

Manganese mutagenesis in yeast

II. Conditions of induction and characteristics of mitochondrial respiratory deficient *Saccharomyces cerevisiae* mutants induced with manganese and cobalt

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SUMMARY

Manganese and cobalt are capable of inducing ρ^- mutations* in non-growing cells of *Saccharomyces cerevisiae*, but their mutagenic action is much stronger in growing cells. At a given concentration cobalt and manganese can be either strongly mutagenic or non-mutagenic, depending on the cell density.

Most of the ρ^- mutants induced with manganese and a considerable proportion of those induced with cobalt are suppressive and/or transmit drug resistance markers, so they must still carry mitochondrial DNA. Cobalt can decrease suppressiveness with low efficiency and eliminate drug resistance markers from established ρ^- clones.

1. INTRODUCTION

Although a number of divalent cations belong to the first group of factors recognized as capable of inducing ρ^- mutations in yeast (Lindgren, Nagai & Nagai, 1958), very little is known about the conditions necessary (Lindgren & Lindgren, 1973), and still less about the properties of such mutants (Putrament, Baranowska & Prazmo, 1973). Whatever the molecular mechanism of ρ^- induction by the divalent cations, it cannot be identical with that of ethidium bromide (Slonimski, Perrodin & Croft, 1968; Goldring, Grossman, Krupnick, Cryer & Marmur, 1970; Goldring, Grossman & Marmur, 1971; Paoletti, Couder & Guerinéau, 1972; Whittaker, Hammond & Luha, 1972) or of ultraviolet irradiation (Waters & Moustacchi, 1974).

* Abbreviations: mitDNA = mitochondrial DNA; C^S/C^B = allelic forms conferring sensitivity/resistance to chloramphenicol; E^S/E^B = allelic forms conferring sensitivity/resistance to erythromycin; Ant^S/Ant^B = sensitivity/resistance to either of the two antibiotics, or to both; Ant^0 = cytoplasmic respiratory-deficient mutants lacking mitDNA which carry Ant^S or Ant^B markers; ρ^+ = 'grande' or respiratory-competent cells; ρ^- = cytoplasmically inherited 'petite' or respiratory-deficient cells; CAP = chloramphenicol; ERY = erythromycin; CFC = colony-forming centres.

The questions we have tried to answer are: (1) is cell growth necessary for ρ^- induction by manganese and cobalt? (2) since both cations are actively taken up by yeast cells (Fuhrmann, 1973; Fuhrmann & Rothstein, 1968), does cell density affect the induction of mutations? (3) is there evidence that manganese- and cobalt-induced ρ^- mutants still possess mitDNA? (4) does cobalt affect mitDNA in established ρ^- clones?

2. MATERIALS AND METHODS

(i) *Strains*

DP1-1B (ρ^+ ω^+ $C^S E_{514}^R \alpha$ *his1 trp1*); IL8-8D (ρ^+ ω^+ $C_{321}^R E_{514}^R$ a *ura1*); IL126-1C (ρ^+ ω^- $C_{321}^R E_{221}^R$ a *ura1*); and 19d/2/16 (ρ^- highly suppressive, α *his1 trp1*) were kindly given by Dr P. P. Slonimski. SBTD-2B (formerly designated 2b (Putrament *et al.* 1973)); $C^R E^S$ *thr2 tyr1 ade5*); M/S,26D (formerly designated 26-4 (*ibid.*)) $C^S E^S$ a *thr2 leu1*); 26-DP (diploid obtained from cross DP1-1B \times M/S-26D).

A number of other strains were used as testers in determinations of suppressiveness and Ant^R transmission.

(ii) *Media*

YEPglu contained 1% yeast extract, 1% peptone, 2% glucose; YEPglyc was YEPglu with 2% glycerol instead of glucose; the YEPglyc medium with 0.1 M final concentration phosphate buffer, pH 6.2, and 0.5% erythromycin or 0.4% chloramphenicol, or with both antibiotics at these concentrations, was used to select E^R mutants, or to test transmission of the Ant^R markers. YEPdif was YEPglyc with 0.2% glucose added. Respiratory-deficient and respiratory-sufficient colonies were distinguished on this medium. Minimal medium contained 0.67% yeast nitrogen base without amino acids but with 2% glucose and was used to test nutritional requirements of the strains. MINDif was minimal medium containing 0.2% glucose and 2% glycerol. It was used to determine suppressiveness of ρ^- mutants in synchronized zygotes.

