INDUCTION OF PETITE MUTANTS IN *SACCHAROMYCES CEREVISIAE* BY ICR-170 AND THEIR GENETIC CHARACTERIZATION

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WE report here the induction of respiration-deficient (petite) mutants in *Saccharomyces cerevisiae* by 2-methoxy-6-chloro-9-(3-[ethyl-2-chloroethyl]aminopropylamino) acridine-2HCl (ICR-170) and a genetic grouping of these mutants.

MATERIALS AND METHODS

The strain employed was S288C, a genetically well-studied respiration-sufficient haploid that forms predominantly single cells and cells with one bud during log-phase growth and single cells in stationary phase. The respiratory genetic makeup of spontaneous and induced petites was determined by tetrad analysis and by complementation tests (PITTMAN *et al.* 1960; SHERMAN and EPHRUSSI 1962) using segregational (*pet* ρ +), cytoplasmic (*PET* ρ —), and double mutant (*pet* ρ —) tester stocks. We thank Professors MAURICE OGUR, ROBERT MORTIMER and FRED SHERMAN for supplying many of the tester cultures.

Formulas are presented elsewhere for the glucose nutrient agar and broth (PITTMAN *et al.* 1960), lactate nutrient agar (OGUR and ST. JOHN 1956), triphenyl tetrazolium chloride (TTC) overlay agar (OGUR, ST. JOHN and NAGAI 1957), and sodium phosphate starvation buffer (BRUSICK 1970). All conditions for growth of cultures, cell starvation, incubation, treatment, and platings were at 30°C.

ICR-170 was kindly supplied by Dr. H. J. CREECH (Institute for Cancer Research, Philadelphia) and designated Sample E in our department. The mutagen was dissolved in glass-distilled water just prior to use, although we find that solutions stored at 5°C remain active over several weeks. All steps involving mutagen were carried out under dim yellow light. Mutagen-containing fluids and materials were treated with 15–30% KOH for 2–3 weeks before discarding or cleaning.

Mutation induction was studied in both log-phase and stationary-phase cells since BRUSICK (1970) observed that the former are highly sensitive to the killing and mutagenic action of ICR-170. Our protocol for growth and treatment of the two cell types is shown in Figure 1. Cells were grown aerobically from a starting inoculum of $1\text{--}2 \times 10^4$ cells/ml to mid-log phase (11–12 hr; 10^7 cells/ml) and to stationary phase (24 hr; 3×10^8 cells/ml), harvested, washed, starved for 12 hr in buffer, and treated as shown. Control and treated suspensions were shaken, sampled at prescribed time intervals (Figure 2), diluted as required in water, and plated on glucose nutrient agar. From 6 to 20 plates were spread per dilution at each time interval. Colony counts were made after plates had incubated 3–4 days. Plates were then separated into two sets for each dilution and treatment period. One set was overlaid with TTC agar and the other set was replica plated onto glucose nutrient agar and lactate nutrient agar. The frequencies of whole-colony mutants and distinctly sectorial colonies obtained by replica plating were comparable to those obtained by TTC overlay. Mutation-dose response curves are based on the percent mutants and "total mutants" (whole-colony mutants plus distinctly sectorial colonies).

Colonies that failed to grow when replica plated onto lactate and which were classified as presumptive whole-colony mutants were collected from master plates, retested, and catalogued as to treatment time and mutagen concentration. About 90% of the whole-colony mutants that

FIGURE 1.—Protocol for growth, starvation, and ICR-170-treatment of log-phase and stationary-phase cells. S = stationary phase; L = log phase; t = time; b = nutrient broth; dH₂O = glass-distilled water; A = sodium phosphate buffer; ICR = ICR-170; C = control (untreated) cell suspension; and I = ICR-170-treated cell suspension.

