SOMATIC SEGREGATION, RECOMBINATION, ASYMMETRICAL
DISTRIBUTION AND COMPLEMENTATION TESTS OF
CYTOPLASMICALLY-INHERITED ANTIBIOTIC-
RESISTANCE MITOCHONDRIAL MARKERS
IN *S. CEREVISIAE*

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ABSTRACT

Genetic analyses of 48-hr-old zygote-daughter-colony cells from crosses between chloramphenicol and erythromycin resistance markers located in mitochondrial DNA demonstrated homoplasmons of parental and recombinant genotypes, and heteroplasmons with recombinant and/or parental genotypes. Although the heteroplasmons were unstable and the homoplasmic components could be segregated by plating on selective media, the heteroplasmic state was often maintained beyond 19 cell divisions when grown on non-selective medium. Homoplasmons of recombinant genotype from repulsion crosses were observed with a frequency of 7.2, 9.0, 11.2 and 11.4 percent; two crosses with the resistance markers in coupling had 5.4 and 11.5 percent recombinants. Under non-selective conditions, the mitochondrial marker derived from the haploid parent of *a* mating type predominated in zygote-daughter-cells; this asymmetrical distribution could be reversed by selective pressure for the marker transmitted with low frequency. The challenge with chloramphenicol and erythromycin of zygotes from crosses of resistance-markers in repulsion revealed that inter-mitochondrial complementation was not occurring.

THE discovery of antibiotic-resistance markers coded for by mitochondrial DNA (THOMAS and WILKIE 1968a) has provided a basis for the genetic analysis of the mitochondrion in *S. cerevisiae*. Genetic recombinants, defined by their ability to grow on medium containing antibiotics selective against both parents, have been found under both anaerobic (THOMAS and WILKIE 1968b) and aerobic conditions (COEN *et al.* 1969). However, these studies have not eliminated the possibility that growth on medium containing antibiotics inhibitory to both parents is the result of inter-mitochondrial complementation. Although the heteroplasmic state required for mitochondrial complementation has been shown to extend beyond the zygote (SAUNDERS *et al.* 1970), it is commonly assumed that cells of 48-hr-old zygote colonies are homoplasmic (THOMAS and WILKIE 1968b; LINNANE *et al.* 1968; COEN *et al.* 1969). In the absence of data relating to the maintenance of the heteroplasmic state and the occurrence of inter-mitochondrial complementation the possibility of true genetic recombination remains uncertain.

The interpretation of genetic data on mitochondrial DNA of yeast is complicated by the asymmetrical distribution of parental markers to cells of the zygote colony (THOMAS and WILKIE 1968b; LINNANE *et al.* 1968; COEN *et al.* 1969). Although this phenomenon has been observed for other cytoplasmic markers in *S. cerevisiae* (Ψ , COX, 1965; *killer*, BEVAN and SOMERS 1969; a plasmagene effecting the uptake of ureidosuccinic acid, LACROUTE 1971), COEN *et al.* (1969) and BOLOTIN *et al.* (1971) use the term *polarity* to refer to the asymmetrical distribution of antibiotic resistance markers. A related definition of polarity was also given as the unequal frequency of reciprocal recombinants found in cells of 48-hr-old zygote colonies. COEN and BOLOTIN have used this concept to evoke the idea of mitochondrial sexuality and to propose a method for mapping antibiotic-resistance mutants (BOLOTIN *et al.* 1971).

Specifically, those markers that have a high frequency of transmission to zygote colony cells are designated ω^+ ; markers that have a low transmission frequency are designated ω^- . The sources of their resistance markers were 3 haploid strains; two strains produced only ω^+ markers whereas a third strain produced only ω^- markers. In a ω^+ by ω^- cross the ω^+ marker found in the maximum recombinant class is placed to the left of the genetic map; using this procedure, they have proposed the map of *CSE* for the cytoplasmic markers of chloramphenicol, spiramycin and erythromycin resistance (BOLOTIN *et al.* 1971).

We have undertaken a genetic analysis of several independently isolated cytoplasmically-inherited chloramphenicol and erythromycin resistance markers since the strains used to derive antibiotic resistance markers were reported to greatly influence the genetic expectations (COEN *et al.* 1969). We report here on (i) the duration of the heteroplasmic state by using media to select for homoplasmons, (ii) an evaluation of the occurrence of mitochondrial complementation by plating zygotes from crosses in coupling and repulsion directly onto various selective media, (iii) the effect of selective pressure on the asymmetrical distribution of parental markers and (iv), the frequency of recombinant homoplasmons. Interpretation of our data by rules suggested for mitochondrial sexuality (BOLOTIN *et al.* 1971) has revealed several inconsistencies.