For platings 2% Difco agar was added to all media.

(iii) *Methods*

In tests for suppressiveness, synchronized zygotes were obtained according to Biliński, Litwińska, Żuk & Gajewski (1973). The zygotes were plated on MINDif medium (Sherman & Ephrussi, 1962). Tests for Ant^R transmission were carried out essentially according to Saunders *et al.* (1971). The replica-cross technique was used as described by Deutsch *et al.* (1974).

Unless otherwise stated, final concentrations of $CoCl_2$ were 2 mM, and $MnCl_2$ or $MnSO_4$ 8 mM in YEPglu medium.

All ρ^- mutants tested were of independent origin.

(iv) *Chemicals*

Erythromycin and chloramphenicol (trade mark Detreomycyna) were Polfa products; $MnCl_2$, $MnSO_4$, $CoCl_2$ and $MgCl_2$ were produced in Poland (Fabryka Odczynników Chemicznych, Gliwice); they were of analytical grade.

3. RESULTS

(i) Conditions of ρ^- induction with manganese and cobalt ions

Cells of strain IL8-8D growing exponentially in YEPglyc medium, were transferred to a final density of 2×10^4 cells/ml into saline containing 2% glucose and 8 mM- MnSO_4 or 2 mM- CoCl_2 , and incubated at 30 °C for 24 h. The survival of cells incubated in manganese solution was below 2%, and about 10% of the

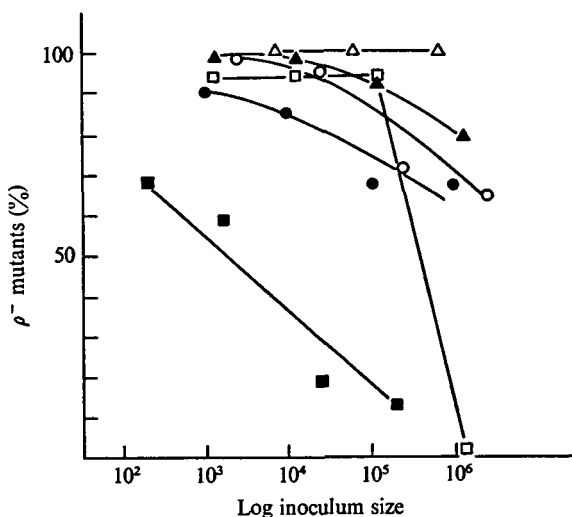


Fig. 1. Relationship between inoculum size and induction of ρ^- mutants by MnCl_2 and CoCl_2 . YEP medium with 8 mM- MnCl_2 or 2 mM- CoCl_2 was inoculated with stationary phase cells as indicated. Strain IL8-8D was incubated with CoCl_2 for 4 days, and with MnCl_2 for 3 days; strain SBTD-2B was incubated with MnCl_2 for 3 days. The remaining cultures were incubated for 2 days. Then suitably diluted samples were plated on YEPdif medium. For each strain and each inoculum size five identical cultures were grown, so that on the curves each point represents a mean from five independent samples. In MnCl_2 -containing cultures after 3 and 4 days incubation the number of cells/ml varied within one range of magnitude. In CoCl_2 -containing cultures even after 2 days incubation the numbers of cells/ml varied within limits similar to or even wider than those presented in Table 2. Out of five cultures of IL8-8D inoculated with 2.5×10^5 cells/ml two contained 36 and 21% ρ^- cells, i.e. much less than in the remaining cultures in this and other experiments. These values were not included in the calculation of the mean percentage of ρ^- . Open symbols: cobalt-containing cultures; closed symbols: manganese-containing cultures. \circ , \bullet , IL8-8D; \square , \blacksquare , DP1-1B; \triangle , \blacktriangle , SBTD-2B.

survivors were mutated to ρ^- . The survival of the cells incubated in cobalt solution was 5–20% in different experiments, and about 20% of the survivors were mutated to ρ^- . Thus cell growth was not absolutely necessary for ρ^- induction by either of the cations. However, when the cations at the same concentrations were added to the YEPglu medium, and the yeast cells incubated for 1–3 days, up to 100% of the surviving cells were mutated to ρ^- (Fig. 1).

