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RECOMBINATION IN 3-FACTOR CROSSES OF CYTOPLASMICALLY INHERITED ANTIBIOTIC-RESISTANCE MITOCHONDRIAL MARKERS IN *S. CEREVISIAE*

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SUMMARY

Four strains carrying different cytoplasmically inherited oligomycin-resistance markers were crossed to a common strain containing cytoplasmically inherited markers for chloramphenicol and erythromycin resistance. The frequencies of cells in 48-hour-old zygote colonies with the phenotypes of (i) the two parental homoplasmons, (ii) the six recombinant homoplasmons, and (iii) heteroplasmons, were determined. All cellular phenotypes, except the oligomycin resistant homoplasmon which comprised approximately 75 per cent. of zygote-colony cells, were found to be present in equal frequency. Recombination between any one of the three possible marker pairs (chloramphenicol-oligomycin, chloramphenicol-erythromycin, erythromycin-oligomycin) was not significantly different from either of the other two marker pairs and was found to be approximately 15 per cent. Thus the recombination data could not be used to construct a map indicating linkage or gene order.

1. INTRODUCTION

RECOMBINATION of cytoplasmically inherited antibiotic-resistance markers has been observed under anaerobic (Thomas and Wilkie, 1968) and aerobic conditions (Coen *et al.*, 1969; Bolotin *et al.*, 1971; Rank and Bech-Hansen 1972*b*). Recombination of antibiotic-resistant markers is most accurately estimated from the frequency of homoplasmic recombinant cells in 48-hour-old zygote colonies (Rank and Bech-Hansen, 1972*b*). Mitochondrial complementation was shown not to occur (Rank and Bech-Hansen, 1972*b*) and thus does not result in an error in the scoring of recombinants. However, heteroplasmic cells in 48-hour-old zygote colonies were shown to exist and can result in an overestimate of multiple-resistant recombinants unless selective media is used to reveal their presence (Rank and Bech-Hansen, 1972*b*). An important property associated with two factor crosses is the asymmetrical distribution of parental markers to cells of the zygote-colony (Bolotin *et al.*, 1971; Rank and Bech-Hansen, 1972*b*). Coen *et al.* (1969) and Bolotin *et al.* (1971) have interpreted the asymmetrical distribution of parental markers and the unequal frequency of reciprocal recombinants in the zygote colonies of two factor crosses as evidence of mitochondrial sexuality. Rank and Bech-Hansen (1972*b*) observed asymmetrical distribution but generally observed an equal frequency of reciprocal recombinants in two factor crosses.

The only report on 3-factor recombination experiments (Thomas and Wilkie, 1968) did not establish an estimate of the variability of parental and recombinant cells between different zygote colonies or an accurate measure of the extent of recombination. We have crossed four strains

carrying different cytoplasmically inherited oligomycin-resistance markers to a common strain carrying cytoplasmically inherited determinants for chloramphenicol and erythromycin resistance. This report gives a statistical estimate of the frequency of heteroplasmic and homoplasmic (two parental and six recombinant) cells found in the 48-hour-old zygote colonies issued from such crosses.

2. Materials and methods

(i) *Yeast strains*

The yeast strains used are listed in table 1. Cytoplasmically inherited markers are enclosed by brackets ([ ]). Markers for chloramphenicol,

Table 1

Yeast strains

Strain	Genotype
OR1-1b	*a ade*1 *ura*; [$chl^{S}ery^{S}oli^{R1}rho+$]*
OR2-5a	*a lys*2; [$chl^{S}ery^{S}oli^{R2}rho+$]
OR3-1a	*a his*; [$chl^{S}ery^{S}oli^{R3}rho+$]
OR4-2b	*a lys*2 *his*; [$chl^{S}ery^{S}oli^{R4}rho+$]
44-5a	α *trp*5; [$chl^{R2}ery^{R3}oli^{S}rho+$]

* Markers enclosed by brackets ([ ]) can be shown to be cytoplasmically inherited.

erythromycin and oligomycin resistance are abbreviated as chl^{R}, ery^{R} and oli^{R}. All antibiotic-resistance markers (oli^{R1}, oli^{R2}, oli^{R3}, oli^{R4}, chl^{R2} and ery^{R3}) were derived from the same sensitive diploid as described previously (Rank and Bech-Hansen, 1972*a*; Rank and Martin, 1972). The [$chl^{R2}ery^{R3}$] strain 44-5a was derived from the same tetrad that gave rise to 44-5d reported earlier (Rank and Bech-Hansen, 1972b). [*rho*+] refers to normal mitochondrial DNA found in respiratory-sufficient strains; [*rho*−] refers to abnormal mitochondrial DNA found in respiratory-deficient strains.