MATERIALS AND METHODS

Yeast strains: The genotype of strains used in this study is indicated in Table 1. As suggested by VON BORSTEL (1969) cytoplasmically-inherited markers are indicated by enclosing them in brackets ([]). Insertion of more than one marker in brackets indicates that all the enclosed markers can be shown to segregate as a unit (except for rearrangements brought about by recombination) but does not necessarily imply molecular linkage. As described by RANK and BECH-HANSEN (1972) and RANK and MARTIN (1972), the cytoplasmically-inherited antibiotic-resistance markers ($[ery^{R2}]$, $[ery^{R3}]$, $[ery^{R10}]$, and $[chl^{R2}]$) were all derived independently from the diploid GR3 as spontaneous mutants that arose on selective medium. The cytoplasmic inheritance of these markers was indicated by their (i) 4:0 segregation in tetrad analysis, (ii) somatic segregation in zygote colonies and (iii), loss upon generation of the $[rho-]$ state.

Strain 62-18a was derived from the tetrad analysis of a cross between MA20 (from the genetic stocks of Dr. R. MORTIMER) and GR62. An α *leu trp5* auxotroph was selected so that crosses could be made to GR24, GR33, GR25, GR25a, and GR25a-5EB. The *pet* phenotype of MA20 prevented the direct scoring of its chloramphenicol and erythromycin phenotypes. How-

TABLE 1
Yeast strains

Strain	Genotype*
GR3	<i>a/a ade1/ade1 his/+ lys2/+ trp2/+ ura/+; [chl^sery^srho+]</i>
GR24	<i>a ade1 trp2 ura; [chl^sery^{R2}rho+]</i>
GR33	<i>a ade1 his lys2 trp2 ura; [chl^sery^{R10}rho+]</i>
GR25	<i>a ade1 his trp2; [chl^sery^{R3}rho+]</i>
GR25a	<i>a ade1 his trp2 ura; [chl^sery^{R3}rho+]</i>
GR25a-5EB	<i>a ade1 his trp2 ura; [rho-(n)]</i>
GR62	<i>a ade1 his lys2; [chl^{R2}ery^srho+]</i>
MA20	<i>a ade2 leu1 lys pet trp5; [chl^sery^srho±]</i>
62-18a	<i>a leu1 trp5; [chl^{R2}ery^srho+]</i>
39-4c	<i>a ade1 leu1 trp; [chl^{R2}ery^{R3}rho+]</i>
44-5d	<i>a his trp; [chl^{R2}ery^{R3}rho+]</i>
25b	<i>a arg; [chl^sery^srho+]</i>
16b	<i>a arg met ade; [chl^sery^srho+]</i>

* When the alleles of auxotrophic markers are not known they are not followed by a subscript.

ever, MA20 was shown to lack cytoplasmically and nuclearly inherited chloramphenicol and erythromycin resistance markers by a tetrad analysis of crosses to sensitive [*rho*+] tester strains. Thus the chloramphenicol resistance marker in 62-18a must have come from GR62. MA20 was also found to be composed of approximately 50% [*rho*—] cells by scoring the respiratory capacity of zygote colonies when crossed to the neutral petite GR25a-5EB.

GR25a is a spontaneously arising uracil auxotroph from GR25. GR25a-5EB is an ethidium bromide induced neutral petite of GR25a; GR25a-5EB was shown to lack any detectable mitochondrial DNA by Dr. J. MARMUR of the Albert Einstein School of Medicine, New York.

39-4c and 44-5d are haploid isolates from stable [*chl^Rery^R*] hybrids of 62-18a by GR25.

25b and 16b are antibiotic sensitive haploids isolated from a sensitive diploid (681-18ES) strain of Dr. D. WILKIE.

Media: YEPD: 1% yeast extract, 2% bactopectone, 2% dextrose and 2% agar; YEPG: as YEPD with 4% glycerol substituted for 2% dextrose; YEPG-Ery, YEPG-Chl and YEPG-Chl-Ery: YEPG supplemented with 2 mg/ml of erythromycin glucoheptonate, 3 mg/ml of chloramphenicol (supplied by Dr. H. MACHAMER of Parke-Davis), and 2 mg/ml of erythromycin and 3 mg/ml of chloramphenicol, respectively; MinD: 0.67% yeast nitrogen base, 2% dextrose and 2% agar; MinG: 0.67% yeast nitrogen base, 4% glycerol and 2% agar; MinG-Ery, MinG-Chl and MinG-Chl-Ery: MinG supplemented with antibiotics as with the YEPG antibiotic media.

Crossing procedure: For the production of 48-hr-old zygote colonies, the strains to be crossed were grown for 48 hr in liquid YEPD, 0.5 ml of each strain was mixed, and the mixture was incubated at 30°C for 3 hr prior to dilutions and spreading on MinD. For complementation tests 1.0 ml of liquid YEPD was added to the copulation mixture, and after incubation at 30°C for 3 hr, the copulation mixture was washed with water, diluted, and spread onto the MinG series of media.