In experiments performed with manganese and growing cells we never observed

cell death even when a 10 mM concentration of the cation was used. On the other hand, when exponentially growing cells of strains DP1-1B, IL8-8D and IL126-1C (inocula about 1×10^5 cells/ml) were incubated for 3 h in YEPglu medium containing 2 mM- CoCl_2 , cell survival of the first two strains was 10 %, and that of the third strain was 27 %.

With similar medium and cation concentrations, the frequencies of ρ^- mutations induced depended on the cell density (Fig. 1). This result was most clear-cut in the case of strain DP1-1B. When the inoculum was 3×10^6 cells/ml, 2 mM cobalt neither inhibited cell growth, nor induced ρ^- mutations; however, when the inoculum was ten times smaller, cell growth was strongly inhibited and almost the entire cell population was mutated to ρ^- . The effects of cell density on manganese mutagenesis were less drastic, but the general tendency was the same. This was

Table 1. *Effects of varying cell density on induction of mitochondrial E^R mutations by 8 mM manganese*

Cells/ml	E^R mutants per $10^6 \rho^+$ CFC
9×10^4	250
9×10^5	23
9×10^6	5
Control	4

Cells growing exponentially in liquid YEPglyc medium were collected by centrifugation, and suspended to the densities indicated in 10 ml of YEPglu medium pH 6 containing 8 mM- MnSO_4 . After 6 h incubation at 30 °C on a shaker the cells were collected, washed once in Ringer solution, resuspended in 1 ml of YEPglu, and plated on YEPglyc medium, pH 6.2, containing 0.5 % erythromycin. The E^R mutant colonies were counted after 10 days incubation.

true for ρ^- induction as well as for induction of mitochondrial erythromycin-resistant mutations by manganese (Table 1).

We found previously (Putrament *et al.* 1973) that when yeast cells were grown in YEPglu medium containing 8 mM manganese and 4 mM magnesium salt, manganese was no longer capable of inducing ρ^- mutations. On the other hand, magnesium even in equimolar concentration with cobalt was incapable of protecting the cells from the mutagenic action of the latter (Table 2).

(ii) *Suppressiveness and Ant^R transmission by ρ^- mutants induced with manganese and cobalt*

Twenty-four manganese-induced and 25 cobalt-induced primary ρ^- clones of strain IL8-8D were subcloned, and 3 secondary clones from each primary clone were treated for suppressiveness and C^RE^R transmission. Thirty-four of the 49 primary clones analysed segregated non-identical secondary clones (Table 3). As seen in Fig. 3, the secondary clones showed a wide range of suppressiveness. Fourteen secondary clones from manganese-induced ρ^- mutants, and 18 from

Table 2. Cell growth inhibition and ρ^- induction by CoCl_2

Culture no.	CFC after 2 days incubation ($\times 10^5$)	ρ^- (%)	No. ρ^- colonies tested for Ant ^R transmission	No. of Ant ^R found
1	16	91	24	3
2	16	71	36	6
3	2	55	12	1
4	25	68	24	6
5	1	90	10	0
6	1	100	24	3
7	9	97	12	0
8	26	81	24	2
9	19	95	24	1
10	38	72	22	4
Total			212	26

YEP medium containing 4 mM- CoCl_2 was inoculated with 2×10^8 stationary phase cells/ml of strain IL126-1C. After 2 h incubation an equal concentration of MgCl_2 was added and the cell culture was divided into ten test tubes and incubated at 30 °C for 2 days. Aliquots of cells from each culture were diluted and plated on YEPdif medium and the percentage of ρ^- was counted after 6 days incubation. The ρ^- mutants to be tested for Ant^R transmission were transferred on YEP medium. Ant^R transmission was tested by the replica-cross technique.