(ii) *Media*

Sensitivity to chloramphenicol, erythromycin and oligomycin in *S. cerevisiae* requires that the energy for growth is derived from a non-fermentable energy source such as glycerol. Thus strains were defined as being resistant to chloramphenicol, erythromycin or oligomycin if they grew on YEPG medium (1 per cent. yeast extract, 2 per cent. bacto-peptone, 2 per cent. agar, 4 per cent. glycerol) supplemented with 3 mg./ml. of chloramphenicol (YEPG-C), 2 mg./ml. of erythromycin (YEPG-E) or 10 μg./ml. of oligomycin (YEPG-O), respectively. Medium with more than one antibiotic contained the above concentrations for each antibiotic. YEPD medium contained 1 per cent. yeast extract, 2 per cent. bacto-peptone and 2 per cent. glucose. Medium selective for hybrid protrophic growth (Min D) but non-selective for antibiotic resistance contained 0·67 per cent. yeast nitrogen base, 2 per cent. dextrose and 2 per cent. agar.

(iii) *Scoring of recombinants*

Recombination of the antibiotic-resistance markers used will be defined as the percentage of recombinant homoplasmic cells in 48-hour-old zygote colonies grown on a medium that is non-selective for recombinant or

parental markers. Parental strains were grown for 48 hours in YEPD, 0·5 ml. of each strain was mixed, and the mixture was incubated at 30° C. for 3 hours prior to dilutions and spreading on Min D. Forty-eight-hour-old zygote colonies were respread on Min D and 72-hour-old zygote-daughter-cell colonies were scored for antibiotic resistance by patching on to YEPG-C,

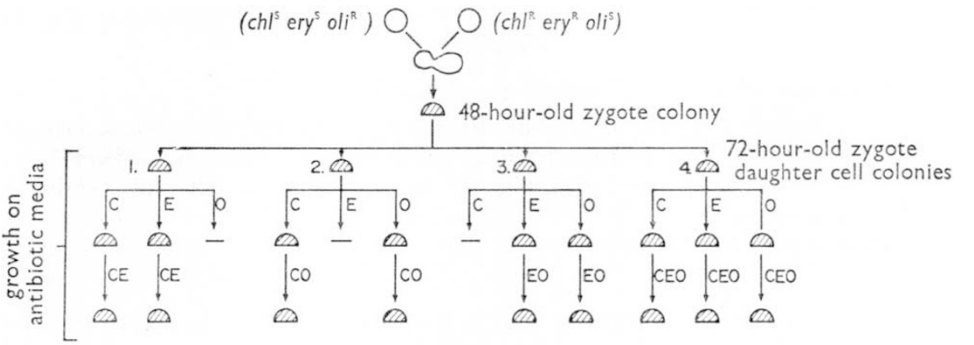

FIG. 1.—Method used to determine the frequency of homoplasmic recombinant cells in 48-hour-old zygote colonies. Zygote-daughter-cell colonies were initially patched on to YEPG media containing either chloramphenicol (C), erythromycin (E) or oligomycin (O). Positive growth on any two, or all three of the media was then sampled on to a medium containing the antibiotics to which resistance was indicated. Zygote daughter cell colonies showing the patterns indicated would be classified as [*chl*^{R}*ery*^{R}*oli*^{S}] (colony 1), [*chl*^{R}*ery*^{S}*oli*^{R}] (colony 2), [*chl*^{S}*ery*^{R}*oli*^{R}] (colony 3) and [*chl*^{R}*ery*^{R}*oli*^{R}] (colony 4) homoplasmons.