Scoring of antibiotic sensitivity or resistance: As indicated in Table 2, the four possible homoplasmons ([*chl^sery^s*], [*chl^Rery^s*], [*chl^sery^R*], [*chl^Rery^R*]) are uniquely defined by their growth response on YEPG, YEPG-Chl, YEYG-Ery and YEPG-Chl-Ery. However, a difficulty is presented if a colony has arisen from a heteroplasmic cell. For example, consider a [*chl^Rery^R*]/[*chl^Rery^s*] heteroplasmic cell. Somatic segregation could produce a colony composed of [*chl^Rery^s*] and [*chl^Rery^R*] cells. A sterile wire loop was used to transfer colony growth onto indicator media. Approximately 5×10^5 colony cells were transferred to indicator media by this method. Sampling of a colony containing [*chl^Rery^s*] and [*chl^Rery^R*] cells by this method would produce a growth response similar to sampling from a colony containing only [*chl^Rery^R*] cells. For the crosses of

cytoplasmic markers in repulsion an attempt was made to estimate the relative frequency of homoplasmic and heteroplasmic cells present in 48-hr-old zygote colonies (containing approximately 10^6 cells). Zygote colonies were streaked onto MinD and 72-hr-old zygote daughter colonies (containing approximately 3.5×10^6 cells) were sampled by a wire loop (5×10^6 cells) onto YEPG, YEPG-Chl, and YEPG-Ery. The latter two media should select for the $[chl^Rery^s]$ and $[chl^sery^R]$ homoplasmons respectively. One-week-old growth (a colony of 1.5×10^8 cells) on both selective media was then challenged by sampling from YEPG-Chl and YEPG-Ery onto YEPG-Chl, YEPG-Ery and YEPG-Chl-Ery (see Figure 1). Growth of cells sampled from YEPG-Chl or YEPG-Ery on *only* YEPG-Chl or YEPG-Ery (respectively) indicated that the zygote colony cell sampled was a $[chl^Rery^s]/[chl^sery^R]$ heteroplasmon. Growth of cells sampled from YEPG-Chl *and* YEPG-Ery on all three indicator media suggested that the zygote colony contained a true $[chl^Rery^R]$ recombinant cell (the assumption is arbitrarily made that recombination occurs sometime prior to the 48-hr-old zygote colony stage).

RESULTS

Somatic segregation: A total of 94 zygote colonies from the crosses of GR24, GR25, GR25a and GR33 by 62-18a was streaked onto MinD and 25 zygote-daughter-cell colonies were sampled as described in MATERIALS AND METHODS onto YEPG-Chl and YEPG-Ery. Of the 2350 zygote-daughter-cell colonies sampled 143 grew on both YEPG-Chl and YEPG-Ery. Subsequent sampling of the week-old colonies growing on YEPG-Chl and YEPG-Ery onto YEPG-Chl, YEPG-Ery and YEPG-Chl-Ery revealed that 65 gave positive growth on each of the 3 indicator media and thus the zygote cells that gave rise to these colonies were true $[chl^Rery^R]$ recombinants. For the remaining 78 zygote-daughter-colonies, that were originally scored as doubly resistant, colonies growing on YEPG-Chl *and/or* YEPG-Ery failed to grow on all of YEPG-Chl, YEPG-Ery or YEPG-Chl-Ery; the 78 zygote-daughter cells that gave rise to these doubly resistant zygote-daughter-cell colonies were classified as being heteroplasmic (Table 3). Sixty-one of the heteroplasmic zygote cells were $[chl^sery^R]/[chl^Rery^s]$ (see colony 2, Figure 1), 6 were $[chl^Rery^R]/[chl^Rery^s]$ (see colony 3, Figure 1), 9 were $[chl^sery^R]/[chl^Rery^R]$ (colony 1, Figure 1) and 2 were $[chl^sery^R]/[chl^Rery^s]/[chl^Rery^R]$ (see colony 4, Figure 1).

In general, the first pass through selective media was sufficient to eliminate the heteroplasmic state. Nevertheless 10 colonies (8 on YEPG-Chl and 2 on YEPG-Ery) on the first set of selective media grew on YEPG-Chl and YEPG-Ery but not on YEPG-Chl-Ery of the second set of selective media; some of the cells of colonies on the first set of selective media must still have been heteroplasmic. When the 20 colonies growing on the YEPG-Chl and YEPG-Ery plates of the

FIGURE 1.—Method used to follow somatic segregation. Cells that gave rise to zygote-daughter colonies 1, 2 and 3 were heteroplasmons of type $[chl^Rery^R]/[chl^sery^R]$, $[chl^Rery^s]/[chl^sery^R]$ and $[chl^Rery^s]/[chl^Rery^R]$, respectively. Zygote-daughter-cell colony 4 was derived from a cell with a $[chl^Rery^R]/[chl^Rery^s]/[chl^sery^R]$ heteroplasmon; the sampling of this colony from YEPG-Ery to new indicator media revealed that the heteroplasmic state was not maintained beyond the first pass through selective medium. Colony 5 was derived from a zygote daughter cell with a recombinant homoplasmon $[chl^Rery^R]$. C, E and CE, in this figure, refer to YEPG-Chl, YEPG-Ery and YEPG-Chl-Ery medium, respectively. Shaded semicircle means growth while — means no growth.