Table 3. Characteristics of IL8-8D primary ρ^- clones induced with manganese and cobalt

Types of secondary clones	No. of primary clones induced with:	
	Manganese	Cobalt
Three secondary clones neutral, Ant ^R	2	4
Three secondary clones neutral, 1 or 2 of them Ant ^R	1	1
One or 2 secondary clones neutral, the remaining suppressive, at least one of them Ant ^R	6	2
Three secondary clones neutral, Ant ^R	2	6
Three secondary clones suppressive, Ant ^R	6	3
One or 2 secondary clones neutral, the remaining suppressive, all Ant ⁰	7	9
Total		
	24	25

All primary clones induced with manganese and ten induced with cobalt were taken from the experiments described in the legend to Fig. 1. The remaining 15 cobalt-induced primary ρ^- clones were induced by incubating the cells for 24 h at 30 °C in 0.1 M phosphate buffer containing 2 % glucose and 2 mM- CoCl_2 .

cobalt-induced ρ^- mutants transmitted C^{RE}R markers. No C^{RE}O or C^OER clones were found. Twenty-eight of the 32 C^{RE}R secondary clones were completely neutral in our tests.

Twenty manganese-induced ρ^- primary clones of strain SBTD-2B showed a wide spectrum of suppressiveness (Fig. 2). In seven of them some cells transmitted

the C^R marker. These clones were subcloned, and the secondary clones thus obtained also differed in respect to suppressiveness. Only one secondary clone transmitted the C^R marker. Twenty cobalt-induced primary ρ^- clones of the same strain were neutral or showed very low suppressiveness (Fig. 2), and none of the 15 secondary clones examined transmitted the C^R marker.

Eleven cobalt-induced ρ^- primary clones of strain DP1-1B/514 were neutral. Two of them transmitted the E^R marker.

As seen in Table 2, in 8 out of 10 samples of ρ^- primary clones induced with cobalt in strain IL126-1C there were 1-6 colonies capable of transmitting Ant^R markers.

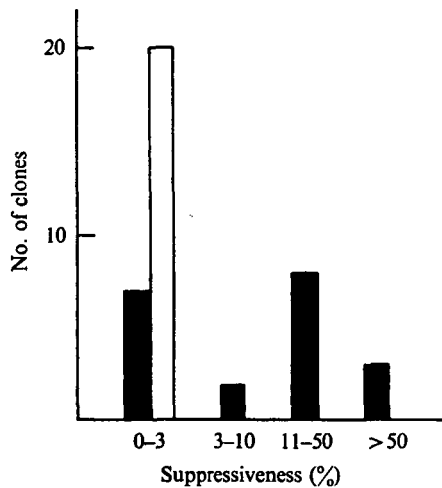


Fig. 2. Suppressiveness of cobalt- and manganese-induced ρ^- primary clones of strain SBTD-2B. The ρ^- mutants were induced in the experiment described in the legend to Fig. 1. ■, ρ^- mutants induced with manganese. □, ρ^- mutants induced with cobalt.

(iii) *Effect of cobalt on ρ^- strains*

Twenty samples of a highly suppressive ρ^- strain 19d/2/16 were incubated for 24 h in YEPglu containing $CoCl_2$ (inoculum 1×10^4 cells/ml). Single-colony isolates from each sample were tested for suppressiveness. Only in two of them did it decrease from 96% to 15% and 30%, respectively. After similar treatment of a highly suppressive ρ^- mutant of strain SBTD-2B, ten subclones retained their high suppressiveness, while in five subclones the suppressiveness decreased below 15%.

A secondary ρ^- clone of strain IL8-8D had 91% of cells transmitting $C^R E^R$ markers. Nineteen samples of this clone were grown for 48 h in YEPglu with cobalt, and after suitable dilution plated on YEPglu. From each sample 14-26 colonies were tested by the replica-cross technique for Ant^R transmission; 40-77% of the colonies failed to produce Ant^R diploids, so the haploid ρ^- cells were Ant^O . Cobalt is therefore capable of eliminating the Ant^R markers from established ρ^- mutants.

