	213	391
	Sor53	195	9	0	0	0	204	335
	Sor54	0	146	82	5	0	233	288
	Sor55	275	16	0	0	0	291	447
	Sor56	295	4	0	0	0	299	392
	Sor57	312	6	0	0	0	318	469
	Sor58	326	9	0	0	0	335	439
	Sor59	349	10	0	0	0	359	517
	Sor60	372	13	0	0	0	385	524

vertants to produce l-sorbose-utilizing mutants, we used 25 subclones derived from the Sou^- revertant Sor52-1, which originated on day 12 from the Sou^+ mutant Sor52. These 25 Sou^- clones, presented in Table 1, were prepared and applied on l-sorbose plates, similar to the 27 clones of the initial strain 3153A. The steps used to generate the strains used in these experiments are summarized as follows: Sou^- (a 3153A subclone) → Sou^+ (Sor52, a first round mutant) → Sou^- (Sor52-1 revertant subclones) → Sou^+ (second round mutants). These so-called second round Sou^+ mutants, derived from the 25 Sou^- subclones of Sor52-1, appeared in a time-dependent manner similar to the Sou^+ mutants derived from the 27 Sou^- subclones of 3153A. However, the daily rates of formation of Sou^+ mutants were consistently about 10 times higher (Table 1; Figure 3B). In fact, on day 6, the scoring of the majority of plates had to be discontinued because of the high numbers of Sou^+ colonies (clones 8–25).

Further experiments were carried out to determine whether other Sou^- revertants would produce higher rates of Sou^+ mutants, as observed for Sor52-1, which originated on day 12 from the Sou^+ mutant Sor52. Tests were performed with one clone from each of two revertants, Sor31-1 and Sor32-1, which derived from two original early mutants, Sor31 and Sor32 of day 4. A similar increase in the rates of formation of Sou^+ mutants was observed (data not presented).

Mathematical analysis of the frequencies of Sou^+ mutants and the fluctuation test: The method of Stewart *et al.* (1990) and the DATAFIT program were used to estimate the number of preexisting Sou^+ mutants and those appearing after contact with the selective plates. These values were deduced from the distribution of Sou^+ mutants from the 27 subclones appearing on day 4 after plating (Table 1). The calculations were performed with numbers from the day 4 colonies because of the inaccuracy of later values due to progressively dying cells (Figures 1 and 2) as well as the differences among the mutants (Table 2). The result of the analysis