TABLE 2

Growth on indicator media of colonies derived from homoplasmic and heteroplasmic cells

Cell genotype	Type	Media			
		YEPG	YEPG-Chl	YEPG-Ery	YEPG-Chl-Ery
[<i>chl^sery^R</i>]	Homoplasmon	+	—	+	—
[<i>chl^Rery^s</i>]	Homoplasmon	+	+	—	—
[<i>chl^sery^s</i>]	Homoplasmon	+	—	—	—
[<i>chl^Rery^R</i>]	Homoplasmon	+	+	+	+
[<i>chl^sery^R</i>]/[<i>chl^Rery^s</i>]	Heteroplasmon	+	+	+	—
[<i>chl^sery^R</i>]/[<i>chl^Rery^R</i>]	Heteroplasmon	+	+	+	+

* +: growth; —: no growth.

TABLE 3

*Mitochondrial marker combinations present in double resistant cells of 48-hour-old zygote colonies**

Cross	Heteroplasmic cells				Homoplasmic cells
	[<i>chl^sery^R</i>] [<i>chl^Rery^s</i>]	[<i>chl^Rery^R</i>] [<i>chl^Rery^s</i>]	[<i>chl^Rery^R</i>] [<i>chl^sery^R</i>]	[<i>chl^sery^R</i>] [<i>chl^Rery^s</i>] [<i>chl^Rery^R</i>]	[<i>chl^Rery^R</i>]
GR24 × 62-18a	7	2	2	0	5
GR25 × 62-18a	16	1	1	0	28
GR33 × 62-18a	30	0	1	0	10
GR25a × 62-18a	8	3	5	2	22

* Forty-eight-hour-old zygote colonies were streaked and individual cells allowed to grow into daughter colonies. Zygote-daughter-cell colonies that grew on both YEPG-Chl and YEPG-Ery were then defined as being double resistant. Samples were then taken from growth on YEPG-Chl and YEPG-Ery and patched onto YEPG-Chl, YEPG-Ery and YEPG-Chl-Ery. Growth patterns on the latter three media (see Figure 1) were used to define the marker combinations present in cells of the original zygote colony.

second set of selective media were again sampled onto YEPG-Ery, YEPG-Chl, and YEPG-Chl-Ery, 3 colonies could still be classified as heteroplasmic as there was growth on YEPG-Chl and YEPG-Ery but not on YEPG-Chl-Ery.

An additional check was carried out to verify that the heteroplasmic components of 48-hr-old zygote cells could be selected by growth on selective antibiotic containing media. Zygote-daughter-cell colonies 7 and 13 from zygote colony 31 of the cross GR25 by 62-18a (Table 5) were classified as being [*chl^sery^R*]/[*chl^Rery^s*] heteroplasmons. Growth from these two colonies that had been selected by YEPG-Chl and YEPG-Ery was subjected to tetrad analyses. The selective effect of the antibiotic media was verified since tetrad analyses of zygote-daughter-cell colonies 7 and 13 growing on YEPG-Chl gave a 4:0 segregation for [*chl^R*]:[*ery^s*] whereas tetrad analyses of growth on YEPG-Ery gave a 4:0 segregation for [*ery^R*]:[*chl^s*] (Table 4).

Recombination of cytoplasmically-inherited antibiotic-resistance markers for crosses in repulsion: Four[*chl^sery^R*] strains (GR24, GR25, GR25a and GR33)

TABLE 4

Tetrad analyses of zygote daughter colonies that grew when sampled onto YEPG-Chl and YPEG-Ery but did not grow on YEPG-Chl-Ery when replicated from YEPG-Chl or YEPG-Ery

Cross	Zygote colony*	Zygote daughter colony	Selective antibiotic medium	Auxotrophic requirements +:—	Antibiotic resistance [chl ^R]:[ery ^R]	Number of tetrads
GR25 by 62-18a	31	7	YEPG-Chl	2:2	4:0	8
GR25 by 62-18a	31	7	YEPG-Ery	2:2	0:4	6
GR25 by 62-18a	31	13	YEPG-Chl	2:2	4:0	7
GR25 by 62-18a	31	13	YEPG-Ery	2:2	0:4	8

* Zygote colony 31 from Table 5.