TABLE 2

Growth of all possible combinations of three resistance markers on selective media

Antibiotic-resistance markers	Media							
	G*	G-C	G-E	G-O	G-CE	G-CO	G-EO	G-CEO
[*chl*^{S}*ery*^{S}*oli*^{S}]	+†	−	−	−	−	−	−	−
[*chl*^{R}*ery*^{S}*oli*^{S}]	+	+	−	−	−	−	−	−
[*chl*^{S}*ery*^{R}*oli*^{S}]	+	−	+	−	−	−	−	−
[*chl*^{S}*ery*^{S}*oli*^{R}]	+	−	−	+	−	−	−	−
[*chl*^{R}*ery*^{R}*oli*^{S}]	+	+	+	−	+	−	−	−
[*chl*^{R}*ery*^{S}*oli*^{R}]	+	+	−	+	−	+	−	−
[*chl*^{S}*ery*^{R}*oli*^{R}]	+	−	+	+	−	−	+	−
[*chl*^{R}*ery*^{R}*oli*^{R}]	+	+	+	+	+	+	+	+

* Abbreviation for YEPG.
† + = Growth; − = No growth.

YEPG-E and YEPG-O (fig. 1). As seen in table 2, all eight possible homoplasmic genotypes are clearly defined by growth patterns on YEPG-C, YEPG-E and YEPG-O. However, except for the [*chl*^{S}*ery*^{S}*oli*^{S}] recombinant, various types of heteroplasmic zygote-colony cells could produce a zygote-daughter-cell colony with a phenotype similar to one of the homoplasmons (Rank and Bech-Hansen, 1972*b*). Thus positive growth on any two, or all three of the media was resampled on to a medium containing all the antibiotics to which resistance was indicated (fig. 1). If positive growth on two or more media was due to a heteroplasmic zygote-colony cell, then passage through one round of selective medium will usually suffice to segregate out the homoplastic components (Rank and Bech-Hansen,

1972*b*). Therefore growth on double or triple antibiotic media, of growth sampled from all single antibiotic media, was taken as evidence for a true recombinant zytogote-colony cell (fig. 1). Heteroplasmic zygote-colony cells were defined as those cells giving rise to doubly or triply resistant growth on the first sample of single selective media but unable to support growth on the second medium containing more than one antibiotic.

3. Results

(i) *Cell types observed in 48-hour-old zygote colonies*

Strains carrying one of the four oligomycin-resistance markers (OR1-1b, OR2-5a, OR3-1a and OR4-2b) were crossed to the common [$chl^{R2}ery^{R3}$] strain 44-5a. The average number of the nine possible cell types (eight homoplasmons plus the heteroplasmon) found among the 25 zygote-colony cells analysed is recorded in table 3. All nine cell types were observed in the

Table 3

The average number of cells found in a sample of 25 cells from zygote colonies of 3-factor crosses of the type [$chl^{S}ery^{S}oli^{R}$] *by* [$chl^{R}ery^{R}oli^{S}$]

Cross	Number of zygote colonies sampled	Parental phenotypes		Recombinant phenotypes						Heteroplasmic cells
		[$chl^{S}ery^{S}oli^{R}$]	[$chl^{R}ery^{R}oli^{S}$]	[$chl^{R}ery^{S}oli^{S}$]	[$chl^{S}ery^{R}oli^{S}$]	[$chl^{R}ery^{S}oli^{R}$]	[$chl^{S}ery^{R}oli^{R}$]	[$chl^{R}ery^{R}oli^{R}$]	[$chl^{S}ery^{S}oli^{S}$]	
OR1-1b by 44-5a	25	13·9	2·8	0·7	0·8	1·3	1·0	2·0	2·2	0·4
OR2-5a by 44-5a	10	17·6	2·9	0·5	0·3	0·8	0·6	0·5	0·6	1·2
OR3-1a by 44-5a	10	17·7	1·3	0·3	0·8	0·6	1·3	0·9	1·7	0·4
OR4-2b by 44-5a	10	17·5	0·5	0·0	1·7	0·3	2·0	0·3	2·7	0·1

zygote colonies of each cross except for the [$chl^{R}ery^{S}oli^{S}$] homoplasmon in the cross of OR4-2b by 44-5a—the latter exception is likely due to sampling error Large variation in the average number of cell types was found between zygote colonies. The most striking aspect of the types of cells observed in all crosses is the high frequency of [$chl^{S}ery^{S}oli^{R}$] homoplasmons; approximately 17 of the 25 cells sampled were of this type for all crosses (table 3). In contrast, homoplasmons for the other parental marker [$chl^{R}ery^{R}oli^{S}$] were observed at a much lower frequency; a range of 0·5 to 2·9 cells was observed in the four crosses (table 3). These data are consistent with the asymmetrical distribution of one of the parental markers previouly observed for two-factor crosses (Rank and Bech-Hansen, 1972*b*).