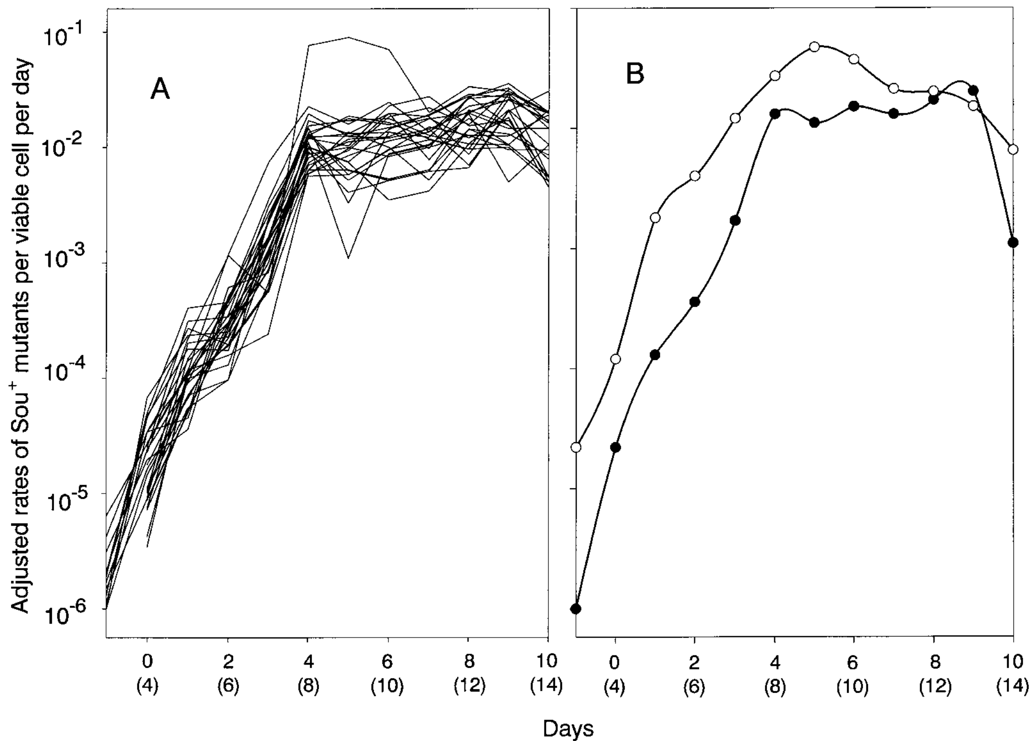

Figure 3.—The adjusted rates of Sou$^+$ mutants per viable cell per day. The adjusted values were calculated from the number of colonies (Table 1) divided by the number of viable cells 4 days prior to the appearance of colonies (Figure 2). Both the deduced time of formation of the mutation (top numbers) and the time of the appearance of the corresponding colonies (bottom numbers, in parentheses) are presented. (A) The adjusted rates of Sou$^+$ mutants of each of the 27 clones of strain 3153A. (B) The averaged adjusted rates from 27 clones of strain 3153A (●) and from 7 clones of the Sou$^-$ revertant Sor52-1 (○).

suggested that among the averages of the 18 mutational events, two of them arose after the contact with the selective medium, and the others were formed before contact. Thus, 2/18 or only ~10% of the day 4 mutants may have arisen by adaptive mutagenesis, in contrast to the vast majority of the mutants appearing after this time.

The rates of spontaneous Sou$^+$ mutation per cell per division that are formed before selection can be approximated by the procedure of Lea and Coulson (1949) and by assuming that the mutants appearing before day 5 preexisted and that the Sou$^-$ and Sou$^+$ mutants have the same growth rate in nonselective YPD medium. An approximate mutation rate of 10^{-7} mutants per cell per generation for the strains 3153A was deduced from data in Table 1. However, this value has to be corrected for the pronounced slower growth of Sou$^+$ mutants in YPD medium. At the other extreme, an approximate mutation rate of 2×10^{-5} mutants per cell per generation can be deduced by assuming that Sou$^+$ mutants do not divide at all in YPD medium, using the relationship established by Ogur *et al.* (1959), where the mutation rate equals the mutant frequency. Thus, 10^{-6} could be considered in the range of the mutation rate, if there is a diminished growth rate of Sou$^+$ mutants.

Electrophoretic karyotypes of Sou$^+$ mutants of strain 3153A: We have compared chromosomal patterns of 20 randomly chosen and independently derived colonies of early- and late-appearing Sou$^+$ mutants, using our precise separation procedures, which involved using several conditions optimized for different sizes of chromosomes (see materials and methods for the isolation of Sou$^+$ mutants, as well as for the conditions of chromosomal separations). The above-mentioned 20 Sou$^+$ mu-

TABLE 3

The number of viable Sou$^-$ cells after incubation for various days on l-sorbose plates as determined by transferring entire agar discs or by washing cells from the surface of the plates

Days of incubation on l-sorbose plates	0	1	2	3
Cells washed from the surface of the plates[a]	1.00×10^6	3.18×10^4	1.33×10^4	1.83×10^3
Transferring agar discs[b]	1.00×10^6	3.75×10^4	1.90×10^4	3.50×10^3

[a] The total numbers of viable cells per plate were determined by suspending cells from three areas, each of 3.1 mm^2, from the l-sorbose plate and spreading the cells on YPD and SD plates, which were subsequently incubated at 37° for 3 days. The number of colonies was used to deduce the number of viable cells on the entire surface of the original plate.

[b] The viable cells were determined by transferring the entire agar discs to YPD plates, which were subsequently incubated for 3 days.