were crossed to the common [chl^Rery^S] strain 62-18a and streaked onto MinD. Forty-eight-hr-old zygote colonies were streaked onto MinD and twenty-five 72-hr zygote-daughter-cell colonies were sampled onto indicator media as outlined in Figure 1. Five zygote-colony cell types ([chl^Sery^R], [chl^Rery^S], [chl^Rery^R], [chl^Sery^S] and heteroplasmons) could be distinguished. Their frequency in zygote colonies from the cross of GR25 by 62-18a is listed in Table 5. The data for all 4 crosses are summarized in Table 6. The following observations appear to be of major importance: (i) a 48-hr-old zygote colony can give rise to zygote-daughter-cell colonies representing all five possible cell types (e.g., zygote colony 4 in Table 5); (ii) the frequency of cell types found in 48-hr-old zygote colonies can vary greatly from zygote colony to zygote colony (e.g., the 25 cells sampled from zygote colony 11 in Table 5 were all [chl^Sery^R] whereas colony 27 scored 24 cells as [chl^Rery^S] and one cell as [chl^Rery^R]); (iii) recombinants (48-hr-old zygote colony cells that are stable [chl^Rery^R] or [chl^Sery^S]) varied from 7.20% for the cross of GR33 by 62-18a to 11.44% for the cross of GR25 by 62-18a—an analysis of 5 tetrads from each of 11 zygote-daughter-cell colonies scored as [chl^Rery^R] from the cross GR25 by 62-18a verified that the isolates were true recombinants since all 55 tetrads produced 4 [chl^Rery^R] ascospore colonies; (iv) although the [chl^Sery^S] recombinant was present in greater frequency than [chl^Rery^R] for all crosses, the difference was statistically significant only for crosses GR24 by 62-18a and GR25 by 62-18a; (v) the cross GR25a-5EB (without detectable mitochondrial DNA) by 62-18a produced 100% [chl^Rery^S] zygote-daughter cell colonies indicating that the information for [chl^Rery^R] of strain GR25a resides in mitochondrial DNA.

Recombination of cytoplasmically-inherited antibiotic-resistance markers for crosses in coupling: One of the 25 zygote-daughter-cell colonies sampled from zygote colony 31 (Table 5) of the cross GR25 by 62-18a was found to be [chl^Rery^R]. A tetrad analysis of growth from the YEPG-Chl plate (from the first set of indicator media) gave rise to the haploid [chl^Rery^R] isolate 39-4c (Table 1). Similarly, a tetrad analysis of growth on the YEPG-Ery plate (from the first set of indicator media) gave rise to the haploid [chl^Rery^R] isolate 44-5d (Table 1). Doubly-sensitive ([chl^Sery^S]) strains, 25b and 16b, were derived from the tetrad analysis of the sensitive diploid strain 681-18ES.

TABLE 5

Number of cells found in 25 cells sampled from 48-hour-old zygote colonies issued from the cross of GR25 by 62-18a ([chl^sery^R] by [chl^Rery^s])

Zygote colony	Homoplasmic cells				Heteroplasmic cells*
	[chl ^s ery ^R]	[chl ^R ery ^s]	[chl ^R ery ^R]	[chl ^s ery ^s]	
1	21	3	0	1	0
2	20	4	0	1	0
3	21	2	0	1	1
4	11	8	1	3	2
5	12	5	4	4	0
6	23	0	1	1	0
7	8	15	2	0	0
8	22	0	0	3	0
9	15	3	0	6	1
10	17	2	4	0	2
11	25	0	0	0	0
12	21	1	3	0	0
13	15	4	0	4	2
14	25	0	0	0	0
15	21	0	1	2	1
16	22	1	0	1	1
17	17	4	0	4	0
18	19	1	0	5	0
19	23	0	1	0	1
20	11	9	0	5	0
21	14	6	3	1	1
22	4	17	4	0	0
23	21	2	1	0	1
24	14	5	0	6	0
25	17	3	0	5	0
26	16	3	0	6	0
27	0	24	1	0	0
28	11	12	1	1	0
29	22	1	0	2	0
30	15	2	0	8	0
31	18	2	1	0	4
32	22	4	0	0	0
33	22	0	0	2	1
34	25	0	0	0	0
35	24	1	0	0	0

* Heteroplasmic combinations found are listed in Table 3.

Twenty-five 48-hr-old zygote colonies from the crosses 39-4c by 25d and 44-5d by 16b were streaked onto MinD. Twenty-five 3-day-old zygote-daughter-cell colonies, from each zygote colony, were then patched onto YEPG-Chl, YEPG-Ery, YEPG-Chl-Ery (Figure 2); the cell types observed are summarized in Table 6. Parental cell types averaged 22.12 of the 25 cells for the cross 25b by 39-4c and 23.64 of the 25 cells for the cross 16b by 44-5d. The recombinants ([chl^sery^R] and [chl^Rery^s]) were 11.52% for the cross 25b by 39-4c and 5.44% for the cross

TABLE 6
The average number of cells found in a sample of 25 cells from zygote colonies
of crosses in coupling and repulsion