Each of the six recombinant homoplasmons were observed with a similar frequency of approximately 4 per cent. (one cell per 25 sampled). There was no obvious difference in the average number of the six different recombinant homoplasmons in the 48-hour-old zygote colony (table 3). Similarly, the number of heteroplasmic cells was similar to the number of cells observed of a given recombinant homoplasmon.

(ii) *Percentage recombination*

Of the six possible recombinant genotypes in the cross of [$chl^{S}ery^{S}oli^{R}$] by [$chl^{R}ery^{R}oli^{S}$]: (i) [$chl^{R}ery^{S}oli^{S}$], [$chl^{S}ery^{R}oli^{S}$], [$chl^{R}ery^{S}oli^{R}$] and [$chl^{S}ery^{R}oli^{R}$]

are recombinants for the chloramphenicol and erythromycin markers, (ii) [*chl*^{s}*ery*^{R}*oli*^{s}], [*chl*^{R}*ery*^{s}*oli*^{R}], [*chl*^{R}*ery*^{R}*oli*^{R}] and [*chl*^{s}*ery*^{s}*oli*^{s}] are recombinants for the chloramphenicol and oligomycin markers and (iii) [*chl*^{R}*ery*^{s}*oli*^{s}], [*chl*^{s}*ery*^{R}*oli*^{R}], [*chl*^{R}*ery*^{R}*oli*^{R}] and [*chl*^{s}*ery*^{s}*oli*^{s}] are recombinants for the erythromycin and oligomycin markers. The average percentage of recombinants observed for the three pairs of markers is summarised in table 4. For any one of the four crosses the percentage recombinants of any one of the three marker pairs ([*chl ery*], [*chl oli*], [*ery oli*]) is not statistically different than the other two pairs of markers. Recombination in the cross OR2-5a by 44-5a was lower than in the other three crosses. The surprising feature of these data is the high percentage of recombination for all pairs of markers in all crosses. The range is from 8·8 to 26·1 per cent. homoplasmic recombinant cells in the 48-hour-old zygote colony. The rather large confidence limits are due to the fact that some zygote colonies had a preponderance of one type of recombinant. For example, the eighth zygote colony from the cross of OR1-1b by 44-5a gave rise to 20 daughter colonies that had a [*chl*^{R}*ery*^{R}*oli*^{R}] recombinant genotype.

TABLE 4

Average percentage recombination found in 48-hour-old zygote colonies from crosses of the type [*chl*^{s}*ery*^{s}*oli*^{R}] *by* [*chl*^{R}*ery*^{R}*oli*^{s}]

	Recombinant markers		
Cross	[*chl ery*]	[*chl oli*]	[*ery oli*]
OR1-1b by 44-5a	14·9 ± 5·2*	26·1 ± 10·3	23·0 ± 8·8
OR2-5a by 44-5a	8·8 ± 5·4	8·8 ± 7·5	8·8 ± 4·8
OR3-1a by 44-5a	12·0 ± 8·5	16·0 ± 14·7	16·8 ± 10·6
OR4-2b by 44-5a	15·6 ± 8·6	19·6 ± 14·9	20·0 ± 5·5

* 95 per cent. confidence limits of the mean.

(iii) *Tetrad analyses of* [*chl*^{R}*ery*^{R}*oli*^{R}] *recombinants*

Triply-resistant isolates from all four crosses were taken from YEPG-CEO plates and subjected to tetrad analyses. Five complete tetrads from each of the crosses of OR1-1b, OR2-5a, and OR4-2b with 44-5a gave the expected 2 : 2 segregation for auxotrophic markers and 4 : 0 segregation on YEPG-C, YEPG-E, YEPG-O and YEPG-CEO. Similarly, a random spore analysis of 22 ascospores from the cross of OR3-1a by 44-5a produced 1 : 1 segregation for auxotrophic markers whereas all ascospores gave a triply antibiotic-resistance phenotype. These results are in accordance with the expectations of cytoplasmic inheritance for antibiotic resistance and suggest that the triply-resistant isolates are true cytoplasmic recombinants.