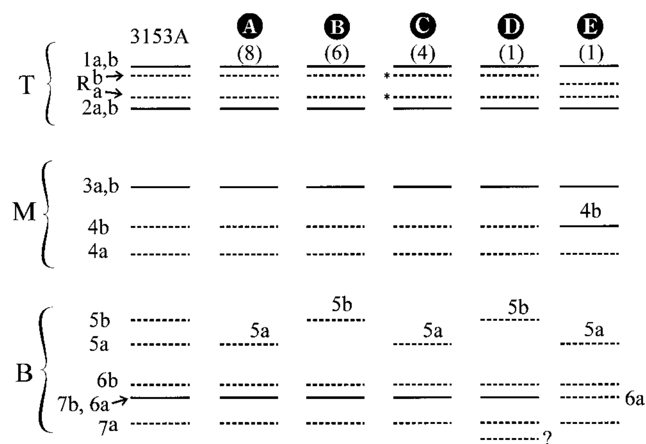

Figure 4.—Schematic representation of the electrophoretic karyotypes of parental strain 3153A and 20 Sou$^+$ mutants appearing on day 4, 8, or 12. A and B represent the two major types of electro-karyotypes lacking either homologue 5a or 5b, as indicated by denoting the remaining homologue. C represents a similar type of electro-karyotype lacking homologue 5a, but also having additional changes in the length of chromosome R, which are not shown, but are denoted by asterisks. D and E depict karyotypes, respectively, with the presence of an unidentified (?) chromosome and with altered numbers of chromosome 4b and 6a, in addition to the loss of chromosome 5a or 5b. The change in the position of chromosome Rb in E is shown. The numbers in parentheses below A–E indicate the number of mutants of each type. The assignment of the eight 3153A chromosomes by using specific probes, the approximate chromosomal sizes, and the rationale for these assignments have been previously published (Rustchenko-Bulgac *et al.* 1990, 1993; Rustchenko-Bulgac 1991). Dotted and solid lines correspond, respectively, to one and two chromosomes. The three groups of chromosome sizes, bottom (B), middle (M), and top (T), can be resolved singly or in combinations with three different running conditions that have been optimized (see materials and methods).

tants were each formed from a different Sou$^-$ subclone to assure that they were derived by independent events. The electrophoretic karyotypes included 9 mutants, Sor31 to Sor38 and Sor40, represented by colonies from day 4, and 10 mutants, Sor51 to Sor60, represented by colonies from day 12. Because of an unusual instability of many mutants that appeared as colonies on day 8 (Table 2), it was technically difficult to prepare a sufficient mass of cells for the electrophoretic analysis. We elected to prepare a single electro-karyotype of a relatively stable mutant, Sor41, and not to examine other mutants of this group.

All of the 20 examined electrophoretic karyotypes had a single common alteration, monosomy of chromosome 5, with the loss of either one of two homologues (Figure 4). We previously proposed the role of this alteration in establishing the Sou$^+$ phenotype (Janbon *et al.* 1998). We suggested that the copy number of a gene that resided on chromosome 5 and that encoded a putative negative regulator was responsible for the Sou$^+$ phenotype. Because the photographs of the gels and autoradiograms with the alteration and identification of chromosome 5 were previously published (Rustchenko *et al.* 1994; Janbon *et al.* 1998), only a schematization of the results is presented (Figure 4). Two out of 20 mutants, Sor38 and Sor51, from days 4 and 12, respectively, had additional changes. Sor38 had a new band in front of chromosome 1a, which may correspond to homologue 5a having a large deletion (Figure 4D). We previously analyzed a similar alteration (Rustchenko *et al.* 1994) and are currently interpreting it as a deletion encompassing the gene encoding the hypothetical negative regulator. Sor51 had a loss of chromosome 7b (data not presented) and presumably a duplication of 4b (Figure 4E). We have not established the identity of this last change or the abnormal band in Sor38. In addition, changes in the length of chromosome R homologues were observed in 5 out of the 20 mutants. Because the length of chromosome R was altered in only a small portion of the Sou$^+$ mutants, these changes were not considered to be relevant to the formation of Sou$^+$ mutants. Also, changes in the length of chromosome R are frequently observed in the so-called morphological mutants, which were detected on glucose medium (Rustchenko-Bulgac 1991; Rustchenko-Bulgac *et al.* 1993).

DISCUSSION

In this work, we took advantage of the natural chromosomal instability that controls assimilation of nutrients in *C. albicans* (Rustchenko *et al.* 1994, 1997). As we have previously demonstrated, the loss of one homologue of chromosome 5 allows the expression of the *SOU1* gene, which resides on a different chromosome, and which is responsible for the assimilation of an alternative carbon source, l-sorbose, probably as a result of a reduction of copy number of a negative regulatory gene, situated on chromosome 5 (Janbon *et al.* 1998). Because we earlier observed the cumulative appearance of Sou$^+$ mutants on l-sorbose plates over an extended period of time (Rustchenko *et al.* 1994), it was reasonable to suggest that the mutants were arising by adaptive mutagenesis, *i.e.*, were formed at higher frequencies in response to the selective condition, consistent with the current usage of this term (see Introduction). The hypothesis was verified in this work by systematically investigating the frequencies and time of occurrence of Sou$^+$ mutants and analyzing the electrophoretic karyotypes of representative mutants. Our results revealed for the first time that adaptive mutagenesis can be caused by chromosomal nondisjunction.