Cross	Type	Number of zygote colonies sampled	Type of zygote colony cell				Percent recombinants
			[<i>chl^hery^R</i>]	[<i>chl^hery^S</i>]	[<i>chl^Rery^R</i>]	[<i>chl^Rery^S</i>]	
GR24 × 62-18a	Repulsion	10	19.80 ± 3.62†	1.20 ± 1.21	0.50 ± 0.70	2.30 ± 1.62	11.20
GR25a × 62-18a	Repulsion	24	17.95 ± 2.78	4.04 ± 1.97	0.92 ± 0.62	1.33 ± 0.64	9.00
GR25 × 62-18a	Repulsion	35	17.54 ± 2.04	4.11 ± 1.86	0.80 ± 0.44	2.06 ± 0.80	11.44
GR25 × 62-18a	Repulsion	10*	0.00	22.00 ± 2.66	3.00 ± 2.65	0.00	12.00
GR33 × 62-18a	Repulsion	25	18.32 ± 2.50	3.68 ± 1.88	0.40 ± 0.34	1.40 ± 0.89	7.20
GR33 × 62-18a	Repulsion	10*	0.00	24.80 ± 0.30	0.20 ± 0.30	0.00	0.80
GR25a-5EB × 62-18a		10	0.00	25.00	0.00	0.00	—
25b × 39-4c	Coupling	25	1.00 ± 0.71	1.88 ± 1.18	5.52 ± 3.06	16.60 ± 3.39	11.52
16b × 44-5d	Coupling	25	0.36 ± 0.31	1.00 ± 0.71	20.36 ± 2.71	3.28 ± 2.12	5.44

* Zygotes were spread onto MinG-Chl and sampled after 7 days of growth.

† 95% confidence limits of mean using a "t" distribution.

FIGURE 2.—One-week growth on antibiotic media of cells sampled from zygote-daughter-cell colonies from a cross of cytoplasmically-inherited chloramphenicol and erythromycin resistance markers in coupling ($[chl^Rery^R]$ by $[chl^Sery^S]$). On these plates, the zygote-daughter-cell colonies 1–25 were from zygote colony 25 of the cross 25b by 39–4c. Zygote-daughter-cell colonies 26–50 and 51–75 were from zygote colonies 1 and 2, respectively, of the cross 16b by 44–5d. Colonies 33, 47, 51, 62, 67 and 68 show a recombinant $[chl^Rery^S]$ phenotype. Letters a, b, c and d refer to parents 25b, 39–4c, 16b and 44–5d, respectively.

16b by 44–5d. In both crosses the $[chl^Rery^S]$ recombinant was more frequent than the $[chl^Sery^R]$ recombinant but the difference was not statistically significant (Table 6).

Asymmetrical distribution of parental antibiotic-resistance markers to the zygote colony: As seen in Table 6, the average number of parental cells is statistically unequal for any cross whether in coupling or repulsion. For the repulsion crosses the ratio of $[chl^Sery^R]$ cells: $[chl^Rery^S]$ cells is 19.80:1.20, 17.95:4.04, 17.54:4.11 and 18.32:3.68 for crosses GR24, GR25a, GR25 and GR33 by 62–18a

respectively. Similarly for the coupling crosses the ratio of $[chl^sery^s]:[chl^Rery^R]$ is 16.60:5.52 and 3.28:20.36 for crosses 25b by 39-4c and 16b by 44-5d respectively. The dramatic difference in inheritance of parental markers of these two crosses is illustrated by Figure 2. It is noteworthy that all parental markers present in strains of mating type *a* were found in a higher frequency in the cells of the zygote colony. The involvement of mitochondrial DNA in the phenomena of asymmetrical distribution of antibiotic-resistance markers is indicated by the cross of GR25a-5EB by 62-18a where the $[chl^Rery^s]$ is present in all 25 zygote cells in contrast to only 4.04 of zygotic cells for the cross GR25a by 62-18a.

Selection pressure for the parental plasmagene that is normally found in lowest frequency can reverse the effect of the asymmetrical distribution. This was indicated by the direct plating of zygotes from crosses GR25 and GR33 by 62-18a on MinD-Chl. Ten one-week-old zygote colonies from each cross were streaked onto MinD and 25 zygote-daughter-cell colonies from each zygote colony were patched onto YEPG-Chl, YEPG-Ery and YEPG-Chl-Ery. The $[chl^Rery^s]$ marker that was found in low frequency when zygotes were plated on MinD was seen to average 22.00 and 24.80 of the 25 cells (Table 6) when the zygotes from these 2 crosses were plated on MinG-Chl. Selection pressure for the $[chl^Rery^s]$ marker by the MinG-Chl media dramatically changed the cellular composition of zygote colonies.

Complementation tests of cytoplasmically-inherited antibiotic-resistance markers: Complementation between $[chl^Rery^s]$ and $[chl^sery^R]$ markers can be evaluated by plating zygotes formed by crossing these markers onto MinG medium containing erythromycin and chloramphenicol. Growth on such medium would require complementation (other than by the production of a $[chl^Rery^R]$ recombinant) since neither the $[chl^Rery^s]$ nor $[chl^sery^R]$ mitochondrion are functional on this medium; complementation would likely involve the mutual exchange of a gene product between $[chl^Rery^s]$ and $[chl^sery^R]$ mitochondria. Copulation mixtures from the crosses GR25 and GR33 by 62-18a and 16b by 44-5d were spread onto MinG, MinG-Chl, MinG-Ery and MinG-Chl-Ery. For any given cross the same dilution, and thus the same number of zygotes, was spread onto the indicated media.