arose were isolated; the remainder lay too close to wild-type colonies to ensure good sampling from pressed colonies on the master plates. Of 729 isolates, 13 showed wild-type phenotype or apparent clonal heterogeneity and 716 retained the lactate-negative phenotype on retesting. Stocks of the latter were subsequently used to determine the genetic makeup of spontaneous and induced petites. Each mutant is negative under TTC-overlay or reduces the dye slowly, as is the case with some segregational petites. Several mutants monitored for oxygen consumption with

FIGURE 2.—Survival curves (upper) and mutation dose-response curves (lower) for log-phase cells and stationary-phase cells treated with $1 \mu\text{g}$ ICR-170/ml. Mutation curves are based on triphenyl tetrazolium chloride overlay data. Normal colonies appear red, whole colony mutants are white, and sectorial colonies are red with white sectors.

glucose as the substrate showed weak or negligible aerobic respiration (WERKHEISER 1971). The mutants, therefore, are not of the lactate-negative, TTC-plus, respiration-sufficient types induced by nitrous acid (KOVAC, LACHOWICZ and SLONIMSKI 1967).

RESULTS AND DISCUSSION

Mutation-induction: Figure 2 presents the survival and mutation curves for log-phase cells and stationary-phase cells exposed to $1 \mu\text{g}$ ICR-170/ml. Log-phase cells are sensitive to both killing and mutation, whereas stationary-phase cells are relatively insensitive to the mutagen. As is evident from the results shown in Figure 2, ICR-170 induced sectorial colonies in log-phase cells (total mutant

frequency minus whole colony mutant frequency). Qualitatively similar results are obtained for log-phase cells treated with 10 μ g ICR-170/ml: at 10 min of treatment (2% survival) we observe that about 40% of the survivors are whole-colony mutants and 15% are sectored colonies. In contrast sectored colonies are rarely encountered among the survivors of treated stationary-phase cells, using either mutagen concentration; consequently, the whole colony mutant frequency closely approximates the total mutant frequency (Figure 2). We consider, therefore, that one property of ICR-170 is the induction of "delayed" mutations in log-phase cells.

It should be noted that the total mutant frequencies for log-phase cells include whole-colony types plus *obviously* sectored colonies. Close inspection of TTC-overlaid plates, however, reveals that the large majority (about 70%) of otherwise "normal" colonies contain mottled patches and/or fine peripheral mutant sectors for treatment times of 15 min or longer, and with either mutagen concentration. Consequently, the mutation curves shown grossly underestimate the total mutational damage by ICR-170.

The results (Figure 2) extend the finding by BRUSICK (1970) on the differential sensitivity of log-phase cells to the mutagenic action of ICR-170. We feel the poor mutagenicity (not specificity) reported for ICR-170 (WILLS 1968; PARKER and SHERMAN 1969) can be accounted for by use of stationary-phase cells rather than log-phase cells. Our protocol (Figure 1) provides a means to compare, in strict parallel fashion, the sensitivity of the two cell types and to obtain log-phase cells having consistently marked sensitivity to ICR-170 induction of petite mutants and auxotrophs.

Grouping of mutants: Each of the 716 mutants, representing 5 separate experiments, was tested for its respiratory genetic makeup by crossing to cytoplasmic (*PET* ρ -), double mutant (*pet5* ρ -), *pet1* . . . *pet6*, and *pet9* (all ρ +) segregational tester stocks. The provisional grouping (Table 1) combines the results obtained with 1 μ g and 10 μ g mutagen/ml, since we find the distribution of mutant classes was basically the same for both concentrations. The distribution of mutant classes for the two cell types, mutagen concentrations, and different treatment periods is tabulated elsewhere (WERKHEISER 1971). All of the spontaneous mutants are cytoplasmic. Of the 499 induced mutants, 417 are cytoplasmic, 71 are segregational, 2 are classified as double mutants, 8 give novel complementation patterns, and one does not mate with either α or *a* testers. A majority of the segregational mutants are non-allelic to the seven tester strains employed; the most frequently occurring identifiable genes were *pet3* and *pet5*. It is of interest that one *pet3* mutant uncovered is also lysine-requiring and loses the functional cytoplasmic factor during substantial vegetative growth, as previously shown for a *pet3* lysine-requiring strain (SHERMAN 1963). Each of the 71 segregational mutants, 31 of the cytoplasmic petites, and both double mutants were crossed to either 8256-N or D52100 (both *pet* ρ +). Diploids of all crosses except one (DW 137 $\times$ 8256) gave good sporulation on acetate sporulation agar and good viability of ascospores. From 8-16 asci with 4 viable spores were employed from each cross for tetrad analysis. Regular (2 *PET* ρ +: 2 *pet* ρ +) segregation