4. DISCUSSION

From the data in table 3 it can be seen that all the expected recombinant homoplasmons for 3-factor crosses were observed with an approximately equal frequency of 0·5 to 3 per cent. (the exception being the [*chl*^{R}*ery*^{s}*oli*^{s}] recombinant for the OR4-2b by 44-5a cross). Grouping these data to gain an estimate of the amount of recombination between the three pairs of markers (table 4) resulted in a surprisingly high percentage of recombination of from 8·8 to 26·1 per cent. A high frequency of recombination for chloroplast DNA has also been observed (Chiang, 1971; Sager and Ramanis,

1971). For any cross the percentage recombination of any one of the three marker pairs ([*chl ery*], [*chl oli*], [*ery oli*]) was not statistically different from the other two. Three of the crosses (OR1-1b, OR3-1a and OR4-2b by 44-5a) gave a similar high percentage recombination of 15 to 20 per cent. for all three marker pairs whereas one cross (OR2-5a by 44-5a) gave a lower percentage for all three marker pairs of approximately 10 per cent.

The absence of any hard data supporting a specific mechanism of plasmagene recombination requires that the recombination data be interpreted by formal genetic analysis. Unfortunately the positioning of any of the three cytoplasmic markers relative to each other by the use of recombination frequencies is impossible since recombination frequencies between marker pairs are equal (table 4). The present data do not enable a distinction between high recombination between three markers widely spaced on a single mitochondrial DNA molecule, or segregation of three different mitochondrial DNA molecules—each carrying a different marker. Certainly there does not appear to be close linkage between any of the three markers.

Asymmetrical distribution of the [$chl^{S}ery^{S}oli^{R}$] parental marker to the zygote-colony cells is evident from the data of table 3 and is consistent with observations using two-factor crosses (Coen *et al.*, 1969; Bolotin *et al.*, 1971). Of the nine cell types scored, this is the only phenotype that is clearly present in a significantly greater number of zygote-colony cells. According to the terminology of Bolotin *et al.* (1971), strains OR1-1b, OR2-5a, OR3-1a and OR4-2b would be designated ω^{+} since the mitochondrial markers of these strains are transmitted with a high frequency to cells of the zygote colony. Conversely, strain 44-5a would be designated by ω^{-}. Since all four crosses reported on here are thus " heterosexual " (ω^{+} by ω^{-}, see Bolotin *et al.*, 1971), the hypothesis of Bolotin *et al.* (1971) predicts that the number of reciprocal recombinants will be unequal. As can be seen in table 3, this expectation was not realised for any of the three pairs of reciprocal recombinants ([$chl^{R}ery^{S}$] *v* [$chl^{S}ery^{R}$], [$chl^{R}oli^{R}$] *v* [$chl^{S}oli^{S}$], and [$ery^{R}oli^{R}$] *v* [$ery^{S}oli^{S}$]) in any of the four crosses. For example, on the cross OR1-1b by 44-5a the [$chl^{R}ery^{S}$] recombinant and its reciprocal [$chl^{S}ery^{R}$] average 2·0 and 1·8 of the 25 zygote-colony cells sampled. The small differences in the frequency of reciprocal recombinants can be the result of sampling error. Thus the relative positioning of markers in these crosses cannot be determined by placing the ω^{+} marker of the maximum recombinant class to the left of the genetic map as suggested by Bolotin *et al.* (1971).

The disagreement between results recorded here and those of Bolotin *et al.* (1971) could perhaps be explained by maintaining the concept of mitochondrial sexuality and assuming the existence of different ω strains. However, it should be noted that the phenomenon of asymmetrical distribution of mitochondrial markers has also been observed for *Paramecium aurelia* (Adoutte and Beisson, 1972) as well as for other cytoplasmically inherited markers in yeast (Cox, 1965; Lacroute, 1971). The plasmagene affecting the uptake of ureidosuccinic acid does not appear to reside in mitochondrial DNA since ethidium bromide-induced [*rho*-] strains maintained the plasmagene (Lacroute, 1971). If this plasmagene does not reside in mitochondrial DNA, then it is unlikely that it resides in the mitochondrion; thus the concept of organelle sexuality to explain asymmetrical distribution

of plasmagenes does not have a general application. Rather it appears that many factors may influence asymmetric distribution and recombination of cytoplasmically inherited markers. Since the plasmagenes of all strains of *a* mating type had a high transmission to the zygote-colony (tables 1 and 4) it appears that nuclear markers may be involved in asymmetric distribution; however, further genetic analyses are required to substantiate this possibility.

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