After five days all four media gave vigorous growth for the cross in coupling (16b by 44-5d). Since growth on MinG-Chl-Ery medium for this cross does not require complementation we can assume that zygotes that transmit a high frequency of $[chl^Rery^R]$ mitochondria (see cross 16b by 44-5d in Table 6) will grow vigorously on double antibiotic medium within 5 days. Similarly the statistically equal number of colonies on all four media (Table 7) serves as a control that the phenotype of $[chl^Rery^R]$ mitochondria is clearly expressed on all media.

Growth on MinG-Ery-Chl medium for the crosses in repulsion was delayed by three to four days and did not appear vigorous until the tenth day. The number of colonies on the media Min, MinG-Chl, and MinG-Ery was statistically greater than the number of MinG-Chl-Ery (Table 7). The delayed and unequal growth on MinG-Chl-Ery can be most simply interpreted as due to the formation of $[chl^Rery^R]$ recombinants rather than being due to inter-mitochondrial comple-

TABLE 7

Number of zygote colonies formed after 10 days growth on selective media

Cross	Type	Media			
		Min	MinG-Chl	MinG-Ery	MinG-Chl-Ery
GR25 $\times$ 62-18a	Repulsion	43.6 $\pm$ 5.4*	31.0 $\pm$ 10.0	24.0 $\pm$ 8.1	3.2 $\pm$ 1.0
GR33 $\times$ 62-18a	Repulsion	74.8 $\pm$ 12.8	71.2 $\pm$ 18.7	49.4 $\pm$ 11.6	10.6 $\pm$ 11.9
16b $\times$ 44-5d	Coupling	58.0 $\pm$ 18.7	56.0 $\pm$ 11.7	49.5 $\pm$ 15.4	43.2 $\pm$ 6.0

* 95% confidence limits of mean using a "t" distribution.

mentation. The delayed growth suggests that very few (if any) recombinants were formed prior to plating on selective media and that recombination and subsequent selective pressure for the recombinant was necessary for vigorous growth on MinG-Chl-Ery. Clonal analysis of six zygote colonies growing on MinG-Chl-Ery revealed that the cells were in fact [*chl^Rery^R*] recombinants.

DISCUSSION

SAUNDERS *et al.* (1970) showed clearly by micromanipulation studies that the heteroplasmic state extends to at least the first few zygotic buds. Others (LINNANE *et al.* 1968; COEN *et al.* 1969) have reported that zygote-daughter cells formed 24-48 hr after mating are homoplasmic. Our results indicate that the heteroplasmic state is fairly common at the 19-20 cell division stage (48-hr-old zygote colonies) and can even persist beyond two passages through selective medium. The discrepancy in results likely stems from the failure of other authors to use a selective method to expose the heteroplasmon. The plating of zygote-daughter-cell colonies onto selective media from crosses in coupling would not likely reveal the heteroplasmic state. Of the six possible bi-heteroplasmons, [*chl^Rery^R*]/[*chl^sery^s*], [*chl^Rery^R*]/[*chl^Rery^s*], [*chl^Rery^R*]/[*chl^sery^R*], [*chl^sery^s*]/[*chl^Rery^s*], [*chl^sery^s*]/[*chl^sery^R*], and [*chl^Rery^s*]/[*chl^sery^R*], only the latter can be defined by plating on YEPG-Chl, YEPG-Ery and YEPG-Chl-Ery. Because both components of the [*chl^Rery^s*]/[*chl^sery^R*] heteroplasmon are recombinants for coupling crosses it is not surprising that it was not observed (Table 6).

A useful operational definition of plasmagene recombination in *S. cerevisiae* is based on the percent of recombinant homoplasmic cells in 48-hr-old zygote colonies grown on medium that is nonselective for recombinant or parental markers. Thus 1% recombinant homoplasmic 48-hr-old zygote cells would represent 1% recombination. In the absence of mitochondrial complementation (Table 7), a recombinant homoplasmon can be clearly defined as one that grows on medium containing antibiotics that prevent the growth of either parent. Recombination is most accurately estimated from crosses in coupling where it was found to be approximately 10% (Table 6). In spite of the uncertainty of the homoplasmic and heteroplasmic state for crosses in coupling, it is apparent that the % of recombination is not appreciably different than for crosses in repulsion (Table 6). Our results, that stable recombinants are formed under aerobic conditions, are

thus in agreement with COEN *et al.* (1970). The concomitant loss of $[chl^s ery^R]$ and mitochondrial DNA by strain 25a-5EB argues strongly that the information for antibiotic resistance plasmagenes resides in mitochondrial DNA, and thus the recombination process involves mitochondrial DNA.