was observed for crosses involving the segregational mutants or the two double mutants, whereas 4 *PET* ρ +: 0 *pet* ρ + segregation was observed for those crosses involving cytoplasmic petites (PITTMAN, D. LIU and WERKHEISER, manuscript in preparation).

The efficacy of the mutagen in producing segregational mutants in log-phase cells can be seen from the following results: With 1 μ g mutagen/ml the frequency of segregational mutants among the survivors was 1.6% (20/1285) at 38% survival and 4.2% (28/662) at 13% survival. At the higher survival, the frequency of cytoplasmic mutants was 18%. With these estimates, the expected proportion of double mutants ($0.18 \times 0.016 = 0.003$) agrees well with the observed frequency of 0.004. The frequencies of segregational and double mutants are considered only fair estimates but the results indicate that segregational mutations and cytoplasmic mutations (ρ -) are induced independently by ICR-170.

Mutants classified as either cytoplasmic or double fail to show revertants when heavily streaked or spread (10^8 cells/plate) on lactate nutrient agar. These mutants, as a group, are under separate genetic and mutation studies. The 71 segregational mutants can be placed into several classes based on functional allelism, leakiness, spontaneous reversion, temperature sensitivity, and reversion on treatment with ICR-170, ultraviolet radiation, and other mutagens. Mutants of a given class can be further separated by one or more criteria that define the other classes. We interpret the different classes to mean that most, if not all, of the segregational mutants arose from non-identical mutations, and we assume that this is the case also for most of the induced cytoplasmic mutants (PITTMAN, D. LIU and WERKHEISER, manuscript in preparation).

BRUSICK (1970) obtained large numbers of auxotrophs in S288C on treatment of log-phase cells with 5 μ g or 10 μ g ICR-170/ml. MUNZ and LEUPOLD (1970) also isolated substantial numbers of auxotrophs for two loci in log-phase cells of *Schizosaccharomyces pombe* treated with 1 μ g ICR-170/ml but with subsequent enrichment of mutants by 2-deoxyglucose. Our studies show that ICR-170 is a highly efficient mutagen for induction of both cytoplasmic and nuclear mutations affecting respiration, and moreover, at a concentration (1 μ g/ml) that may otherwise be considered by some as biologically tolerable. In addition, we find that a significant number of ICR-170-induced mutational events occur after several cell divisions. Whether these events are bona fide delayed mutations or reflect the further activity of any bound ICR-170 molecules during clonal growth of survivors is under study.

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TABLE 1

Summary of genetic grouping of 716 petite mutants

Growth phase	Treatment	Number of mutants	Genetic constitution of mutants			
			Segregational (<i>pet</i>)	Cytoplasmic (ρ —)	Double (<i>pet</i> ρ —)	Unknown
Log	0	111	0	111	0	0
Log	ICR-170	413	69	333	2	9
Stationary	0	106	0	106	0	0
Stationary	ICR-170	86	2	84	0	0
Total number of mutants		716	71	634	2	9

Symbols: *pet*, respiratory segregational gene; ρ —, nonfunctional extrachromosomal factor. Most of the 71 *pet* mutants are functionally nonallelic to seven standard *pet* tester stocks.

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