By following a standard fluctuation test used in this type of experiment, we isolated l-sorbose-utilizing mutants from 27 independent clones of a parental strain 3153A (Table 1). The continuous appearance of colonies representing mutants over the course of 2 wk, as well as their time-dependent occurrence indicative of

their formation after contact with the selective medium, as revealed in the reconstruction experiment (Tables 1 and 2, respectively), was reminiscent of earlier work with other organisms, including the fungus *S. cerevisiae* (Steele and Jinks-Robertson 1992). However, accurate calculations with results of the fluctuation test for distinguishing preexisting and adaptive mutants were hindered by the progressive death of Sou$^-$ cells on l-sorbose medium (Figures 1 and 2), which did not occur at a uniform rate and was not identical with different clones. In addition, there were qualitative differences among the Sou$^+$ mutants (Table 2), including unstable mutants having a high proportion of cells that did not grow on l-sorbose medium after replating.

Nevertheless, crucial evidence for adaptive mutagenesis came from the comparison of the daily rates of formation of Sou$^+$ mutants (see results). As presented in Figure 3, there is a striking increase of four orders of magnitude between the rates deduced to occur immediately after contact with the selective medium, day 0, and those of days 4 and 8, clearly suggesting that the vast majority of the mutational events appeared gradually after contact with the selective condition. Taken together with the time-dependent manner of the appearance of the mutants, which was shown by the reconstruction experiment (Table 2), the increased rates of formation of at least the late mutants are indicative of adaptive mutagenesis.

We stress that the increased rates of formation of Sou$^+$ mutants cannot be caused simply by residual growth or turnover of Sou$^-$ cells on the l-sorbose plates. The results of comparing the agar discs and cell suspensions (Table 3) indicated the lack of net growth of Sou$^-$ cells on the selection plate and the absence of microcolonies. It is reasonable to suggest that there is a turnover during the death of the Sou$^-$ cells, in which the rate of death exceeds the rate of growth, and that this turnover is required for producing Sou$^+$ mutants. However, the enormously high rate of formation of $\sim 10^{-2}$ Sou$^+$ mutants per viable cell per day clearly established that these mutants arose by adaptive mutagenesis. A very approximate mutation rate under nonselective conditions was deduced to be 10^{-6} Sou$^+$ mutants per cell per division for the strains 3153A. Considering the uncertainties of the assumptions used in the calculation, this value is close to the rate of 10^{-5} per cell per generation for nondisjunction in *S. cerevisiae* (Page and Snyder 1993). The high rate of 10^{-2} cannot be explained even if one assumes that the dying Sou$^-$ cells are turning over with a reasonable generation time. With a normal mutation rate of 10^{-5} per cell per generation, one would have to assume that the dying cells are dividing every minute to accumulate the number of Sou$^+$ mutants observed during the sharp increase of the rates.

The comparison of the karyotypic alterations among nine mutants whose colonies appeared on day 4, one from day 8, and ten from day 12, with all mutants derived as independent mutational events, could not be used to distinguish between preexisting and adaptive mutants, because the same chromosomal alteration, monosomy of chromosome 5, was found in all chromosomal patterns (Figure 4), consistent with the earlier reports (Rustchenko *et al.* 1994; Janbon *et al.* 1998). However, it has not been excluded that nondisjunction of a chromosome 5 homologue can occur by different mechanisms and that preexisting and adaptive mutants occur by different processes. Also it has not been excluded that chromosomes other than chromosome 5 undergo nondisjunction during formation of Sou$^+$ mutants by adaptive mutagenesis. In fact, chromosomal alterations, other than chromosome 5, occurred in 2 out of 20 strains, or 10% of the strains, excluding chromosome R alterations, which are considered to be due mainly to changes in the number of rDNA units (Rustchenko *et al.* 1993). The high proportion of altered chromosomes, other than chromosome 5, indicates that a general chromosomal instability may be associated with the Sou$^+$ selection or with stressful conditions.