According to the terminology of BOLOTIN *et al.* (1971) strains GR24, GR25a, GR25, GR33, 25b and 44-5d would be designated ω^+ since the markers of these strains had a high distribution to cells of the zygote colony. Conversely 62-18a, 39-4c and 16b would be designated ω^- since the markers of these strains occur in low frequency among the cells of zygote colonies. Because all of the crosses listed in Table 6 can be defined as being heterosexual (ω^+ by ω^-) the expectations are that the two recombinant classes would be unequal (BOLOTIN *et al.* 1971). Of the six ω^+ by ω^- crosses listed in Table 6 only two of them (GR24 and GR25 by 62-18a) gave statistically different frequencies of reciprocal recombinants. Thus our data do not support the concept of polarity of recombination in heterosexual crosses (COEN *et al.* 1969; BOLOTIN *et al.* 1971). Another difficulty was encountered when we attempted to map the $[chl^R]$ and $[ery^R]$ markers by placing the ω^+ marker found in the maximum recombinant class to the left of the genetic map (BOLOTIN *et al.* 1971). For all the crosses except 25b by 39-4c this rule results in a gene order of $[chl\ ery]$. However, the cross 25b by 39-4c results in a gene order of $[ery\ chl]$ since the ω^+ marker for the maximum recombinant class ($[chl^R\ ery^s]$) is $[ery^s]$. An inconsistency was also found in the sex of the recombinants since we observed both ω^+ (44-5d) and ω^- (39-4c) recombinants whereas all the recombinants of BOLOTIN *et al.* (1971) were ω^+ . Similarly our observations that (i) ω^+ and ω^- strains were isolated from the same diploid (GR3) and (ii) that there is a prolonged maintenance of the heteroplasmic state, are hard to reconcile with the concept of sexual polarity of the mitochondrion. Both observations suggest the independent coexistence of opposite sexes within a common cell which seems incompatible with the expected mating between opposite sexes. Certainly the proposed sexuality is not compatible with a sex mechanism similar to F^+ and F^- bacterial strains where the F^+ phenotype is rapidly spread throughout the population. Mitochondrial sexuality also fails to explain the production of recombinants (BOLOTIN *et al.* 1971) in homosexual crosses (ω^+ by ω^+ or ω^- by ω^-) since such mitochondria would not be expected to mate.

Genetic polarity has been defined (JACOB and WOLLMAN 1961) as the frequency with which a given allele of an unselected character appears among the recombinants. Using this definition it was possible to note differences in the frequency of unselected markers in reciprocal crosses between different sexual strains of bacteria. It is perhaps premature to suggest that the unequal frequency of reciprocal two-factor recombinants (COEN *et al.* 1969; BOLOTIN *et al.* 1971) can be taken as evidence of mitochondrial sexuality. A meaningful application of this concept will require multifactor reciprocal crosses and the correlation of unselected markers with different recombinants. In any event we did not usually observe an unequal frequency of reciprocal recombinants in heterosexual crosses (Table 6).

Asymmetrical distribution of cytoplasmic markers to progeny cells is of wide-

spread occurrence; distribution ranges from the virtually 100% marker association of the maternal parent in some species to the more variable distribution of antibiotic-resistance markers in *S. cerevisiae*. In *S. cerevisiae* and other species the asymmetrical distribution is referred to as suppressiveness (JINKS 1964a and 1964b) when the phenotype is scored by observation of the intact zygote colony. For example, the suppressiveness of the $[rho^-]$ marker in *S. cerevisiae* is defined as the frequency of $[rho^-]$ zygote colonies issued from a $[rho^-]$ by $[rho^+]$ cross. The striking similarity between suppressiveness and asymmetrical distribution of antibiotic-resistance markers is underlined by the observation that some of the zygote colonies sampled from the cross of GR25 by 62-18a were 100% $[chl^s ery^R]$ (Table 5—zygote colonies 14 and 34). Thus it appears likely that suppressiveness and asymmetrical distribution share at least some elements of a common system. Any general mechanism of sexuality to explain these phenomena is met with the difficulty that some of these markers do not appear to reside in organelles; for example, the Ψ factor (COX 1965) and the plasmagene affecting the uptake of ureidosuccinic acid (LACROUTE 1971). Rather it is more likely that these phenomena are caused by the complex interaction of cytoplasmic and nuclear genes. Certainly the observation that *a* mating type is associated with the high transmission of antibiotic resistance markers (Table 6) is suggestive of the involvement of nuclear loci. However, an understanding of the genetic control of asymmetrical distribution will require detailed genetic analyses of crosses between strains with high and low plasmagene transmission to the zygote colony.

Lack of complementation between plasmagenes of $[chl^R ery^s]/[chl^s ery^R]$ zygotes is consistent with the concept of mitochondria as discrete organelles. The unitary nature of the mitochondrion is also indicated by the preponderance of zygote colony cells containing only the parental plasmagene. However, overriding the relative independence of the mitochondrion are the phenomena of recombination, asymmetrical distribution and selective pressure, any of which can drastically alter the mitochondrial composition of descendent cells.

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