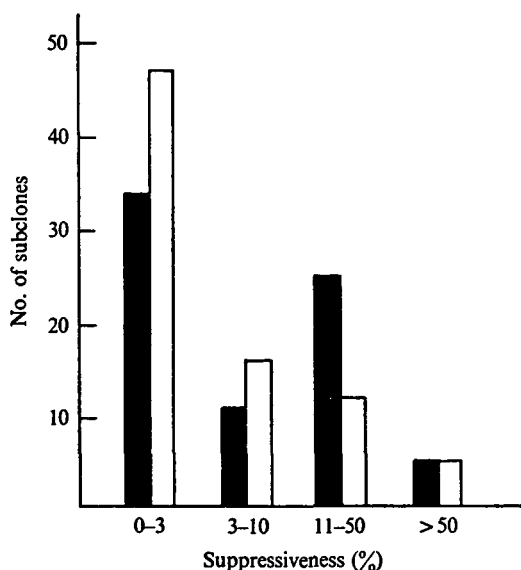


Fig. 3. Suppressiveness of ρ^- cobalt- and manganese-induced secondary clones of strain IL8-8D. All manganese-induced and ten cobalt-induced primary ρ^- clones were obtained in the experiment described in the legend to Fig. 1. For the origin of the remaining 15 cobalt-induced ρ^- primary clones see legend to Table 3. Filled blocks: ρ^- mutants induced with manganese. Open blocks: ρ^- mutants induced with cobalt.

4. DISCUSSION

Our results show that both cations studied are capable of inducing ρ^- mutations in conditions which favour cation uptake. At the same time they prevent cell growth, and, presumably, mitDNA replication. However, both are much more effective when added to a growth medium. Therefore the possibility cannot be excluded that there may be more than one mechanism responsible for ρ^- induction by either cation, one mechanism operating predominantly in non-growing, and another in growing cells.

Cobalt is toxic for yeast cells irrespective of treatment conditions, whereas manganese is toxic when present in saline-glucose, but not in YEPglu medium. This might be due to the slowing-down of Mn^{2+} uptake by other divalent cations, particularly Mg^{2+} and Zn^{2+} (Fuhrmann, 1973; Fuhrmann & Rothstein, 1968). It also seems possible that there is an efflux of manganese, but not of cobalt, when the yeast cells are incubated in a rich growth medium (Silver & Kralovic, 1969; Silver, Johnseine & King, 1970; Silver, Johnseine, Whitney & Clark, 1972; Eisenstadt *et al.* 1973; Fisher *et al.* 1973; Nelson & Kennedy, 1971).

So far manganese, cobalt, and presumably a number of other divalent cations (Lindegren *et al.* 1958) are the only known factors which are necessary for normal cell growth in trace amounts. They are actively taken up by the cells, tolerated by them up to a certain increased intracellular level, and become mutagenic only when this level reaches some, as yet unknown, value. Indeed, we know of no other

mutagen which at the same concentration in the incubation medium would be strongly mutagenic towards a diluted cell culture and completely non-mutagenic towards a ten times higher concentration of cells in the mutagenic medium (Fig. 1).

In view of this peculiarity of manganese and cobalt it would be extremely difficult to study the kinetics of ρ^- induction by either cation, in particular the relationship between the dose of the mutagen and suppressiveness or loss of the Ant^R marker (Deutsch *et al.* 1974). In so far as comparisons are possible, the established ρ^- mutants induced with either cation do not differ strikingly from those of spontaneous origin, or from those induced with eufavin and small concentrations of ethidium bromide (Saunders *et al.* 1971). Many of them show suppressiveness and/or transmit Ant^R markers and thus must carry mitDNA (Michaelis *et al.* 1971; Thomas & Wilkie, 1968). Most of the primary clones segregate out secondary clones which are non-identical as regards suppressiveness and Ant^R transmission. Thus, whatever the primary lesions in mitDNA resulting from manganese and cobalt treatment, the subsequent events leading to the formation of ρ^- mutants do not seem to differ from those occurring after ultra-violet or ethidium bromide treatment (Deutsch *et al.* 1974).

In our tests 28 secondary ρ^- clones of strain IL8-8D were neutral, yet capable of transmitting Ant^R markers. This extends the conclusion drawn by Moustacchi (1972) that neutral ρ^- mutants can still carry mitDNA.

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