There are two phenomena that remain to be explained, the unstable mutants arising as colonies on day 8 (Table 2), and the increased formation of Sou$^+$ mutants from Sou$^-$ revertants, such as Sor52-1 (Table 1; Figure 3B). The replating of the day 8 colonies revealed heterogenous classes of mutants that could be assigned to two types: (i) relatively stable and (ii) highly unstable. Relatively stable Sou$^+$ mutants (i), which grew up after 3–4 days, represented by Sor41, Sor46, Sor47, and Sor49, were similar to the mutants from days 4 and 12. Highly unstable mutants (ii) contained a significant number of cells that no longer grew on l-sorbose medium, for example, Sor42, Sor43, and Sor50. In the extreme case, no growth was observed for the Sor44 mutant. The majority of the mutants were relatively stable, typically represented by the day 4 and day 12 mutants, and these apparently were formed 4 days prior to their appearance (Figures 2 and 3). However, the time of formation of the highly unstable mutants is unknown, as is the portion of mutants that failed to grow. The uncertainty of the time of formation of these mutants adds to the variability among different clones in the first days of appearance and raises questions about the deduced number of preexisting mutants in the first day of colony appearance (see results). The occurrence of microscopic colonies on l-sorbose plates, which arose throughout the 2-wk period (data not presented), as well as diminished numbers of colonies appearing on l-sorbose medium in comparison with SD medium (Table 2), implies that this high instability is indicative of reversion to Sou$^-$ phenotype. At this time, we cannot explain the large portion of highly unstable mutants that arose in the early period after cell plating. They could be the result of additional mutations that enhance nondisjunction or other chromosomal rearrangements, analogous to mutators that enhance spontaneous mutation rates of DNA alterations

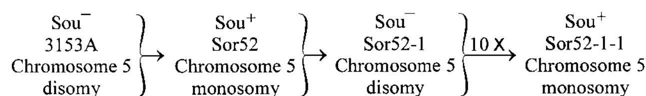

Figure 5.—The relationship between disomy/monosomy of chromosome 5 and the Sou$^-$/Sou$^+$ phenotypes.

in prokaryotic and eukaryotic cells. Although it is unclear how these mutants arise, their high frequency of occurrence, $\sim10^{-3}$ (Table 2), suggests that they were formed by adaptive mutagenesis. According to our results, both highly unstable Sou$^+$ mutants and mutators may have been formed by adaptive mutagenesis. These unstable mutants may arise by a mechanism that differs from the mechanism responsible for forming relatively stable mutants.

Thus, taken together, our results suggest that an uncertain portion of the day 4 colonies corresponds to preexisting mutants and that the day 12 colonies were formed exclusively by adaptive mutagenesis.

In this work we found that the revertant Sou$^-$ strain, Sor52-1, differed from original Sou$^-$ strain 3153A by acquiring an $\sim$10 times higher ability to produce second round Sou$^+$ mutants (see Table 1 and Figure 3B), although the mutants arise by the same karyotypic alteration, the loss of one homologue of chromosome 5 (Janbon *et al.* 1998), as schematically presented in Figure 5. This higher number could not be explained by a difference in viability because the death rates of Sor52-1 and 3153A were similar. It remains to be seen whether the higher rate of Sou$^+$ mutant formation is related to the fact that Sor52-1 and other Sou$^-$ revertants are homozygous for one or the other chromosome 5 homologues. Alternatively, one can consider mutations increasing the rate of nondisjunction, similar to mutators that affect the rate of gene mutations during selection for the utilization of lactose (Mao *et al.* 1997), especially in light of the fact that numerous genes in *S. cerevisiae* affect chromosomal stability (Page and Snyder 1993). Another possibility is that the higher numbers of Sou$^+$ mutants produced by Sor52-1 are due to stabilization of some of the Sou$^+$ mutants, in which there is no reversion to Sou$^-$ as described above for day 8 mutants.

Mitotic nondisjunction is well tolerated in fungi and plants (Fincham and Day 1971). Despite an apparent immediate deleterious effect of monosomy, such condition has significant evolutionary potential, allowing the expression of recessive alleles and introducing phenotypic variation in a population. Furthermore, duplication of a monosomic chromosome by a second nondisjunction event readily occurs, restoring the normal copy number of genes, but fixing the expression of the recessive alleles. Moreover, increased mutability, as observed in *C. albicans*, in conjunction with a monosomic condition, enhances the ability to produce desirable new traits in changing environments.

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